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

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Abstract

Drug allergies occur when hapten-like drug metabolites conjugated to serum proteins, through their interactions with specific immunoglobulin E (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 antibody 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 (RBL) 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 seperate 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 antibody and inhibited degranulation in cellular degranulation assays using RBL 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.

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Author Contributions: PED synthesized and characterized all compounds used in this study, performed most in vitro experiments and analyzed this data, made all figure and wrote the manuscript. BB and PED conceived of the idea for the study, designed the molecules and designed all experiments. TK helped in experimental design and manuscript writing and editing. BK (Baksun Kim) performed affinity measurements for cHBI compounds and aided with in vitro studies. BK (Byunghee Koh) and AAQ performed all in vivo murine experiments and performed analysis. MK designed in vivo experiments, analyzed in vivo data and helped to edit the manuscript.

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INTRODUCTION

Immediate immunoglobulin E (IgE) mediated hypersensitivity reactions caused by drugs (drug allergies) are a type of adverse drug reaction (ADR) that afflicts over 2 million people per year in the US and can trigger severe and life-threating 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, here 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 crosslinking of drug-hapten specific IgE, which are present on the surfaces of mast cells and basophils. These crosslinking 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 crosslinking 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 inhibit 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, HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate), Fmoc-Lys(IvDde)-OH, Fmoc-Arg(pfb)-OH, 10 kDa 0.5 mL centrifugal filters, and BSA were purchased from EMD Millipore.

DMF (N,N-Dimethylformamide) (>99.8%), DCM (dichloromethane) (>99.8%), DIEA (N,N-Diisopropylethylamine), methanol, hydrazine, piperidine, TFA (trifluoroacetic acid), TIS (triisopropylsilane), Tryptamine, 2-Naphthaleneacetic Acid, ethylene diamine, biotin, BOC_2O (Di-tert-butyl carbonate), DMAP (4-(Dimethylamino)pyridine), Succinic anhydride, CS_2 (Carbon disulfide), BDI (butane diisthiolcyanate), THF (Tetrahydrafuran), TPP (triphenylphosphine), DIAD (diisopropylazocarboxylate), MeI (methyl iodine), DNFB (2,4-Dinitro-1-fluorobenzene), acetonitrile, acetic acid, methanol, carbonate-bicarbonate buffer,

Tween 20, IBA (Indole-3-butyric acid),Biotin and PBS (phosphate buffered saline), Bicarbonate-carbonate buffer (Bicarb), OVA (ovalbumin), Step-HRP (streptavidin conjugated to HRP), PCMB (p-chloromercuribenzoic acid) were purchased from Sigma Aldrich.

High Binding and Non-Binding 96 well plates were purchased from Corning. Minimum Essential Media, Penicillin-Strep solution, L-glutamine, and Amplex Red ELISA kits were purchased from Life Technologies. Bovine Serum Albumin was purchased from Gemini Biosciences. 96 well Tissue Culture plates were purchased from Falcon. EG_2 (Fmoc-N-amido-$dPEG_2$-acid) and EG_8 (Fmoc-N-amido-$dPEG_8$-acid) were purchased from Quanta biodesign. FITC (Fluorescein Isothiocyanate) 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 $IgG^{Penicillin}$ (monoclonal antibody 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 figure S-2 and S-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. $cHBI^{dansyl}$ ($C_{63}H_{94}N_8O_{17}S_2$) was determined to be 1299.61 Da (1298.62 Da expected); HBI^{dansyl} ($C_{49}H_{71}N_5O_{12}S$) was determined to be 970.20 Da (969.19 Da expected); $cHBI^{Pen}$ ($C_{85}H_{135}N_{17}O_{22}S_3$)was determined to be 1842.92 Da (1841.91 Da expected); HBI^{BPO} ($C_{78}H_{115}N_{13}O_{25}S$) was determined to be 1666.71 Da (1665.70 Da expected); Pen ($C_{41}H_{58}N_{10}O_{14}S$) was determined to be 947.39 Da (946.39 Da expected); Biotin-$cHBI^{dansyl}$ ($C_{86}H_{133}N_{13}O_{23}S_3$) was determined to be 1812.87 Da (1811.88 Da expected); Biotin-$cHBI^{Pen}$ ($C_{102}H_{162}N_{18}O_{27}S_4$) was determined to be 2201.08 Da (expected 2200.07 Da); FITC-$cHBI^{dansyl}$ ($C_{104}H_{142}N_{12}O_{30}S_2$) was determined to be 2104.96 Da (expected 2103.94 Da) $cHBI^{DNP}$ ($C_{62}H_{92}N_{10}O_{22}S$) was determined to be 1361.62 Da (expected 1360.61 Da); Dansyl-ITC ($C_{53}H_{88}N_8O_{17}S_2$) was determined to be 1174.46 Da (expected 1173.45 Da).

In Solution Conjugation of cHBI Molecules

Before ELISA analysis of cHBI-antibody conjugates, we performed an in-solution-conjugation of cHBI molecules and antibodies allowing ITC moieties to react with primary amines on antibody 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 37ºC. Note that IgG^{Pen} was used due to the lack of commercially avaliable Pen specific IgE. Additionally, IgG^{Pen} is specific for Penicillin G with an intact beta-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 antibodies three times in PBS. Purified antibodies 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 in order 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) while 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 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 hours at 37ºC. 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 43800 and 30950 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 hours. Pen-protein conjugates were filtered in a similar manner. In order 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 while Pen-OVA had 8 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 non-binding plate. DNP labeled molecules were then titrated into wells and fluorescence (Ex. 280, Em. 335) was read using a SpectraMax M5 spectrophotometer. As molecules bound their respective antibodies, DNP quenched the fluorescence of tryptophan on antibodies. The drop in fluorescence was compared to a PBS control and a control with tryptamine diluted to the same initial fluorescence value as the antibody dilutions.

ELISA Assay

Binding of cHBI molecules to antibodies was observed using a direct ELISA. Prior to ELISA test, antibodies or BSA were incubated at 100 nM concentrations with biotin labeled cHBI molecules at various concentrations in PBS at 37ºC for 5 hours, then unbound cHBI was removed using membrane filtration with a 10 kDa molecular weight cutoff (Thermo Scientific). After filtration, protein concentration was determined by absorbance, using ε= 200,000 cm^{-1} M^{-1} for IgE and 150,000 for IgG and 40,900 for 43,820 for BSA at 280 nm. 100 μL of 2 nM antibody or BSA molecules previously reacted with cHBIs that were labeled with biotin were incubated for 2 hours in bicarbonate buffer on a high binding 96 well plate. Plates were washed with an AquaMax 2000 plate-washer to remove unbound antibody. Wells were blocked with a 5% BSA, 0.2% Tween 20 solution in PBS for 1 hour, washed and

incubated with an streptavidin conjugated to HRP for 1 hour 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 RBL-2H3 cells using a Guava easyCyte 8HT in order to demonstrate dansyl cHBI molecule attachment under more physiological conditions. RBL-2H3 cells split at 500,000 cells per mL into a 24 well dish (0.5 mL each) and allowed to attach to plate overnight. Following morning, 0.5 μg of IgE^{DNP} or IgE^{dansyl} was added and allowed to incubate for 24 hours. Cells were then washed twice with sterile PBS, and incubated with fresh media with FITC- $cHBI^{dansyl}$ between 0-1000 nM for 16 hours. Cells were then washed again with PBS and given fresh media, then chilled on ice for 30 minutes. 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 every 48-72 hours at a 1:3 dilution into fresh RBL-2H3 media. Plates for experiments were prepared at roughly 500,000 cells per 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) allergen was added to stimulate degranulation. Briefly, 50,000 cells were incubated in a 96 well tissue culture plate and either mixtures of monoclonal antibodies (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 hours. 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 cHBI's by control without cHBI for same allergen concentration. For experiments in figure 4C, after incubating with inhibitors for 24 hours, cells were washed and allowed to incubate in cell culture media between 24-72 hours 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 due to 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 co-housed 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 by i.v. with cHBI molecules either, 16 hours or simultaneously with hapten-BSA challenge (i.e. co-administered with hapten-BSA).

Murine Anaphylaxis Model

For the systemic anaphylaxis model,(11) mice were sensitized with 20 μg Pen-OVA adsorbed to alum i.p. on day 0 and 7. Subsequently, 100 μg Pen-BSA was used to challenge mice i.p. on day 14. Inhibitor (10nM) or PBS was injected via i.v one hour before challenge. Data is combined from two independent experiment (n = 6 for PBS treated and n = 9 for cHBI treated). No difference was observed between animals treated with 10nM of $cHBI^{Pen}$ 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-μm sections of whole ears were stained with H&E. Routine histological techniques were used to paraffin embed ears, and 5-μm 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 4 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's t test. A p value ≤ 0.05 was considered statistically significant. Calculations were performed using the Prism 6.0 software program.

RESULTS

Design of Covalent Heterobivalent Inhibitor (cHBI)

In this study, we engineered cHBI (Figure 1A) to inhibit mast cell degranulation by irreversibly inhibiting the binding interactions between drug-hapten specific antibody and the haptenized-drug molecules, thereby inhibiting drug allergy reactions. The cHBI design has three components: i) a drug-hapten used to target the antigen binding site (ABS), ii) a small molecule ligand to target the nucleotide binding site (NBS), and iii) reactive functional group (RFG) to form irreversible covalent bonds with the target IgE (Figure 1A). The NBS is an underutilized conserved binding site located proximal to the ABS between the heavy and light chain of all immunoglobulins (Figure 1B).(8, 12) In our laboratory, we have identified several small molecule ligands, including indole-3 butyric acid (IBA) and 2-naphteleneacetic acid (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 (Figure 1C).

NBS ligand and Hapten selection for the cHBIs

To aid in the development cHBIs as inhibitors for penicillin drug allergies by screening potential ABS and NBS ligands, we first evaluated the inhibitors that lacked the covalently reactive RFG moiety (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 2-naphteleneacetic acid (Napht) as the NBS ligand with a 1.8±0.3 μM K_d for IgE by fluorescence quenching experiments (Figure 2A).

As the ABS targeting ligand in our design, we incorporated a drug-hapten moiety derived from penicillin G. It is important to note while 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 benzylpenicilloyl (Pen) group (Figure 2B). Hence, in our studies we used Pen to target the ABS of IgE^{Pen}. Due to commercial or otherwise unavailability of IgE^{Pen}, we used a monoclonal penicillin G specific IgG (IgG^{Pen}) antibody to measure binding constants of the penicillin-based molecules. We first measured the K_d of Pen for IgG^{Pen} to be 20±4 μM by fluorescence quenching (Figure 2B). Next, we synthesized heterobivalent molecules that displayed Pen and an NBS ligand (HBI^{Pen}, see methods for further details on HBI synthesis). The structure of HBI^{Pen} is given in Table 1. We determined the binding of HBI^{Pen} to IgG^{Pen} to be 0.96±0.11 μM, an over 20 fold improvement over monovalent Pen binding to IgG^{Pen} alone (Figure 2B). Furthermore, we synthesized a HBI with a similar design using dansyl as the ABS ligand and Napth as the NBS ligand (HBI^{dansyl}; Table 1). We then measured the binding affinity of HBI^{dansyl} to an anti-dansyl IgE (IgE^{dansyl}) using fluorescence 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) (Figure 2C). Combined, the HBI^{Pen} and HBI^{Dansyl} binding studies demonstrated how heterobivalent binding of the HBIs to the target antibodies improves the overall inhibitor avidity, while maintaining specificity.

Reactive Functional Group (RFG) selection

In addition to NBS and ABS ligands, the most critical design factor for the cHBI is the presence of a RFG that can form covalent bonds after cHBI non-covalently 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, isothiocynate (ITC), as the RFG in the cHBIs design (Figure 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* half-life of over 50 hours.(21, 22) Since the ITC functionalities typically have fairly slow reaction kinetics under physiological conditions, the pre-association 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 Figure 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 antibodies and observed at least one potential lysine within 2 nm of the lip of the NBS pocket in each case (Figure S-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 1) to test our hypothesis (see methods and supplementary figure 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. In order to quantify conjugation of cHBI molecules to the antibodies, cHBIs were synthesized with biotin tags and their conjugation to the target antibody was measured. Specifically, we incubated IgG^{Pen} with a biotin tagged $cHBI^{Pen}$ (Biotin-$cHBI^{Pen}$) for 16 hours at 37º, removed unbound Biotin-$cHBI^{Pen}$ via membrane filtration and then measured modified IgG^{Pen} via ELISA (Figure 3A). The fluorescence increased in a concentration dependent manner between 1 nM and 100 nM of $cHBI^{Pen}$ while conjugation to a BSA control remained minimal, demonstrating the specificity of $cHBI^{Pen}$ (Figure 3A). Similar results were observed using dansyl and a biotinylated $cHBI^{dansyl}$ (Biotin-$cHBI^{dansyl}$, Figure 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 fetal bovine serum (10%). For this, rat basophilic leukemia (RBL) cells were primed with IgE^{dansyl} to allow for binding of the IgE to the RBL FcεRI cell surface receptors. Next, the RBL cells were incubated with FITC-$cHBI^{dansyl}$ ($cHBI^{dansyl}$ synthesized with a fluorescein tag). Binding of FITC-$cHBI^{dansyl}$ to IgE^{dansyl} on the cell surface was analyzed with flow cytometry. The data indicated that $cHBI^{dansyl}$ molecules bind effectively to IgE^{dansyl} on the surfaces of RBL cells in a dose dependent manner (Figure 3C). Negative control experiments where RBL-2H3 cells were primed with IgE^{DNP} showed significantly less binding at these concentrations (Figure 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 antibodies, 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 where 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 figure 3D, when $cHBI^{dansyl}$ (1 μM) 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 μg/mL). Similar control experiments were performed where 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 (Figure 3D). Additional control experiments were also performed where $cHBI^{dansyl}$ 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 (Figure S-4A).

We further tested the effects of varying incubation times and concentrations of $cHBI^{dansyl}$ (Figure 3E). As shown in Figure 3E, inhibition of degranulation increased with increasing concentrations of $cHBI^{dansyl}$ and with increasing incubation times. At doses near 1000 nM, >50% inhibition was observed in as short as a 10 minute incubation time and >95% inhibition was observed after a 1.5 hour cHBI incubation at the same 1000 nM dose, which we expect to correspond to a clinically feasible milligram scale dose, although further pharmacokinetic data is required to verify this (Figure 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 hours as before, but after washing away the unbound $cHBI^{dansyl}$, cells were incubated in cell culture media for 24-72 hours before being challenged with dansyl-BSA. As demonstrated in figure 3F, $cHBI^{dansyl}$ was a potent inhibitor even after 72 hours of incubation, due to the formation of irreversible covalent bond between cHBI and target IgE.

cHBIs Inhibit Degranulation in RBL cells primed with Mouse Sera

In order to further evaluate cHBIs in a more physiologically relevant system, we primed RBL cells with serum from mice challenged with penicillin haptenized ovalbumin protein (Pen-OVA). Briefly, mice were sensitized with two i.p. injections of 20 μg Pen-OVA at one-week intervals (on day 1 and day 7) in order to generate a polyclonal population of IgE antibodies 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 (Figure S-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 ($p < 10^{-4}$, Figure 4A). In a separate experiment, RBL cells were primed with Pen-OVA sensitized serum containing IgE^{Pen}, washed and then incubated with varying concentrations of $cHBI^{Pen}$ and challenged with 200 ng/mL Pen-BSA. As demonstrated by figure 4B, $cHBI^{Pen}$ significantly inhibited degranulation at 100 nM of $cHBI^{Pen}$ 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 benzyl penicilloyl) 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} (Figure 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 day 14, mice (5 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 hours after challenge. As demonstrated in figure 5A, Pen-OVA sensitized mice that were co-administered with 1 nmol or 10 nmol of cHBI^{Pen} during Pen-BSA allergen challenge demonstrated a significant reduction in ear swelling when compared to a control with no cHBI^{Pen} injection($p<0.01$, Figure 5A). Histopathological analysis confirmed an increase in dermal and intradermal tissue thickness for mice challenged without cHBI^{Pen} when compared to mice injected with either 1 or 10 nmol per mouse (Figure 5B, Figure S-4C). Mice were also sensitized with dansyl-OVA in a similar fashion, and we observed a significant decrease in ear swelling using 1 nmols of cHBI^{dansyl} further demonstrating the inhibitory potential of cHBIs ($p<0.05$, Figure 5C). This was also confirmed with histological analysis (Figure 5D, Figure S-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 non-specifically 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 non-specific interactions.

Next, we tested if cHBIs demonstrated long lasting inhibitory effects *in vivo*. Pen-OVA sensitized mice (N=5) were injected with 1 nmol of cHBI^{Pen} and then challenged with Pen-BSA, either immediately or after 16 hours. 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 to control mice without the inhibitors ($p <0.05$, Figure 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 nMols of cHBI^{Pen}, then challenged with i.p. administration of 100μg of Pen-BSA 1 hour 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 two hours after allergen exposure. The no inhibitor control group (N=6) experienced a severe drop in body temperature, indicating anaphylaxis, while cHBI^{Pen} injected mice (N=9) had a significantly smaller change in body temperature at 20, 30, 45, 60, 90 and 120 mins (Figure 5F, $p<0.05$). We calculated the area under the curve (AUC) for the data in figure 6F for both cHBI^{Pen} treated mice and the control group and calculated a significantly smaller absolute value of AUC for cHBI^{Pen} treated mice (Figure 5G, $p<0.05$). Furthermore, as seen in Figure 5H, there was a significant drop in plasma IL-6 concentration for cHBI^{Pen} treated mice compared to 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 prospect 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: i) $cHBI^{Pen}$ to inhibit allergic reactions against penicillin G, and ii) $cHBI^{dansyl}$ to inhibit allergic reactions to a yet another small molecule, dansyl, in order 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/antibody interaction with minimal off target effects. Here, we established that cHBIs specifically and irreversibly bound target antibodies while having minimal off target interactions with non-specific antibodies 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. Since cHBI is designed to recognize the Fab and not discriminate by Fc region, it can target all antibody isotypes, and not only the IgE (Figure 2A and 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 where RBL cells were primed with serum taken from mice sensitized to either Pen or dansyl (which contained a polyclonal antibody 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), while 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. While these studies were performed exclusively in mice, we expect similar effects in humans due to 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 SPT 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 beta-lactam antibotics.(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 antibodies. This point is demonstrated in figure 2B, where incorporating penicilloyl in the bivalent design of HBI^{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 anti-penicillin IgG, suggesting that cHBIs can still covalently bind and inhibit minor determinant-specific IgE that has relatively low affinity (Figure 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 (1) the first successful design of an inhibitor that inhibits specific IgE/allergen interactions and (2) a potential therapeutic for a clinically relevant drug allergy. Currently, the only FDA approved IgE inhibitor is the monoclonal antibody omalizumab, which is pan-specific 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 non-specific targeting of all IgE.(32, 33) We anticipate that the cHBIs, due to 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 (due to 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 hours was tested *in vivo*. Additionally, there is a concern that cHBIs could affect the normal immunological functions of immunoglobulins after long term use. While we show little off target antibody 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 utilized 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Key Points:

- cHBIs display a drug metabolite, an antibody targeting moiety and a reactive group
- cHBI specifically target drug allergy IgE and prevent drug induced degranulation
- cHBIs to penicillin were synthesized and prevent penicillin allergies in vivo.

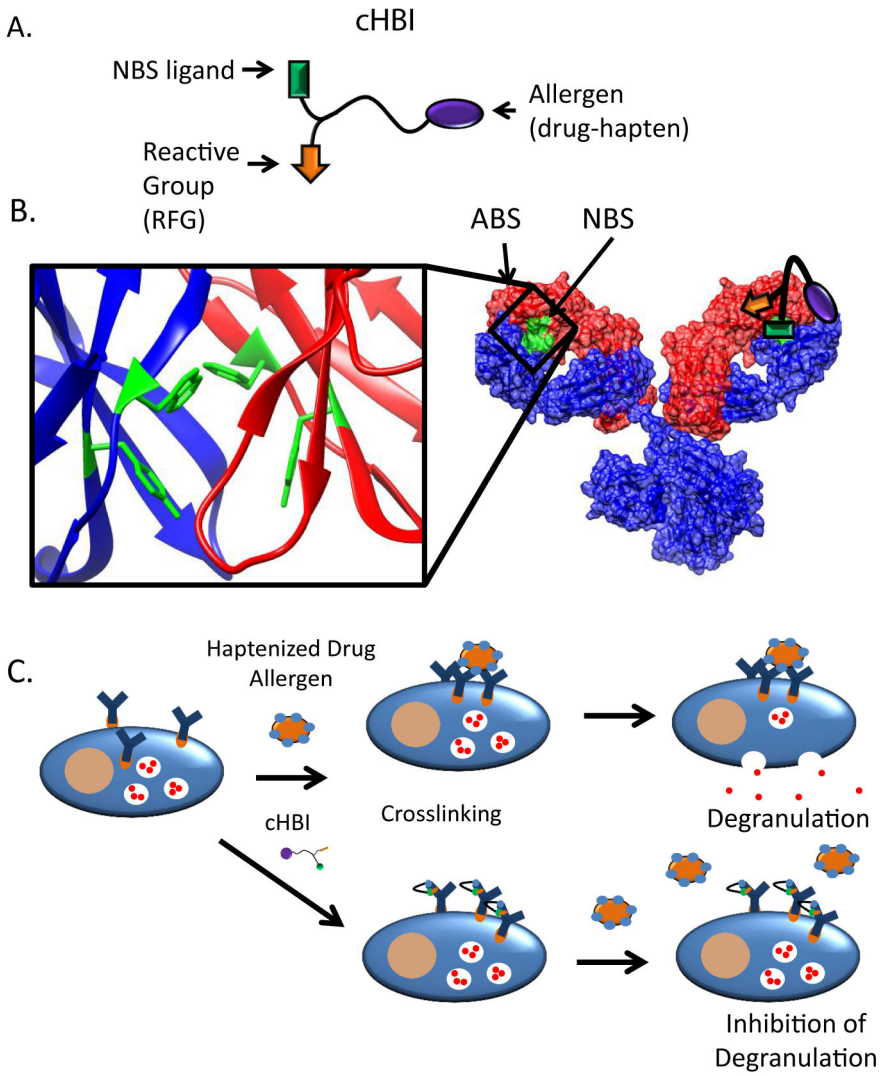


Figure 1.

Covalent Heterobivalent Inhibitor (cHBI) molecular design and mechanism of action. (A) Schematic illustration of cHBI structure with its three major components: Antigen binding site (ABS) targeting ligand (drug-hapten), nucleotide binding site (NBS) ligand, and chemically reactive functional group (RFG) to enable irreversible covalent bond formation (B) A crystal structure of an immunoglobulin (PDB: 1IGY) demonstrating the location of the NBS (with NBS residues colored in green) between the heavy chain (in blue) and light chain (in red) is shown. Schematic of cHBI binding to the antibody 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 crosslinks 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 crosslinking and degranulation.

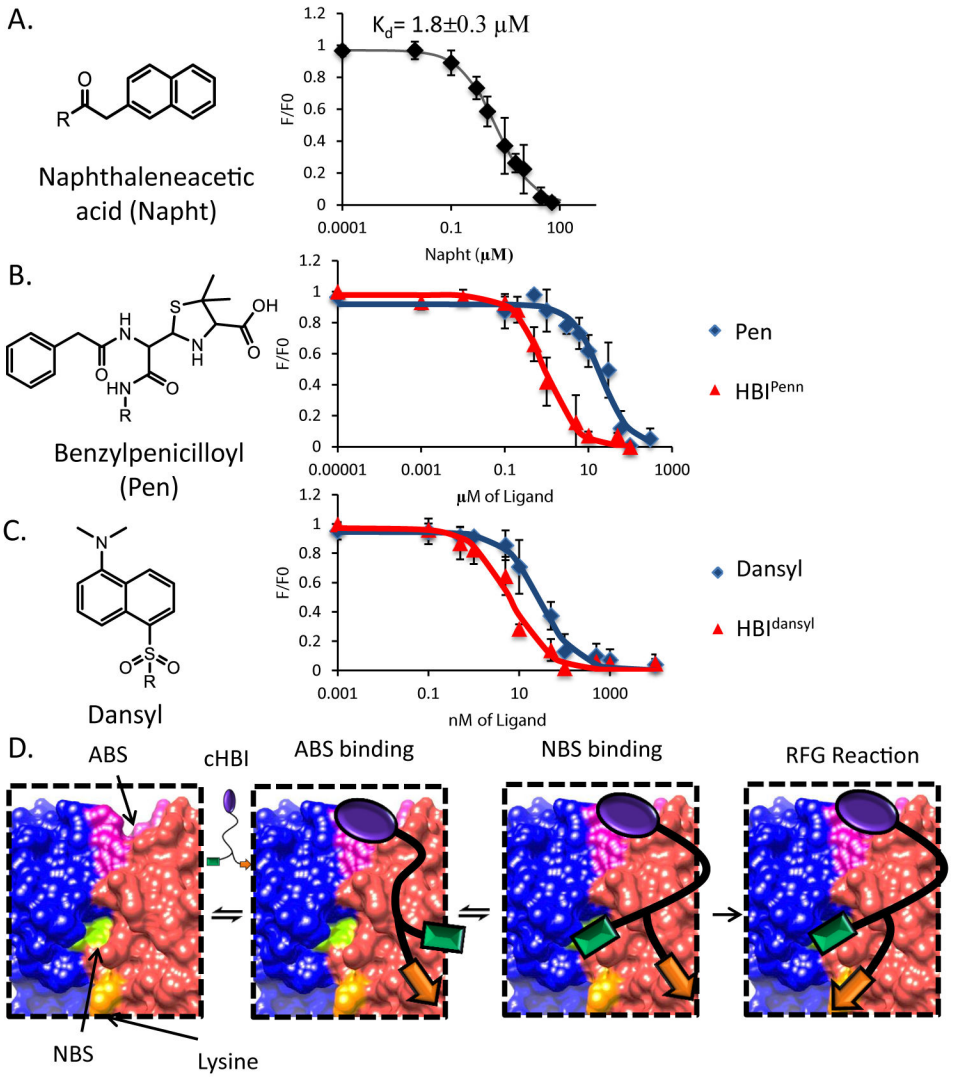


Figure 2.

Chemical Design of Covalent Heterobivalent Inhibitors (cHBI) and IgE-binding studies. (A) Structure of Naphthaleneacetic acid (Napht) as an NBS ligand is shown. The binding of Napht to IgE antibody was determined by fluorescence quenching experiments (Kd = 1.8 µM). (B) Structure of Benzylpenicilloyl (Pen), the most common penicillin hapten, is shown. The binding of Pen to IgG^{Pen} and the binding of the heterobivalent inhibitor engineered to inhibit Pen- IgGPen interactions (HBI^{Pen}) are shown. Binding was measured by fluorescence quenching experiments (Kd(Pen)= 20±4 µM, Kd(HBI^{Pen})= 0.96±0.11 µM) (C) Structure of dansyl and binding of both monovalent dansyl (dansyl) and the engineered heterobivalent dansyl (HBI^{dansyl}) to IgEdansyl is shown. Binding was observed through florescence quenching experiments (Kd(dansyl)= 29.9±10 nM, Kd(HBI^{dansyl})= 6.4±2.5 nM). Error bars represent ±SD of technical triplicate experiments. (D) Crystal structure of a typical antibody binding pocket with cartoon depicting the cHBI covalent binding to antibodies. First, cHBIs bind hetero-bivalently to the target antibody via its ABS and NBS

targeting domain with enhanced avidity. Next, cHBIs react covalently with the lysine side chains proximal to the NBS via its RFG (the ITC moiety) forming an irreversible bond.

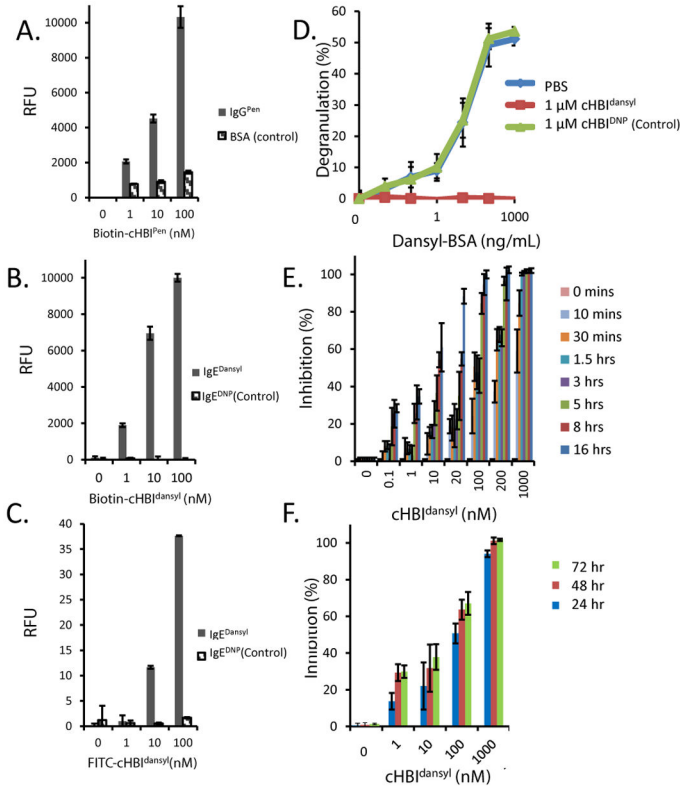


Figure 3.

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 hours at 37ºC 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 assay. (B) Biotin-cHBI^{dansyl} binds to IgE^{dansyl}. Only negligible binding was observed to IgEDNP which was used as isotype control (dashed bars). IgE^{dansyl} or IgE^{DNP} was incubated for 16 hours at 37ºC with Biotin-cHBI^{dansyl}, 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 assay. (C) In a cellular binding assay, FITC-cHBI^{dansyl} selectively bind to the IgE^{dansyl} present on the surface of RBL-2H3 cells. RBL-2H3 cells were primed with IgE^{dansyl} and then incubated with FITC-cHBI^{dansyl}, washed and analyzed with flow cytometry. As a control, IgEDNP, which targets a different small molecule, was used in place of IgE^{dansyl}. All cellular binding experiments were performed on ice. RFU- Relative Fluorescence Units. (D) RBL cells were primed with IgE^{dansyl}, and incubated for 5 hours with 1μM of cHBI^{dansyl}. 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 beta-hexoamidase assay. Red points indicate cHBI^{dansyl} incubated cells. Control experiments were performed with PBS (blue) or cHBI^{DNP} (green) in place of cHBI^{dansyl}. (E) IgE^{dansyl} sensitized RBL cells were incubated with cHBI^{dansyl} 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 to PBS control is shown. (F) RBL cells were primed with IgE^{dansyl} and then incubated with $cHBI^{dansyl}$ at varying concentrations for 16 hours. 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 ± SD of biological triplicate experiments.

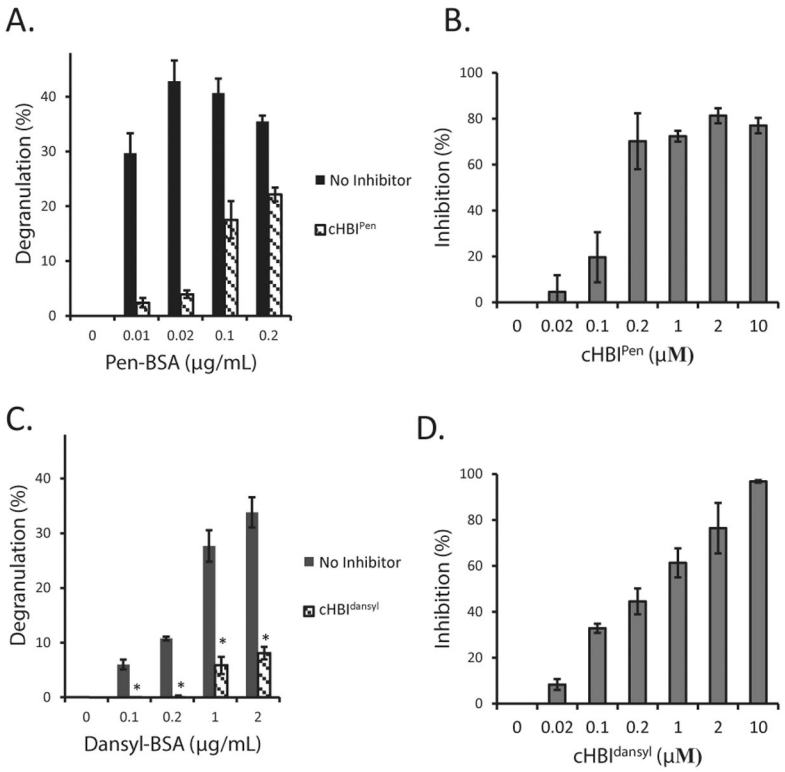


Figure 4.

cHBIs inhibit degranulation ex vivo. (A) 5 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, overnight incubation then washed). RBL-2H3 cells were then incubated with 500 nM of $cHBI^{Pen}$ (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, 5 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 of $cHBI^{dansyl}$ (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 µg/mL dansyl-BSA. In all experiments, degranulation was observed with a standard beta-hexoamidase assay. Data represent the mean ± SD of biological triplicate experiments. *indicates $p<0.0001$

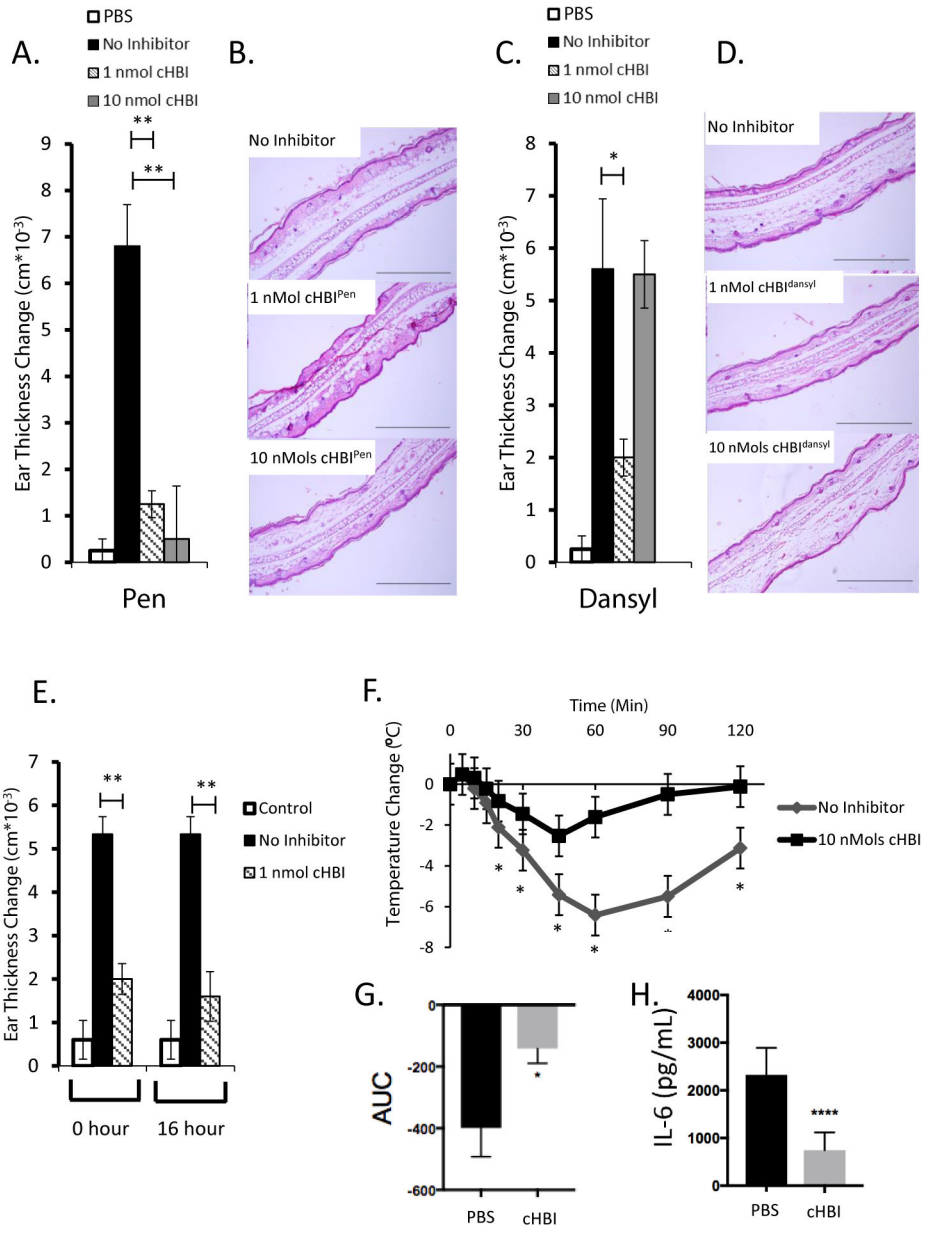


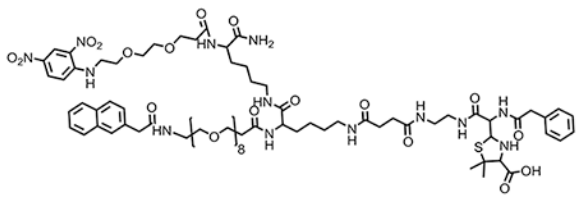
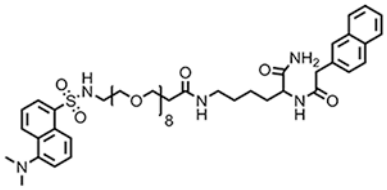
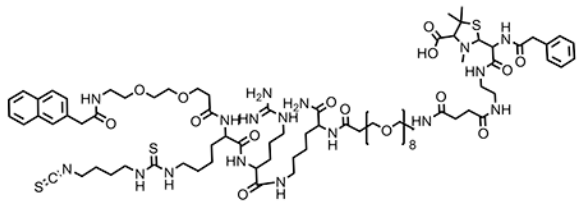
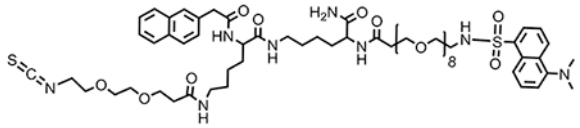
Figure 5.

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 days 14, mice were challenged with intradermal injections Pen-BSA in the presence cHBI^{Pen} (1nM, 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; the scale bar represents 200 μm. (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 cHBIdansyl (1nM, N=5, or 10 nM, N=4) or absence of cHBIdansyl (no inhibitor, N=5) as a control. (D) Immunohistological images of ear tissue in the presence or absence of

cHBI^{dansyl} is shown; the scale bar represents 200 μm. (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 days 14, mice were injected with cHBI^{Pen} (1nM) or PBS (no inhibitor) control. The control experiment also included a PBS treatment in place of Pen-OVA. Mice were then challenged with 20 μg of Pen-BSA either immediately (0 hr) or 16 hours after cHBI injection and ear swelling was measured. Data represents mean of 4 or 5 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 nMols of cHBI^{Pen} (N=9) or PBS (no inhibitor) (N=6) and then challenged with an i.p. injection of 20 μg Pen-BSA one hour later. Temperature drop was then measured over the course of 120 minutes. (G) The area under the curve (AUC) for data in part F was calculated. (H) IL-6 was measured from mice analyzed with the systemic anaphylaxis model. After 120 minutes, mice were sacrificed and their plasma was analyzed via ELISA for the presence of IL-6. Error bars indicate ± SEM * $p<0.05$; ** $p<0.01$, ****$p<0.0001$

Table 1.

List of Compounds Synthesized in this Study- Structures of and molecular weights of heterobivalent molecules for penicillin (HBI^{Pen}) and dansyl (HBI^{dansyl}), and covalent heterobivalent inhibitors for penicillin ($cHBI^{Pen}$) and dansyl ($cHBI^{dansyl}$) are shown.

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