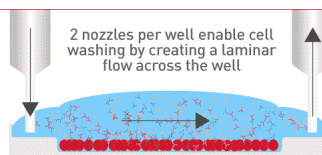


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Covalent Heterobivalent Inhibitor Design for Inhibition of IgE-Dependent Penicillin Allergy in a Murine Model

Peter E. Deak,* Baksun Kim,* Byunghye Koh,[†] Amina Abdul Qayum,[†] Tanyel Kiziltepe,* Mark H. Kaplan,[†] and Basar Bilgicer^{*,‡,§}

Drug allergies occur when hapten-like drug metabolites conjugated to serum proteins, through their interactions with specific IgE, trigger allergic reactions that can be life threatening. A molecule termed covalent heterobivalent inhibitor (cHBI) was designed to specifically target drug hapten-specific IgE to prevent it from binding drug-haptenated serum proteins. cHBI binds the two independent sites on a drug hapten-specific Ab and covalently conjugates only to the specific IgE, permanently inhibiting it. The cHBI design was evaluated via ELISA to measure cHBI-IgE binding, degranulation assays of rat basophil leukemia cells for in vitro efficacy, and mouse models of ear swelling and systemic anaphylaxis responses for in vivo efficacy. The cHBI design was evaluated using two separate models: one specific to inhibit penicillin G-reactive IgE and another to inhibit IgE specific to a model compound, dansyl. We show that cHBI conjugated specifically to its target Ab and inhibited degranulation in cellular degranulation assays using rat basophil leukemia cells. Furthermore, cHBIs demonstrated in vivo inhibition of allergic responses in both murine models. We establish the cHBI design to be a versatile platform for inhibiting hapten/IgE interactions, which can potentially be applied to inhibit IgE-mediated allergic reactions to any drug/small-molecule allergy. *The Journal of Immunology*, 2019, 203: 000–000.

Immediate IgE-mediated hypersensitivity reactions caused by drugs (drug allergies) are a type of adverse drug reaction that afflicts over 2 million people per year in the United States and can trigger severe and life-threatening anaphylaxis (1). Drug allergies are unpredictable, can occur to very commonly used antibiotics such as sulfa drugs and penicillins, and currently have

no preventative therapies (2). In light of this need, in this study, we present the development of a unique allergy inhibitor platform that can be used to prevent IgE-mediated allergic reactions triggered by small-molecule drugs such as penicillin.

Severe drug allergy reactions are due to a process called haptenization, in which multiple copies of a drug molecule covalently bind to a carrier protein, decorating the protein with modified versions of the drug, known as drug haptens (2, 3). The multivalently presented haptens on the surface of the protein trigger the multivalent cross-linking of drug hapten-specific IgE, which are present on the surfaces of mast cells and basophils. These cross-linking events then trigger the degranulation of mast cells and basophils (4, 5).

Among numerous drug allergies, β -lactam antibiotic allergies (e.g., penicillin and penicillin derivatives) are of particular concern given their wide usage. β -Lactam rings are reactive with primary amines and can readily haptenize serum proteins and initiate cross-linking of IgE on mast cells and basophils, causing allergic reactions (6). In this paper, we describe the rational design, synthesis, and in vitro and in vivo evaluation of a new class of allergy inhibitor molecules we call covalent heterobivalent inhibitors (cHBIs), developed to specifically and permanently inhibit the binding interactions between drug haptens and their respective IgE, hence inhibiting the allergic response. In this study, we synthesized a cHBI that specifically inhibits allergic responses to penicillin G (a β -lactam antibiotic) by covalently binding penicillin G-specific IgE and thereby preventing degranulation responses. Finally, to demonstrate that our platform can be used to develop cHBI inhibitors for a broad class of small-molecule drugs in addition to penicillin G, we have further validated our approach by using another small molecule that is frequently used as a hapten, dansyl (7).

Materials and Methods

Materials

NovaPEG Rink Amide resin, 5 (6)-carboxy-fluorescein, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU), Fmoc-Lys(IvDde)-OH, Fmoc-Arg(pfb)-OH, 10-kDa 0.5-ml centrifugal filters, and BSA were purchased from EMD Millipore.

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P.E.D. synthesized and characterized all compounds used in this study, performed most in vitro experiments, and analyzed these data, made all figures, and wrote the manuscript. B.B. and P.E.D. conceived of the idea for the study, designed the molecules, and designed all experiments. T.K. helped in experimental design and manuscript writing and editing. B. Kim performed affinity measurements for cHBI compounds and aided with in vitro studies. B. Koh and A.A.Q. performed all in vivo murine experiments and performed analysis. M.H.K. designed in vivo experiments, analyzed in vivo data, and helped to edit the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ABS, Ag binding site; AUC, area under the curve; biotin-cHBI^{dansyl}, biotinylated cHBI^{dansyl}; biotin-cHBI^{Pen}, biotin-tagged cHBI^{Pen}; cHBI, covalent heterobivalent inhibitor; FITC-cHBI^{dansyl}, cHBI^{dansyl} synthesized with a fluorescein tag; HBI, heterobivalent inhibitor; ITC, isothiocyanate; Napht, 2-naphthaleneacetic acid; NBS, nucleotide binding site; Pen, benzylpenicilloyl-DNP; Pen-OVA, penicillin-haptenized OVA protein; RBL, rat basophil leukemia; RFG, reactive functional group.

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N,N-dimethylformamide (DMF) (>99.8%), dichloromethane (DCM) (>99.8%), N,N-diisopropylethylamine (DIEA), methanol, hydrazine, piperidine, trifluoroacetic acid (TFA), triisopropylsilane (TIS), tryptamine, 2-naphthaleneacetic acid (Napht), ethylene diamine, biotin, BOC₂O (di-tert-butyl carbonate), 4-(dimethylamino)pyridine (DMAP), succinic anhydride, CS₂, butane diisothiolcyanate (BDI), tetrahydrofuran (THF), triphenylphosphine (TPP), diisopropylazocarboxylate (DIAD), methyl iodine (MeI), 2,4-dinitro-1-fluorobenzene (DNFB), acetonitrile, acetic acid, methanol, carbonate-bicarbonate buffer, Tween 20, indole-3 butyric acid (IBA), biotin and PBS, bicarbonate-carbonate buffer (Bicarb), OVA, streptavidin conjugated to HRP, and p-chloromercuribenzoic acid (PCMB) were purchased from Sigma-Aldrich.

High-binding and nonbinding 96-well plates were purchased from Corning. MEM, penicillin-streptomycin solution, L-glutamine, and Amplex Red ELISA kits were purchased from Life Technologies. BSA was purchased from Gemini Biosciences. Ninety-six-well tissue culture plates were purchased from Falcon. Fmoc-N-amido-dPEG₂ acid (EG₂) and Fmoc-N-amido-dPEG₈ acid (EG₈) were purchased from Quanta BioDesign. FITC was purchased from Toronto Research Chemistry. Anti-dansyl IgE (clone 27–74) and anti-human cyclinA IgE (clone BF683) were purchased from BD Biosciences. Mouse IgG^{Penicillin} (mAb clone P2B9) was purchased from Abcam. Anti-DNP IgE (clone SPE-7) was purchased from Sigma-Aldrich.

Synthesis of inhibitors and ligands. Molecules were synthesized using solid-phase peptide synthesis, as described previously (8), with several modifications. See Supplemental Figs. 2 and 3 for further details.

The following molecules used in this study were analyzed using high-resolution mass spectroscopy using Bruker micrOTOF II mass spectrometer. Note that all molecular weights have +1 mass increase from addition of a proton. cHBI^{dansyl} (C₆₃H₉₄N₈O₁₇S₂) was determined to be 1299.61 Da (1298.62 Da expected); HBI^{dansyl} (C₄₉H₇₁N₅O₁₂S) was determined to be 970.20 Da (969.19 Da expected); cHBI^{Pen} (C₈₅H₁₃₅N₁₇O₂₂S₃) was determined to be 1842.92 Da (1841.91 Da expected); HBI^{BFO} (C₇₈H₁₁₅N₁₃O₂₅S) was determined to be 1666.71 Da (1665.70 Da expected); benzylpenicilloyl-DNP (Pen) (C₄₁H₅₈N₁₀O₁₄S) was determined to be 947.39 Da (946.39 Da expected); biotinylated cHBI^{dansyl} (biotin-cHBI^{dansyl}) (C₈₆H₁₃₃N₁₃O₂₃S₃) was determined to be 1812.87 Da (1811.88 Da expected); biotin-tagged cHBI^{Pen} (biotin-cHBI^{Pen}) (C₁₀₂H₁₆₂N₁₈O₂₇S₄) was determined to be 2201.08 Da (expected 2200.07 Da); cHBI^{dansyl} synthesized with a fluorescein tag (FITC-cHBI^{dansyl}) (C₁₀₄H₁₄₂N₁₂O₃₀S₂) was determined to be 2104.96 Da (expected 2103.94 Da); cHBI^{DNP} (C₆₂H₉₂N₁₀O₂₂S) was determined to be 1361.62 Da (expected 1360.61 Da); dansyl-isothiocyanate (ITC) (C₅₃H₈₈N₈O₁₇S₂) was determined to be 1174.46 Da (expected 1173.45 Da).

In-solution conjugation of cHBI molecules. Before ELISA analysis of cHBI-Ab conjugates, we performed an in-solution conjugation of cHBI molecules and Abs, allowing ITC moieties to react with primary amines on Ab proteins. cHBI^{Pen} or cHBI^{dansyl} at various concentrations were incubated with either IgE^{dansyl} or IgE^{DNP} (as a control) or IgG^{Pen} or BSA (as a control) at 1-μM concentrations for various incubation times in either PBS (pH 7.4) or bicarbonate-carbonate buffer (pH 9.6) at 50 μl total volumes at 37°C. Note that IgG^{Pen} was used due to the lack of commercially available Pen-specific IgE. Additionally, IgG^{Pen} is specific for penicillin G with an intact β-lactam ring, not penicilloyl, which explains the lower affinity it has for penicilloyl-displaying molecules. After reaction, excess cHBI molecules were removed using membrane filtration with 10-kDa 0.5-ml centrifugal filters (Millipore) by washing Abs three times in PBS. Purified Abs were analyzed with a SpectraMax M5 spectrophotometer at 280 nm using an extinction coefficient of 200,000 cm⁻¹ M⁻¹ for IgE^{DNP} and IgE^{dansyl} and 150,000 cm⁻¹ M⁻¹ for IgG^{Pen}.

Synthesis of BSA and OVA drug-hapten conjugates. Protein-hapten conjugates were prepared to sensitize mice for allergen challenges and to trigger in vitro degranulation, as previously described, with some modification (9). Two different haptens, penicillin and dansyl chloride, were used with two different protein carriers, OVA and BSA. OVA conjugates were injected into mice to sensitize animals (see in vivo method section), whereas BSA conjugates were used to trigger degranulation and perform allergen challenges. Dansyl was conjugated to OVA and BSA by dissolving 20 mg of BSA or OVA in 3 ml of bicarbonate-carbonate buffer (pH 9.6) and then adding 20 mg of dansyl chloride that was dissolved in DMF. These compounds reacted under mild stirring over 24 h at 37°C. After reaction, products were passed through a 0.22-μm filter and filtered using a 10-kDa membrane filtration to remove excess dansyl. Protein concentration and hapten labeling was determined using a dansyl extinction coefficient of 3400 cm⁻¹ M⁻¹ at 335 nm, and an extinction coefficient of 43,800 and 30,950 cm⁻¹ M⁻¹ at 280 nm for BSA and OVA, respectively, and a dansyl correction factor of 0.39 to correct for dansyl absorbance at 280 nm. Using

the ratios of absorbance at 335/280 nm, we determined dansyl-BSA to have 18 dansyl per protein and dansyl-OVA to have 12 dansyl per protein.

For penicillin conjugates, we performed a similar addition of hapten to protein, except using 200 mg of penicillin G salt and allowing reaction to take place over 72 h. Pen-protein conjugates were filtered in a similar manner. To determine conjugation efficiency, we used a penmaldate assay from Levine et al. (6) We determined Pen-BSA to have 12 Pen groups per protein, whereas penicillin-haptenized OVA protein (Pen-OVA) had eight Pen groups per protein.

Fluorescence quenching. Briefly, 200 μl of 40 nM dilutions of either IgE^{dansyl} or IgG^{Pen} were placed in a 96-well nonbinding plate. DNP-labeled molecules were then titrated into wells, and fluorescence (excitation: 280, emission: 335) was read using a SpectraMax M5 spectrophotometer. As molecules bound their respective Abs, DNP quenched the fluorescence of tryptophan on Abs. The drop in fluorescence was compared with a PBS control and a control with tryptamine diluted to the same initial fluorescence value as the Ab dilutions.

ELISA. Binding of cHBI molecules to Abs was observed using a direct ELISA. Prior to ELISA test, Abs or BSA were incubated at 100-nM concentrations with biotin-labeled cHBI molecules at various concentrations in PBS at 37°C for 5 h, then unbound cHBI was removed using membrane filtration with a 10-kDa m.w. cutoff (Thermo Fisher Scientific). After filtration, protein concentration was determined by absorbance, using ε = 200,000 cm⁻¹ M⁻¹ for IgE, 150,000 for IgG, and 40,900 for 43,820 for BSA at 280 nm. A total of 100 μl of 2 nM Ab or BSA molecules previously reacted with cHBIs that were labeled with biotin were incubated for 2 h in bicarbonate buffer on a high-binding 96-well plate. Plates were washed with an AquaMax 2000 plate washer to remove unbound Ab. Wells were blocked with a 5% BSA, 0.2% Tween 20 solution in PBS for 1 h, washed, and incubated with a streptavidin conjugated to HRP for 1 h in blocking buffer. Plate was washed again, and an Amplex Red Kit was used to quantify ELISA signal using a SpectraMax M5 spectrophotometer according to manufacturer's instruction.

Flow cytometry. Flow cytometry was performed on rat basophil leukemia (RBL)-2H3 cells using a Guava easyCyte 8HT to demonstrate dansyl cHBI molecule attachment under more physiological conditions. RBL-2H3 cells split at 500,000 cells/ml into a 24-well dish (0.5 ml each) and allowed to attach to plate overnight. The following morning, 0.5 μg of IgE^{DNP} or IgE^{dansyl} was added and allowed to incubate for 24 h. Cells were then washed twice with sterile PBS and incubated with fresh media with FITC-cHBI^{dansyl} between 0 and 1000 nM for 16 h. Cells were then washed again with PBS, given fresh media, and then chilled on ice for 30 min. Cells were washed with PBS and incubated in 1.5% BSA in PBS, scraped, and analyzed.

Tissue culture and degranulation assays. RBL-2H3 cells were passaged every 48–72 h at a 1:3 dilution into fresh RBL-2H3 media. Plates for experiments were prepared at roughly 500,000 cells/ml in either 0.5-ml or 100-μl wells on tissue culture plates. Degranulation assays were performed with some modifications based on a procedure in Handlogten et al. (9) All of these degranulation assays followed this basic procedure (1): RBL cells previously primed with IgE (either from monoclonal sources or mouse sera from mouse sensitization below) were incubated with cHBIs for varying amounts of time (2), cells were washed to remove any unbound or unconjugated cHBIs (3), and allergen was added to stimulate degranulation. Briefly, 50,000 cells were incubated in a 96-well tissue culture plate, and either mixtures of mAbs (with 25% IgE^{dansyl} and 75% orthogonal IgE^{cyclinA}) to a final concentration of 1 μg/ml or dilutions of mouse sera were added for 24 h. Cells were then washed with sterile PBS, and cHBI compounds were added at various dilutions for varying time points. Cells were then washed with tyrodes buffer, and degranulation was triggered using either dansyl-BSA or Pen-BSA as previously described (9). Percent inhibition was calculated by dividing percent degranulation with cHBIs by control without cHBI for the same allergen concentration. For experiments in Fig. 4C, after incubating with inhibitors for 24 h, cells were washed and allowed to incubate in cell culture media between 24 and 72 h before testing degranulation response.

In vivo experiments. BALB/c female mice (7–8 wk) were obtained from Harlan Biosciences (Indianapolis, IN). BALB/c mice were chosen because of their marked increase in IgE-mediated mast cell responses (10). Mice were maintained in pathogen-free conditions, and studies were approved by the Indiana University Institutional Animal Care and Use Committee. All control mice were cohoused with experimental mice.

Ear swelling murine model. Mice were sensitized using haptenized OVA proteins as previously described (8), except using dansyl-OVA or Pen-OVA as the sensitizing agent. Mice were injected by i.p. on days 1 and 7 with 20 μg of haptenized OVA. On day 14, mice were challenged via intradermal injection with 20 μg of hapten-BSA conjugates in one ear with a PBS control in the other ear, and ear swelling was measured and expressed

as a change before and after allergen challenge. Ear tissue was collected 2 h after challenge for histological examination (8). Mice were injected i.v. with cHBI molecules either 16 h or simultaneously with hapten-BSA challenge (i.e., coadministered with hapten-BSA).

Murine anaphylaxis model. For the systemic anaphylaxis model (11), mice were sensitized with 20 μ g of Pen-OVA adsorbed to alum i.p. on day 0 and 7. Subsequently, 100 μ g of Pen-BSA was used to challenge mice i.p. on day 14. Inhibitor (10 nM) or PBS was injected via i.v. 1 h before challenge. Data are combined from two independent experiments ($n = 6$ for PBS treated and $n = 9$ for cHBI treated). No difference was observed between animals treated with 10 nM cHBI^{Pen} and PBS (data not shown).

Ear histological analysis. Ear biopsies from mice were fixed in 10% formalin for 24 h and then transferred to 70% ethanol. Ears were paraffin embedded, and 5- μ m sections of whole ears were stained with H&E. Routine histological techniques were used to paraffin-embed ears, and 5- μ m sections of whole ears were stained with H&E. Quantitative digital morphometric analysis of ear thickness was performed using the application program ImageJ. A minimum of four measurements were analyzed. The same area for each ear was captured, and dermal thickness was calculated in inches per pixel.

Statistics. Statistical significance was determined using a Student *t* test. A *p* value ≤ 0.05 was considered statistically significant. Calculations were performed using the Prism 6.0 software program.

Results

Design of cHBI

In this study, we engineered cHBI (Fig. 1A) to inhibit mast cell degranulation by irreversibly inhibiting the binding interactions between drug hapten-specific Ab and the haptenized drug

molecules, thereby inhibiting drug allergy reactions. The cHBI design has three components: 1) a drug hapten used to target the Ag binding site (ABS), 2) a small-molecule ligand to target the nucleotide binding site (NBS), and 3) reactive functional group (RFG) to form irreversible covalent bonds with the target IgE (Fig. 1A). The NBS is an underused, conserved binding site located proximal to the ABS between the H and L chain of all Igs (Fig. 1B) (8, 12). In our laboratory, we have identified several small-molecule ligands, including indole-3 butyric acid (IBA) and Napht, that specifically bind the NBS with low-micromolar affinities (13–17). The cHBI is designed to first associate bivalently to the allergen-specific IgE via ABS and NBS sites, followed by a covalent reaction with the proximal lysine residues via RFG, irreversibly preventing allergen/IgE interactions (Fig. 1C).

NBS ligand and hapten selection for the cHBIs

To aid in the development of cHBIs as inhibitors for penicillin drug allergies by screening potential ABS and NBS ligands, we first evaluated the inhibitors that lacked the covalently RFG moiety heterobivalent inhibitor (HBI). The HBIs were designed to simultaneously target the NBS and ABS of penicillin-specific IgE (IgE^{Pen}) in a heterobivalent fashion, but without the ability to form covalent bonds, their complexes with IgE eventually dissociated (9). After screening a library of potential NBS-targeting compounds, we selected Napht as the NBS ligand with a 1.8 ± 0.3 μ M K_d for IgE by fluorescence quenching experiments (Fig. 2A).

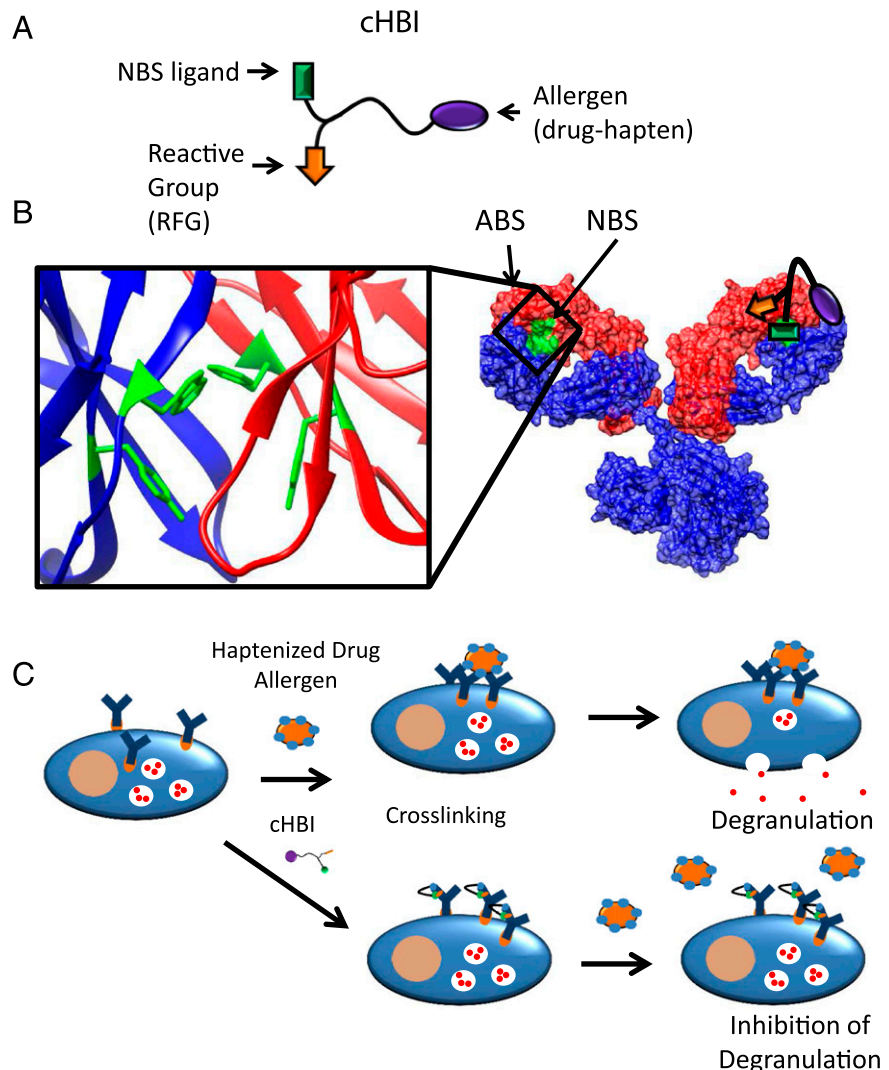


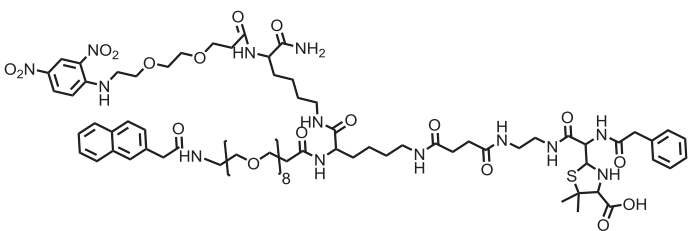
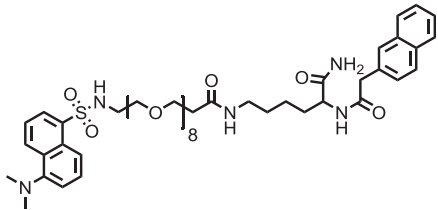
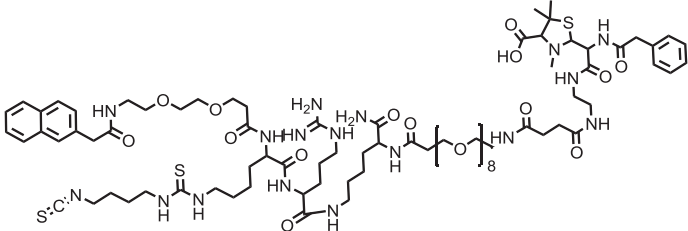
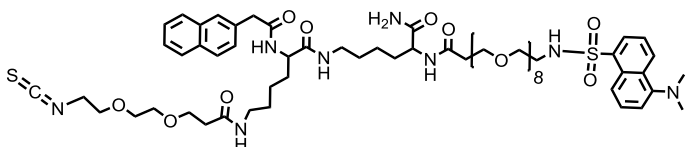
FIGURE 1. cHBI molecular design and mechanism of action. **(A)** Schematic illustration of cHBI structure with its three major components: ABS-targeting ligand (drug hapten), NBS ligand, and chemically RFG to enable irreversible covalent bond formation. **(B)** A crystal structure of an Ig (Protein Data Bank: 1IGY) demonstrating the location of the NBS (with NBS residues colored in green) between the H chain (in blue) and L chain (in red) is shown. Schematic of cHBI binding to the Ab via the NBS and ABS is also shown. **(C)** Schematic illustration of the cHBIs inhibiting allergen-IgE interactions. Without cHBI (as seen in the top), haptenized drug multivalently cross-links drug hapten-specific IgE bound to mast cells, triggering degranulation. In the presence of cHBIs (bottom), the binding of the haptenized drug to the IgE is inhibited, thereby preventing cross-linking and degranulation.



As the ABS-targeting ligand in our design, we incorporated a drug-hapten moiety derived from penicillin G. It is important to note, although frequently described as an allergy to penicillin G, the actual drug hapten, which binds IgE, is a conjugate of penicillin G with a primary amine, forming a Pen group (Fig. 2B). Hence, in our studies, we used Pen to target the ABS of IgE^{Pen}. Because of commercial or otherwise unavailability of IgE^{Pen}, we used a monoclonal penicillin G-specific IgG (IgG^{Pen}) Ab to measure binding constants of the penicillin-based molecules. We first measured the K_d of Pen for IgG^{Pen} to be $20 \pm 4 \mu\text{M}$ by

fluorescence quenching (Fig. 2B). Next, we synthesized heterobivalent molecules that displayed Pen and an NBS ligand (HBI^{Pen}, see *Materials and Methods* for further details on HBI synthesis). The structure of HBI^{Pen} is given in Table I. We determined the binding of HBI^{Pen} to IgG^{Pen} to be $0.96 \pm 0.11 \mu\text{M}$, an over 20-fold improvement over monovalent Pen binding to IgG^{Pen} alone (Fig. 2B). Furthermore, we synthesized an HBI with a similar design using dansyl as the ABS ligand and Napht as the NBS ligand (HBI^{dansyl}; Table I). We then measured the binding affinity of HBI^{dansyl} to an anti-dansyl IgE (IgE^{dansyl}) using fluorescence

Table I. List of compounds synthesized in this study

Compound	Structure	Exact Mass (Da)
HBI ^{Pen}		1665.78
HBI ^{dansyl}		969.20
cHBI ^{Pen}		1841.91
cHBI ^{dansyl}		1298.62

Structures of and molecular weights of heterobivalent molecules for penicillin (HBI^{Pen}) and dansyl (HBI^{dansyl}) and cHBIs for penicillin (cHBI^{Pen}) and dansyl (cHBI^{dansyl}) are shown.

quenching and calculated a nearly 5-fold increase in observed K_d for HBI^{dansyl} (6.4 ± 2.5 nM) over monovalent dansyl (29.9 ± 10 nM) (Fig. 2C). Combined, the HBI^{Pen} and HBI^{dansyl} binding studies demonstrated how heterobivalent binding of the HBIs to the target Abs improves the overall inhibitor avidity while maintaining specificity.

RFG selection

In addition to NBS and ABS ligands, the most critical design factor for the cHBI is the presence of an RFG that can form covalent bonds after cHBI noncovalently binds to the target IgE, thereby permanently inhibiting IgE/allergen interactions through covalent inhibition. One of the major challenges of covalent inhibitors is that the RFG must bind rapidly but only with intended targets and without significant levels of off-target conjugation. We have chosen an amine-reactive chemical moiety, ITC, as the RFG in the cHBIs design (Fig. 2D). ITC compounds are frequently found in nature and are reportedly nontoxic (18–20). ITC groups form thiourea bonds with primary amines, such as lysines, in elevated pH solutions (>9) but react rather slowly under physiological pH (7.4), having an in vivo $t_{1/2}$ of over 50 h (21, 22). Because the ITC functionalities typically have fairly slow reaction kinetics under physiological conditions, the preassociation with the ABS and the NBS sites is necessary to increase the effective molarity of ITC for the proximal lysine residues, which in turn enhances the kinetics and specificity of covalent bond formation. As shown in Fig. 2D, we predict that allergen-specific IgE inhibition via cHBIs is a

three-step process. First, a monovalent binding interaction by either the hapten or NBS ligand forms with the target IgE, followed by the second binding event, which results in the stable heterobivalent cHBI-IgE complex. Finally, because the resulting complex provides an increased effective molarity for the ITC moiety to the lysine located in proximity to the NBS, formation of the covalent bond is expedited. We have assessed the crystal structures of several Abs and observed at least one potential lysine within 2 nm of the lip of the NBS pocket in each case (Supplemental Fig. 1). We envisioned cHBIs armed with these design parameters to provide selective and potent inhibition of target IgE–allergen interactions. Hence, we synthesized cHBIs specific to Pen (cHBI^{Pen}) or dansyl (cHBI^{dansyl}) (Table I) to test our hypothesis (see *Materials and Methods* and Supplemental Figs. 2 and 3 for further detail on chemical synthesis).

cHBIs bind to the target IgE specifically and form irreversible bonds

After synthesizing cHBIs, we evaluated their ability to form covalent bonds specifically with their target IgE. To quantify conjugation of cHBI molecules to the Abs, cHBIs were synthesized with biotin tags, and their conjugation to the target Ab was measured. Specifically, we incubated IgG^{Pen} with a biotin-cHBI^{Pen} for 16 h at 37°, removed unbound biotin-cHBI^{Pen} via membrane filtration, and then measured modified IgG^{Pen} via ELISA (Fig. 3A). The fluorescence increased in a concentration-dependent manner between 1 and 100 nM cHBI^{Pen}, whereas

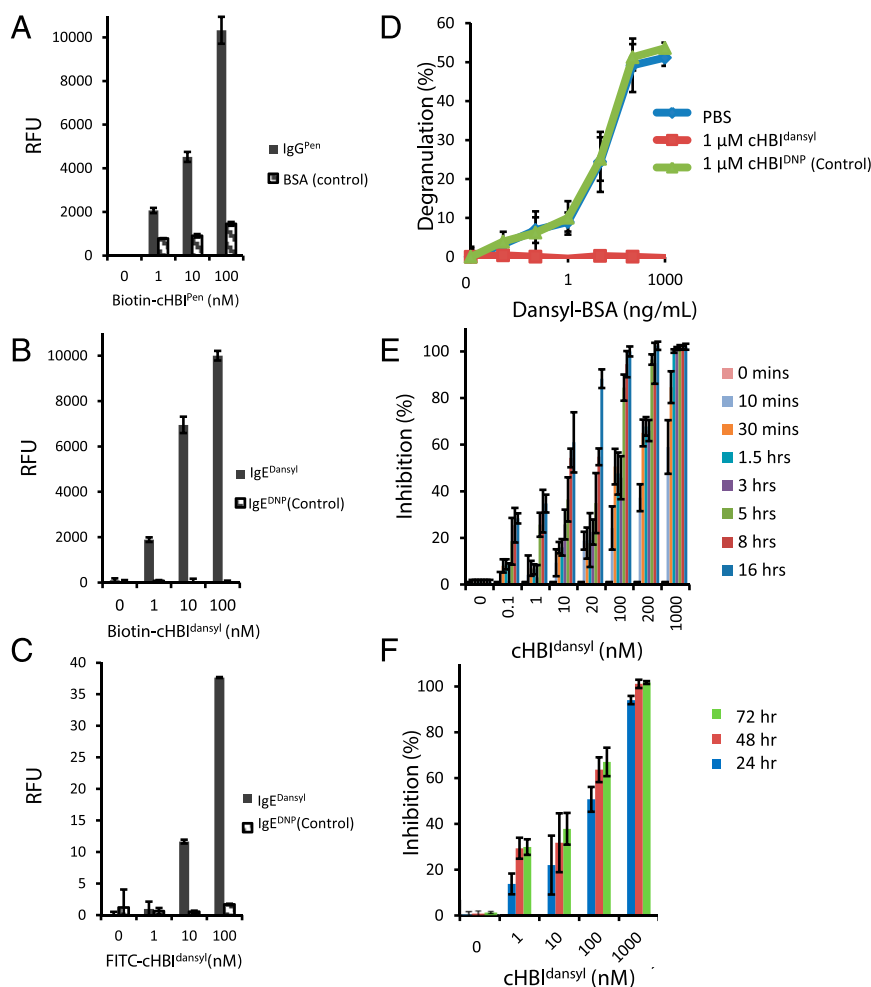


FIGURE 3. cHBI molecules bind and inhibit target IgE in vitro and in cellular assays. **(A)** Biotin-cHBI^{Pen} selectively binds to an IgG^{Pen} (black bars). Only negligible binding was observed with a BSA control (dashed bars). IgG^{Pen} or BSA was incubated for 16 h at 37°C with biotin-cHBI^{Pen} purified with membrane filtration to remove unbound cHBI, and binding was monitored by measuring fluorescence from an Amplex Red substrate after reaction with streptavidin-HRP conjugate in an ELISA. **(B)** Biotin-cHBI^{DNP} binds to IgE^{DNP}. Only negligible binding was observed to IgE^{DNP}, which was used as isotype control (dashed bars). IgE^{DNP} or IgE^{DNP} was incubated for 16 h at 37°C with biotin-cHBI^{DNP} purified with membrane filtration to remove unbound cHBI, and binding was monitored by measuring fluorescence from an Amplex Red substrate after reaction with streptavidin-HRP conjugate in an ELISA. **(C)** In a cellular binding assay, FITC-cHBI^{DNP} selectively bind to the IgE^{DNP} present on the surface of RBL-2H3 cells. RBL-2H3 cells were primed with IgE^{DNP} and then incubated with FITC-cHBI^{DNP}, washed, and analyzed with flow cytometry. As a control, IgE^{DNP}, which targets a different small molecule, was used in place of IgE^{DNP}. All cellular binding experiments were performed on ice. **(D)** RBL cells were primed with IgE^{DNP} and incubated for 5 h with 1 μ M cHBI^{DNP}. Cells were washed to remove unbound inhibitors and were challenged with varying concentrations of dansyl-haptenized BSA (dansyl-BSA). Degranulation was observed using a standard β -hexoamidase assay. Red points indicate cHBI^{DNP} incubated cells. Control experiments were performed with PBS (blue) or cHBI^{DNP} (green) in place of cHBI^{DNP}. **(E)** IgE^{DNP}-sensitized RBL cells were incubated with cHBI^{DNP} at various concentrations and for various durations. Cells were washed to remove unbound inhibitors and were challenged with 100 ng/ml dansyl-BSA. Inhibition of degranulation (inhibition %) when compared with PBS control is shown. **(F)** RBL cells were primed with IgE^{DNP} and then incubated with cHBI^{DNP} at varying concentrations for 16 h. Cells were washed to remove unbound inhibitors, incubated in cell culture media for varying periods of time (0, 24, 48, and 72 h), washed a second time, and then challenged with 100 ng/ml dansyl-BSA. Inhibition of degranulation is shown. Data represent the mean $\pm$ SD of biological triplicate experiments. RFU, relative fluorescence units.

conjugation to a BSA control remained minimal, demonstrating the specificity of cHBI^{Pen} (Fig. 3A). Similar results were observed using dansyl and a biotin-cHBI^{DNP}, Fig. 3B.

Finally, we validated that the specific binding and conjugation of the cHBIs to the IgE can also occur in a relevant cellular assay system in a crude biological mixture such as cell culture media containing FBS (10%). For this, RBL cells were primed with IgE^{DNP} to allow for binding of the IgE to the RBL Fc ϵ RI cell surface receptors. Next, the RBL cells were incubated with FITC-cHBI^{DNP}. Binding of FITC-cHBI^{DNP} to IgE^{DNP} on the cell surface was analyzed with flow cytometry. The data indicated that cHBI^{DNP} molecules bind effectively to IgE^{DNP} on the surfaces of RBL cells in a dose-dependent manner (Fig. 3C).

Negative-control experiments in which RBL-2H3 cells were primed with IgE^{DNP} showed significantly less binding at these concentrations (Fig. 3C, $p < 0.005$). Taken together, these results validate that cHBIs covalently and specifically bind to membrane-bound target IgE in cellular assays.

cHBIs demonstrate inhibition of degranulation in cellular assays

After confirming that cHBI molecules form specific covalent bonds with their target Abs, next, we evaluated the ability of cHBI to inhibit mast cell degranulation in cellular assays. We tested the cHBIs with a modified version of a well-established degranulation assay using RBL cells in which cHBIs are incubated with primed

RBL cells, washed to remove any unconjugated inhibitor, and then challenged with a haptenized BSA protein (dansyl-BSA) (23, 24). As demonstrated in Fig. 3D, when cHBI^{dansyl} (1 μ M) was incubated with IgE^{dansyl}-primed RBL cells, degranulation responses were completely inhibited over a wide range of allergen concentration (dansyl-BSA: 0.01 ng/ml–2 μ g/ml). Similar control experiments were performed in which RBL cells were primed with IgE^{dansyl} and then incubated with cHBI^{DNP} (a cHBI with a different small-molecule specificity) did not yield any inhibition when cells were challenged with dansyl-BSA, verifying the specificity of these inhibitors (Fig. 3D). Additional control experiments were also performed in which cHBI^{dansyl} was synthesized, omitting one or two of the three moieties of the cHBI design (NBS ligand, hapten, or ITC). In these experiments, we observed significant decrease in inhibition of degranulation when any of one the three moieties were absent from the cHBI design (Supplemental Fig. 4A).

We further tested the effects of varying incubation times and concentrations of cHBI^{dansyl} (Fig. 3E). As shown in Fig. 3E, inhibition of degranulation increased with increasing concentrations of cHBI^{dansyl} and with increasing incubation times. At doses near 1000 nM, $>IC_{50}$ was observed in as short as a 10-min incubation time, and $>95\%$ inhibition was observed after a 1.5-h cHBI incubation at the same 1000 nM dose, which we expect to correspond to a clinically feasible milligram scale dose, although further pharmacokinetic data are required to verify this (Fig. 3E) (25). Next, we tested if cHBI demonstrated long-lasting inhibitory effects in cellular assays, given that they were designed as irreversible inhibitors. Primed RBL cells were incubated with cHBI^{dansyl} for 16 h as before, but after washing away the unbound cHBI^{dansyl}, cells were incubated in cell culture media for 24–72 h before being challenged with dansyl-BSA. As demonstrated in Fig. 3F, cHBI^{dansyl} was a potent inhibitor even after 72 h of incubation because of the formation of irreversible covalent bond between cHBI and target IgE.

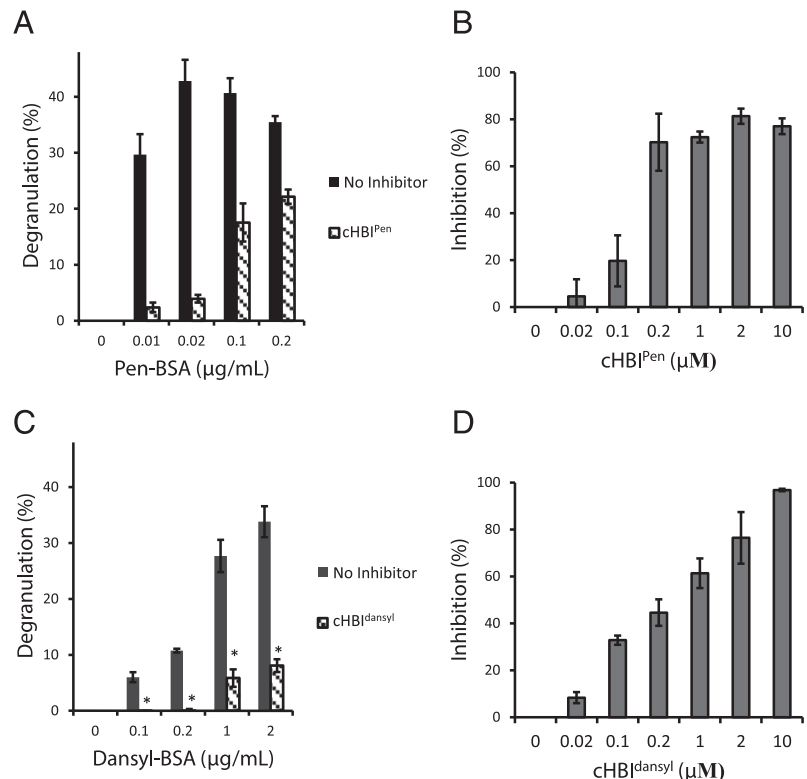
cHBIs inhibit degranulation in RBL cells primed with mouse sera

To further evaluate cHBIs in a more physiologically relevant system, we primed RBL cells with serum from mice challenged with Pen-OVA. Briefly, mice were sensitized with two i.p. injections of 20 μ g of Pen-OVA at 1-wk intervals (on day 1 and day 7) to generate a polyclonal population of IgE Abs specific to Pen (IgE^{Pen}). On day 14, sera were pooled from five mice. Before performing experiments with cHBIs, we first confirmed the presence of IgE^{Pen} in the serum and that this response is IgE mediated (Supplemental Fig. 4B). Next, RBL cells were incubated in a 90/10% mixture of cell culture media to Pen-OVA-sensitized mouse serum overnight, washed, and tested using an RBL cell assay. Cells incubated with cHBI^{Pen} had a significant inhibition of degranulation at all concentrations of Pen-BSA challenge ($p < 10^{-4}$, Fig. 4A). In a separate experiment, RBL cells were primed with Pen-OVA-sensitized serum containing IgE^{Pen}, washed, and then incubated with various concentrations of cHBI^{Pen} and challenged with 200 ng/ml Pen-BSA. As demonstrated by Fig. 4B, cHBI^{Pen} significantly inhibited degranulation at 100 nM cHBI^{Pen} and inhibited over 75% of degranulation responses at 200 nM ($p < 0.01$). One plausible explanation for why cHBI^{Pen} did not completely eliminate degranulation is that IgE specific to other minor determinants (other than Pen) of penicillin G were generated when the mice were sensitized (26). These two experiments were also repeated using dansyl, priming RBL cells with mouse serum sensitized with dansyl-OVA, incubating the cells with cHBI^{dansyl}, and challenging them with dansyl-BSA. Inhibition of degranulation was observed at all concentrations of dansyl-BSA and cHBI^{dansyl} (Fig. 4C, 4D).

cHBIs inhibit degranulation in vivo

After the ex vivo validation of cHBIs, we tested their inhibitory potential in vivo using a mouse allergy model. Mice were sensitized with Pen-OVA on day 1 and 7 to allow for IgE^{Pen} development. On

FIGURE 4. cHBIs inhibit degranulation ex vivo. **(A)** Five BALB/c mice were challenged with Pen-OVA. Their serum was pooled and then used to sensitize RBL-2H3 cells (in a mixture of 10% serum with 90% cell culture media, followed by overnight incubation and then washed). RBL-2H3 cells were then incubated with 500 nM cHBI^{Pen} (black bar) or with a PBS control (hashed bar) in cell culture media overnight. Cells were washed to remove unbound inhibitors and challenged with Pen-BSA at varying concentrations. **(B)** RBL-2H3 cells primed with serum from Pen-OVA-challenged mice were washed and then incubated with varying concentrations of cHBI^{Pen} (black bar) overnight, washed again to remove unbound cHBI, and challenged with 200 ng/ml Pen-BSA. **(C)** Similarly, five BALB/c mice were sensitized with dansyl-OVA. Their serum was pooled and then used to prime RBL-2H3 cells. RBL-2H3 cells were washed and then incubated with 500 nM cHBI^{dansyl} (black bar) or with a PBS control (hashed bar) overnight, washed again to remove unbound inhibitor, and challenged with dansyl-BSA at varying concentrations. **(D)** RBL-2H3 cells sensitized with dansyl-OVA-challenged serum were washed and then incubated with varying concentrations of cHBI^{dansyl} (black bar) overnight, washed again to remove unbound cHBI, and challenged with 2 μ g/ml dansyl-BSA. In all experiments, degranulation was observed with a standard β -hexoamidase assay. Data represent the mean $\pm$ SD of biological triplicate experiments. * $p < 0.0001$.



day 14, mice (five mice per group) were challenged with 20 μg of Pen-BSA in the presence or absence of cHBI^{Pen} via injection into the ear. An additional group of control mice were injected with PBS in place of Pen-BSA. The allergic response was monitored by measuring ear swelling 2 h after challenge. As demonstrated in Fig. 5A, Pen-OVA-sensitized mice that were coadministered with 1 or 10 nmol cHBI^{Pen} during Pen-BSA allergen challenge demonstrated a significant reduction in ear swelling when compared with a control with no cHBI^{Pen} injection ($p < 0.01$, Fig. 5A). Histopathological analysis confirmed an increase in dermal and intradermal tissue thickness for mice challenged without cHBI^{Pen} when compared with mice injected with either 1 or 10 nmol per mouse (Fig. 5B, Supplemental Fig. 4C). Mice were also sensitized with dansyl-OVA in a similar fashion, and we observed a significant decrease in ear swelling using 1 nmol cHBI^{dansyl}, further demonstrating the inhibitory potential of cHBIs ($p < 0.05$, Fig. 5C). This was also confirmed with histological analysis (Fig. 5D, Supplemental Fig. 4C). However, the inhibitory effect of cHBI^{dansyl} did not persist at the 10-nmol dose. We speculate that this is an artifact of our experimental design. We coadministered the cHBI^{dansyl} and dansyl-BSA, mixing them in the same syringe at very high (0.1 mM) concentrations, likely allowing the cHBI to react nonspecifically with dansyl-BSA and further labeling the dansyl-BSA with dansyl groups while depleting the cHBI^{dansyl}. We further speculate that this was not the case with cHBI^{Pen}, as penicillin is significantly less hydrophobic than dansyl, leading to fewer nonspecific interactions.

Next, we tested if cHBIs demonstrated long-lasting inhibitory effects in vivo. Pen-OVA-sensitized mice ($n = 5$) were injected with 1 nmol cHBI^{Pen} and then challenged with Pen-BSA, either immediately or after 16 h. At both time points, mice injected with cHBI^{Pen} retained their resistance to allergic reactions as shown by a significant drop in ear swelling when compared with control mice without the inhibitors ($p < 0.05$, Fig. 5E).

Finally, cHBIs were evaluated with a murine systemic anaphylaxis model (11). Mice were sensitized as before with Pen-OVA, injected via i.v. with 10 nmol cHBI^{Pen}, and then challenged with i.p. administration of 100 μg of Pen-BSA 1 h after cHBI^{Pen} injection, and their temperature monitored with a rectal probe. The change in body temperature for these mice was monitored over the course of 2 h after allergen exposure. The no-inhibitor control group ($n = 6$) experienced a severe drop in body temperature, indicating anaphylaxis, whereas cHBI^{Pen}-injected mice ($n = 9$) had a significantly smaller change in body temperature at 20, 30, 45, 60, 90, and 120 min (Fig. 5F, $p < 0.05$). We calculated the area under the curve (AUC) for the data in Fig. 5G for both cHBI^{Pen}-treated mice and the control group and calculated a significantly smaller absolute value of the AUC for cHBI^{Pen}-treated mice (Fig. 5G, $p < 0.05$). Furthermore, as seen in Fig. 5H, there was a significant drop in plasma IL-6 concentration for cHBI^{Pen}-treated mice compared with the no-inhibitor control group, indicating a reduction in systemic inflammation. Altogether, these results demonstrate that cHBIs can block active allergic responses in vivo and have promising clinical potential.

Discussion

In this study, we described the design, synthesis, and characterization of cHBI, a novel and multifaceted inhibitor of drug-hapten and IgE interactions that has promising prospects as a therapy for drug allergies. cHBIs were rationally designed to irreversibly inhibit the binding interactions between the drug hapten and its specific IgE of the major allergy determinant, thereby effectively and specifically preventing allergic reactions.

We have shown the synthesis and characterization of cHBIs with two distinct specificities: 1) cHBI^{Pen} to inhibit allergic reactions against penicillin G and 2) cHBI^{dansyl} to inhibit allergic reactions to yet another small molecule, dansyl, to demonstrate the broad applicability of the cHBI design to any small-molecule drug allergy. Each chemical moiety in the cHBI design was carefully chosen to facilitate specific inhibition of drug hapten/Ab interaction with minimal off-target effects. In this study, we established that cHBIs specifically and irreversibly bound target Abs while having minimal off-target interactions with nonspecific Abs or other proteins. Importantly, in a physiologically relevant in vitro allergy model, cHBIs irreversibly bound to cell surface-bound IgE, thereby preventing degranulation from RBL cells.

Before we started the experiments, one potential concern was related to sequestration of cHBI by allergen-specific IgG in vivo, making it unavailable to inhibit specific IgE. Because cHBI is designed to recognize the Fab and not discriminate by Fc region, it can target all Ab isotypes and not only the IgE (Figs. 2A, 3A). Our results, however, established that any binding to other isotypes did not diminish anaphylaxis-blocking activity in vitro and in vivo experiments. During in vitro experiments, we demonstrated that cHBIs effectively inhibited degranulation in which RBL cells were primed with serum taken from mice sensitized to either Pen or dansyl (which contained a polyclonal Ab mixture to the drug hapten) and then challenged with drug-haptenized proteins. Although these in vitro results are impressive, the model cell line used (RBL), although useful for reproducibility of experiments across multiple experiments, cannot recapitulate the complexity of mast cell responses in vivo. Rather than use more sophisticated cell culture models, such as bone marrow-derived mast cells, we opted to directly assess cHBI effectiveness using mouse models. We performed an extensive in vivo evaluation of cHBIs using two distinct murine models. These results demonstrated that the concentration of cHBI was sufficient to bind sIgE and prevent allergic responses even when IgG was present. Although these studies were performed exclusively in mice, we expect similar effects in humans because of the conserved nature of IgE and the NBS (9).

Another concern for this study was the fact that we designed cHBIs to target only the well-known major determinant of the penicillin allergy while not taking minor allergy determinants into account. Although literature reports have shown skin prick test reactions to minor penicillin allergy determinants, our results established that a single cHBI can still be effective in mice (27). We believe this is due to the high degree of homology between the major penicillin determinant, penicilloyl, and minor determinants such as penicilloate and penilloate and the high cross-reactivity between β -lactam antibiotics (28, 29). Consequently, all IgE is expected to adopt a moderate binding affinity toward penicilloyl, which results in cHBI^{Pen} achieving a level of inhibition for these Abs. This point is demonstrated in Fig. 2B, in which incorporating penicilloyl in the bivalent design of cHBI^{Pen} improved the anti-penicillin IgG's moderately low affinity of 20 μM by 20-fold to 1 μM , due to avidity. Additionally, covalent conjugation was achieved at nanomolar concentrations of cHBI^{Pen} with the same antipenicillin IgG, suggesting that cHBIs can still covalently bind and inhibit minor determinant-specific IgE that has relatively low affinity (Fig. 3A). Furthermore, it is important to emphasize that the versatile cHBI platform can accommodate any number of potential ABS ligands in separate designs, as demonstrated by DNP and dansyl examples, and cHBIs specific for the minor determinant sIgE can be easily generated.

This study is particularly significant because it is, to our knowledge, (1) the first successful design of an inhibitor that inhibits specific IgE/allergen interactions and (2) a potential

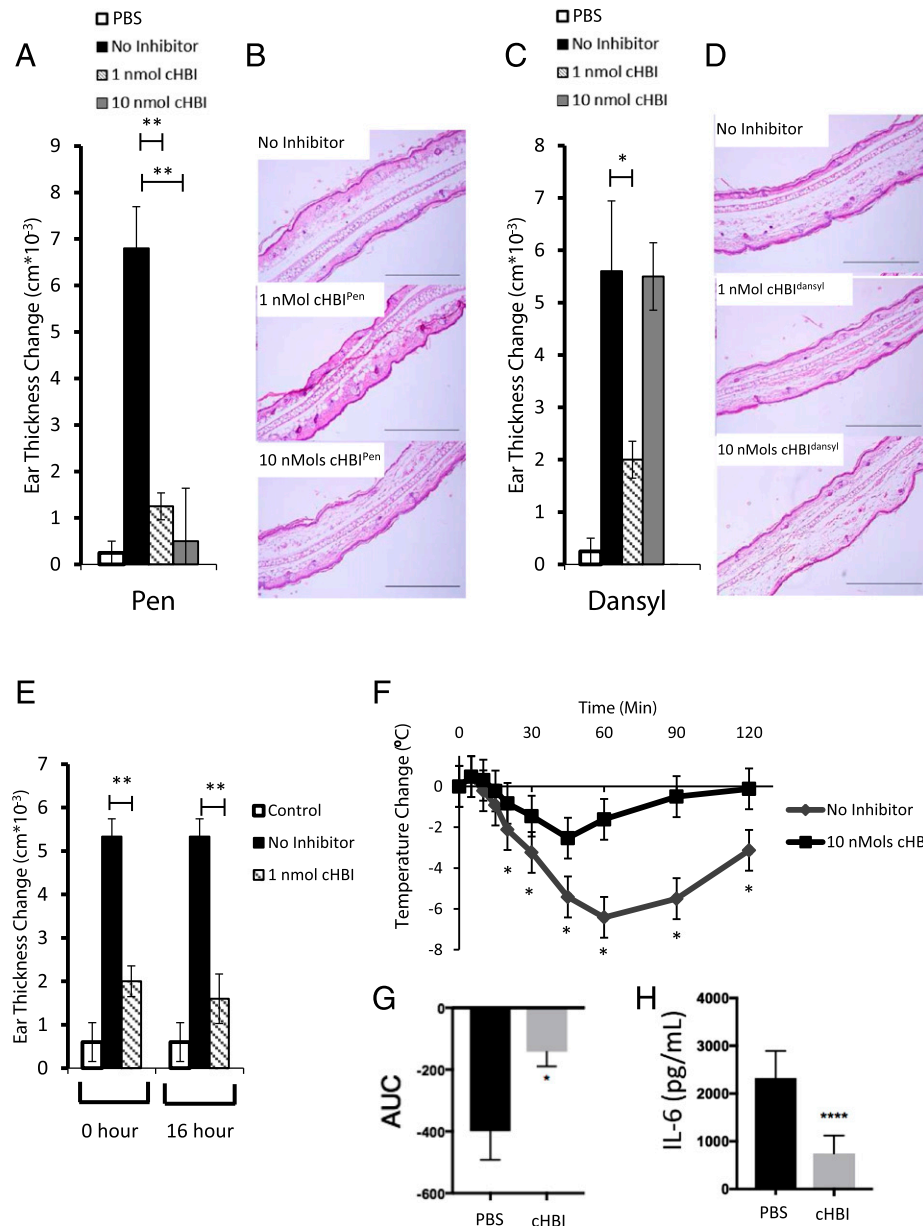


FIGURE 5. cHBIs inhibit allergic responses in vivo. **(A)** BALB/c mice were sensitized twice with two i.p. injections of Pen-OVA at 1-wk intervals on day 1 and day 7. On day 14, mice were challenged with intradermal injections of Pen-BSA in the presence cHBI^{Pen} (1 nM, $n = 4$ or 10 nM, $n = 4$) or absence of cHBI^{Pen} (no inhibitor, $n = 5$). The control experiment included a PBS treatment ($n = 4$) in place of Pen-OVA. **(B)** Immunohistological images of ear tissue in the presence or absence of cHBI^{Pen} is shown. Scale bar, 200 μ m. **(C)** Similarly, BALB/c mice were sensitized twice with two i.p. injections of dansyl-OVA on days 1 and 7. On day 14, mice were challenged with intradermal injections of dansyl-BSA in the presence cHBI^{Dansyl} (1 nM, $n = 5$ or 10 nM, $n = 4$) or absence of cHBI^{Dansyl} (no inhibitor, $n = 5$) as a control. **(D)** Immunohistological images of ear tissue in the presence or absence of cHBI^{Dansyl} is shown. Scale bar, 200 μ m. **(E)** BALB/c mice were sensitized twice with two i.p. injections of Pen-OVA at 1-wk intervals on day 1 and day 7. On day 14, mice were injected with cHBI^{Pen} (1 nM) or PBS (no inhibitor) control. The control experiment also included a PBS treatment in place of Pen-OVA. Mice were then challenged with 20 μ g of Pen-BSA either immediately (0 h) or 16 h after cHBI injection, and ear swelling was measured. Data represent mean of four or five mice per group. **(F)** cHBIs were tested in a systemic anaphylaxis model. Mice were similarly sensitized to Pen-OVA and then injected with either 10 nmol of cHBI^{Pen} ($n = 9$) or PBS (no inhibitor) ($n = 6$) and then challenged with an i.p. injection of 20 μ g of Pen-BSA 1 h later. Temperature drop was then measured over the course of 120 min. **(G)** The AUC for data in part F was calculated. **(H)** IL-6 was measured from mice analyzed with the systemic anaphylaxis model. After 120 min, mice were sacrificed, and their plasma was analyzed via ELISA for the presence of IL-6. Error bars indicate $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

therapeutic for a clinically relevant drug allergy. Currently, the only FDA-approved IgE inhibitor is the mAb omalizumab, which is panspecific for all IgE and has only been approved for use in chronic asthmatic conditions and chronic idiopathic urticaria (30, 31). Additionally, omalizumab has been linked to a trend in cardiac toxicity and increased risk of parasitic infections, presumably due to its nonspecific targeting of all IgE (32, 33). We anticipate

that the cHBIs, because of their allergen-selective design and controlled covalent inhibition, have the potential to overcome these issues in the clinic.

We anticipate that cHBI^{Pen} will be long lasting, as its inhibitory characteristics (because of irreversible covalent bonds) should persist throughout the course of a mast cell or basophil IgE lifetime, which would be ideal for clinical use. However, longer-term

use studies would be necessary to confirm this, as inhibition up to only 16 h was tested in vivo. Additionally, there is a concern that cHBIs could affect the normal immunological functions of Igs after long-term use. Although we show little off-target Ab binding, it would be crucial to track long-term, off-target cHBI binding in vivo for future studies. Another potential usage for cHBIs would be for aiding in drug desensitization. Rapid drug desensitization is an effective technique for reducing allergic reactions to a drug in the short term (34, 35). cHBIs could be used to improve the effectiveness of this technique by reducing the allergic side effects. Finally, this study obtained similarly promising results with a different small-molecule hapten, dansyl, which demonstrates that the cHBI design can be used to develop inhibitors to any small-molecule drug compound such as other penicillin derivatives, sulfa drugs, or chemotherapeutics.

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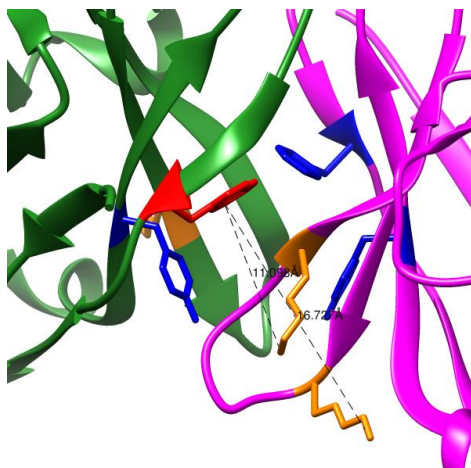
Disclosures

The authors have no financial conflicts of interest.

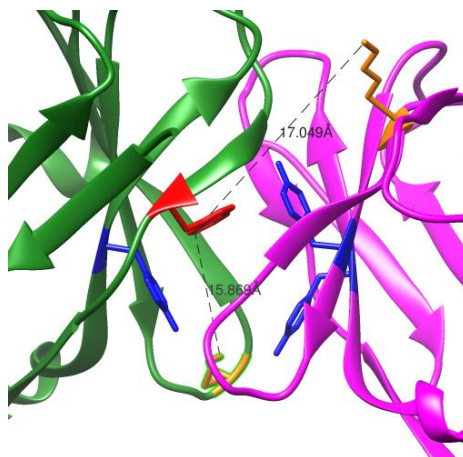
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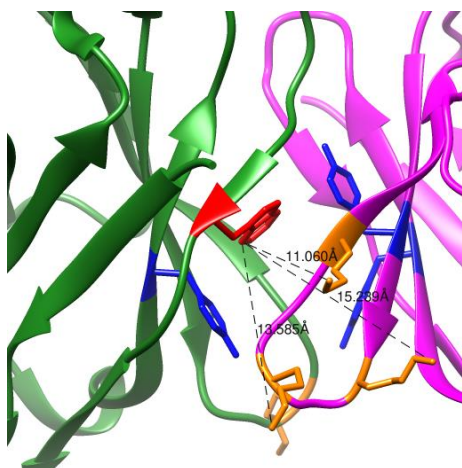
A.



B.



C.



D.

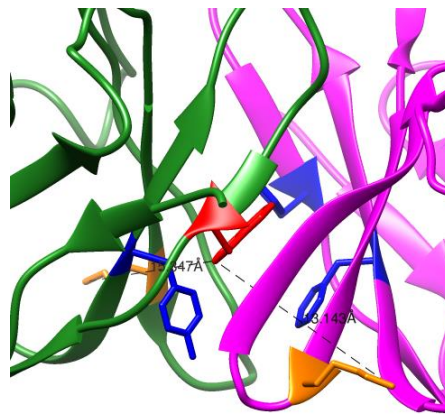


Figure S-1. Antibodies contain reactive lysines near their NBS. The crystal structures of several antibodies (with protein databank numbers in parenthesis) are shown with lysines that could potentially react with chBI molecules located within 0.2 nm of the NBS pocket are colored in orange. The light chain is shown in purple and the heavy chain in green. The following antibodies are shown: (A) Rituximab (PDB: 2OSL) (B) Cetuximab (PDB: 5HYQ), (C) Trastuzumab (PDB: 4HKZ), (D) Anti-DNP IgE SPE-7 (PDB: 1O AQ).

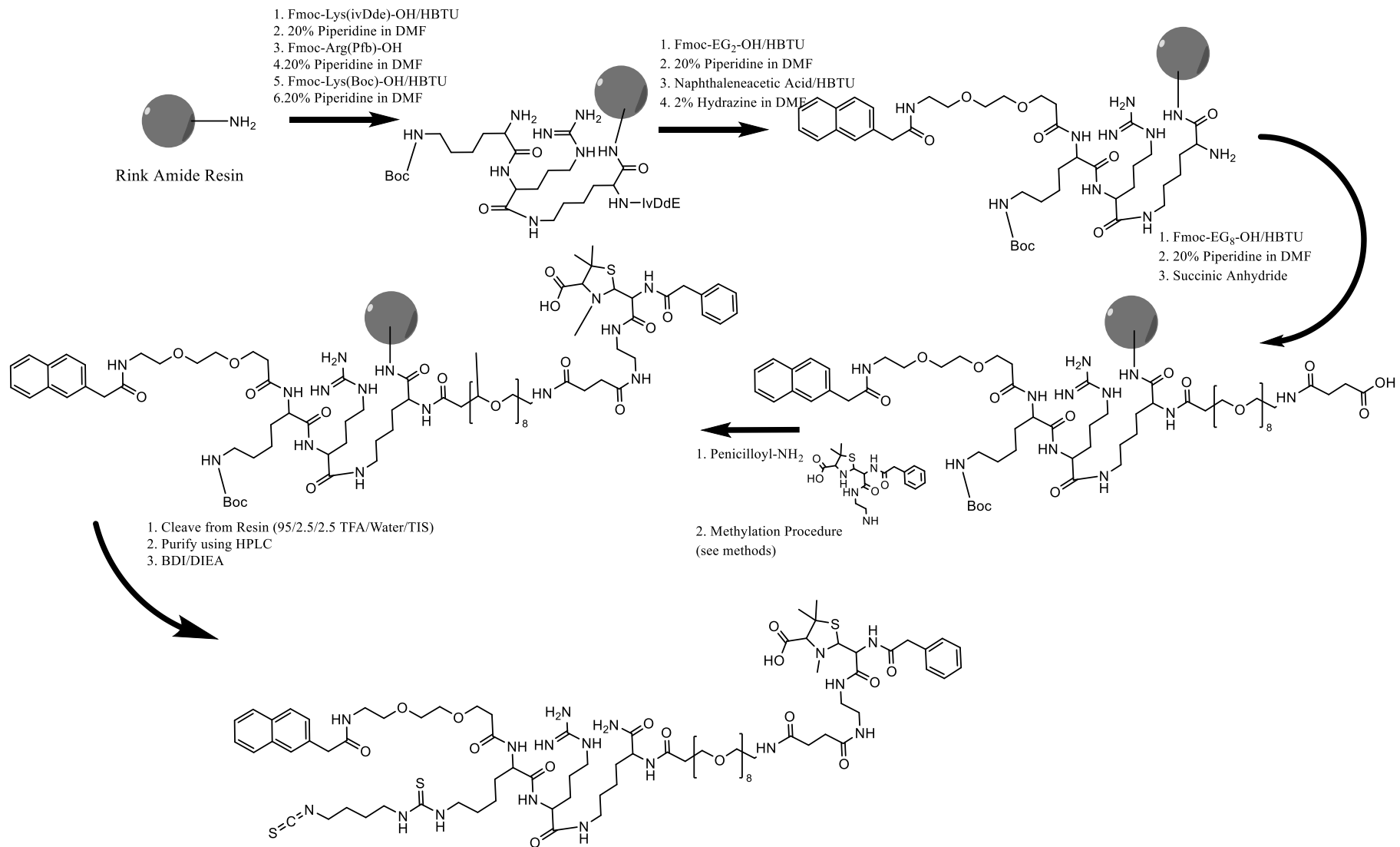


Figure S-2. Synthetic Scheme of cHBI^{Pen}

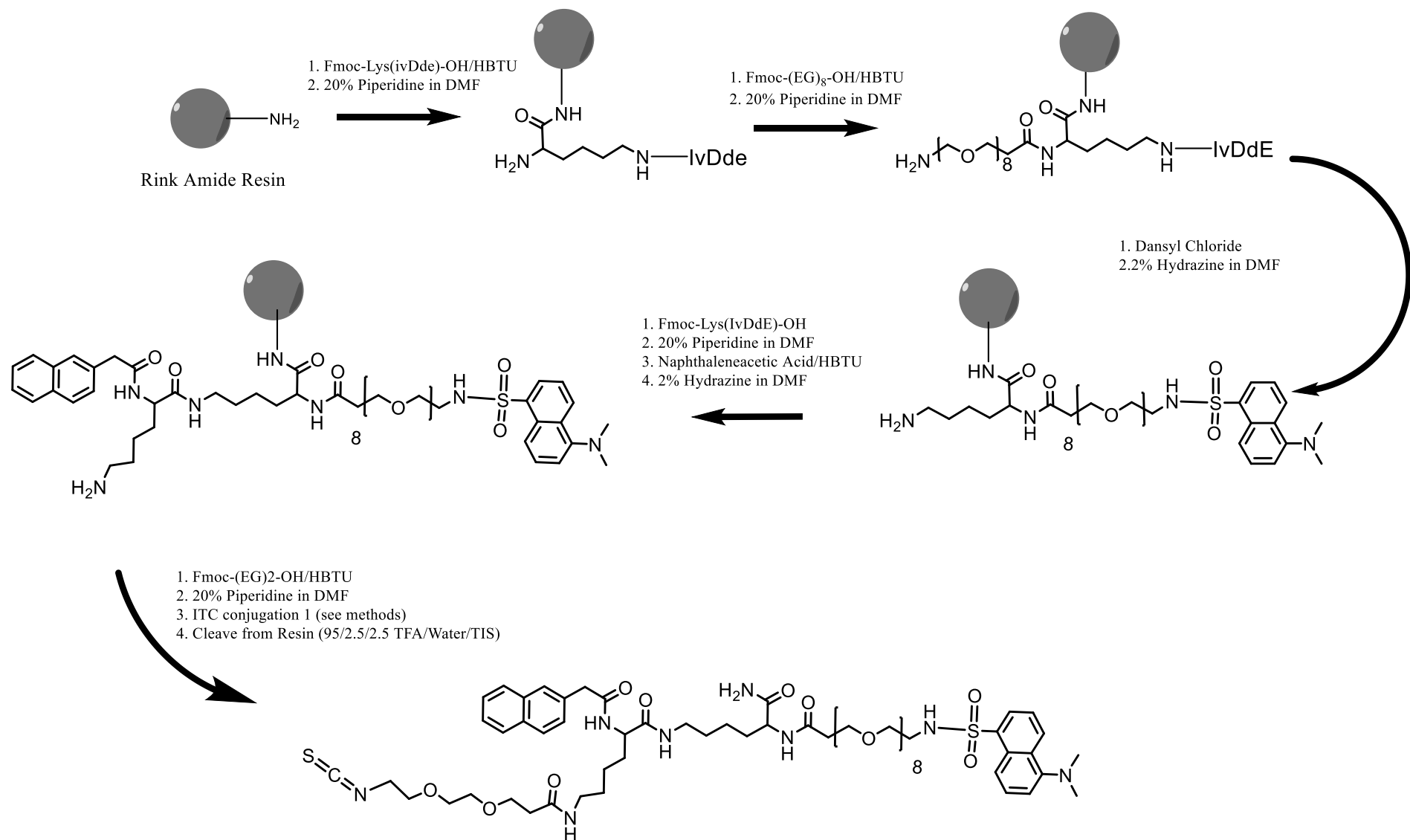


Figure S-3. Synthetic Scheme of cHBI^{dansyl}

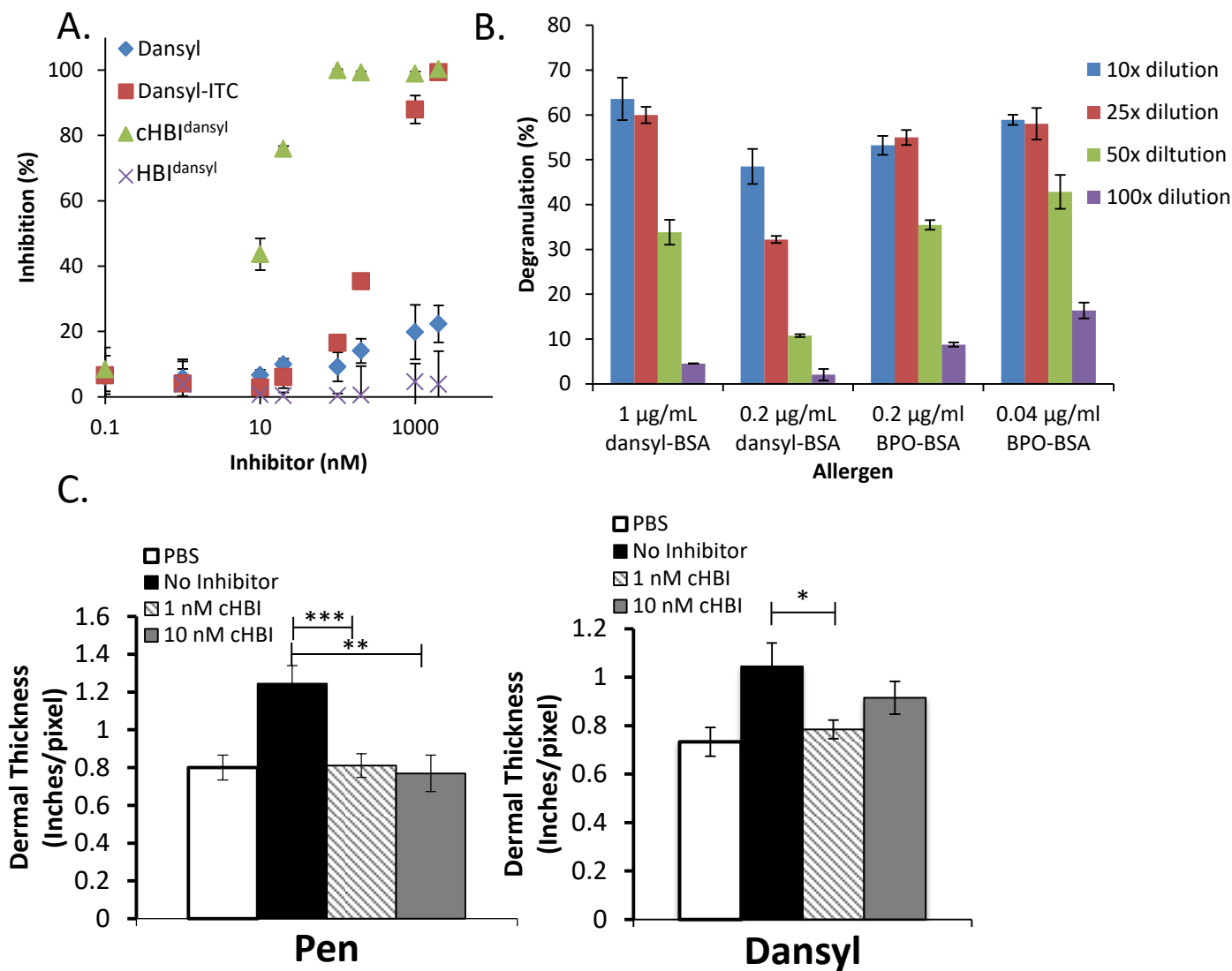


Figure S-4. Further Characterization of cHBI compounds. (A) cHBIs require an ABS ligand, a NBS ligand and ITC for optimal inhibition. Inhibitor molecules were synthesized by omitting either a NBS ligand but retaining ABS ligand and ITC (Dansyl-ITC), by omitting the ITC moiety but retaining the ABS and NBS ligands (HBI^{Dansyl}), or by omitting both NBS ligand and ITC and retaining only the ABS ligand (Dansyl). The inhibitory effects of these molecules were compared to cHBI^{Dansyl}. Briefly, RBL-2H3 cells were primed with IgE^{Dansyl} and incubated with each of these molecules for 16 hours. Cells were then washed and degranulation was triggered with 1000 ng/mL dansyl-BSA. (B) Determination of optimal dilution of mouse sensitized serum for degranulation assays. RBL degranulation assays were used to determine the optimal dilution of mouse serum taken from Pen and dansyl sensitized mice for analysis of cHBIs. Briefly, mice (N=5) were sensitized via 20 µg injections of Pen-OVA on day 1 and day 7. On day 14, mice were sacrificed and their serum pooled. The Pen-OVA sensitized serum was then diluted at various ratios and incubated with RBL cells for 16 hours. Cells were then challenged with two different concentrations of Pen-BSA. The strongest degranulation responses were seen at a 1:25 dilution of mouse serum in media, which was used in studies from figure 5A and 5B. In a similar experiment, mice (N=5) were injected with 20 µg of dansyl-OVA on day 1 and day 7 and serum pooled and tested with RBL-2H3 cells. The highest degranulation responses were seen at a 1:10 dilution of serum with cell culture media. This dilution was used for experiments seen in figure 5C and 5D. (C) Morphometric analysis of *in vivo* histology images. Ear biopsies from mouse experiment from figure 6A-D were fixed and stained with H&E. Ear thickness was measured digitally in ImageJ application using 100X images of stained slides. The same area in each slide image was analyzed with a minimum of 4 dermal measurements and calculated as inches of dermal tissue per pixel. Data represents the mean ± SEM of five mice from two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001