Allosteric Noncompetitive Small Molecule Selective Inhibitors of CD45 Tyrosine Phosphatase Suppress T-Cell Receptor Signals and Inflammation In Vivo

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ABSTRACT

CD45 is a receptor-like member of the protein tyrosine phosphatase (PTP) family. We screened in silico for small molecules binding at a predicted allosteric pocket unique to the CD45 intracellular domain, and validated inhibitors by in vitro phosphatase assays. Compound 211 exhibited a CD45 IC50 value of 200 nM and had >100-fold selectivity over six related PTPs. The relevance of the allosteric pocket was verified through site-directed mutagenesis. Compound 211 has a noncompetitive mechanism of action, and it is extremely effective at preventing dephosphorylation of substrate Lck phosphotyrosine (pY)-505 versus preventing dephosphorylation of Lck pY-393. In cultured primary T cells, compound 211 prevents T-cell receptor–mediated activation of Lck, Zap-70, and mitogen-activated protein kinase, and interleukin-2 production. In a delayed-type hypersensitivity reaction in vivo, compound 211 abolished inflammation. This work demonstrates a novel approach to develop effective allosteric inhibitors that can be expanded to target the corresponding allosteric domains of other receptor PTPs.

Introduction

CD45 is a transmembrane receptor–like protein tyrosine phosphatase (PTP), expressed on cells of hematopoietic origin. The function of CD45 is to regulate signal transduction and T-cell activation in response to antigen stimulation at the T-cell receptor (TCR) (Justement, 1997). CD45 exists as a variably glycosylated extracellular domain with no known physiologic ligand (Streuli et al., 1987), and a cytoplasmic portion with tandem D1 and D2 domains (Glover and Tracey, 2000). D1 is the catalytically active domain, because it harbors the cysteine residue involved in the enzymatic reaction that hydrolyzes phosphate from tyrosine residues. D2 is catalytically inactive (Streuli et al., 1990).

CD45 regulates the phosphorylation of the Src-family kinases such as Lck and Fyn, as well as ζ-immunoreceptor tyrosine-based activation motif of the CD3 component of the TCR. In T cells, proper activation of Lck causes rapid Ca2+ fluxes and the phosphorylation of Zap-70 and Erk, which then stimulate the production of interleukin-2 (IL-2). These are standard readouts for T-cell activation and CD45 activity (Thomas and Brown, 1999; Alexander, 2000). CD45-deficient T cells have diminished proliferation and cytokine production in response to TCR stimulation (Pingel and Thomas, 1989). Furthermore, mutations in the human CD45 gene have been associated with autoimmune diseases, such as severe combined immunodeficiency and multiple sclerosis (Jacobsen et al., 2000; Kung et al., 2000). All of these events implicate CD45 as playing a key role in lymphocyte development and activation (Trowbridge and Thomas, 1994).

Full activation of Lck requires the dephosphorylation of an inhibitory phosphotyrosine (pY)-505. Dephosphorylation of Lck pY-505 by CD45 facilitates Lck autophosphorylation at an activating tyrosine, Lck phosphotyrosine 393 (pY-393). The activating Lck pY-393 is also under CD45 regulation. Thus, CD45 essentially functions as a rheostat to keep Lck in a “primed” state, ready for activation (Hermiston et al., 2003) but avoiding sustained hyperactivation (D’Oro et al., 1996).

Drug inhibitors of CD45 would disrupt the rheostat and could cause immunosuppression. Competitive CD45 inhibitors that block substrates from docking (Beers et al., 1997) and substrate analogs that poison the catalytic pocket have been...
reported (Urbanek et al., 2001), with an in vitro IC50 value of approximately 0.2 \( \mu \)M and activity in antiproliferative assays. One competitive inhibitor (a benzimidazole derivative) had effects on histamine release from rat peritoneal mast cells (Hamaguchi et al., 2001). Unfortunately, these CD45 inhibitors are not clinically useful. One general problem with competitive inhibitors is their poor selectivity because many phosphatases share substrates and/or the structure of the catalytic pocket (Blaskovich, 2009) and, because they usually are charged, this prevents efficient entry into cells.

As an alternative, allosteric inhibitors of CD45 could have different mechanisms of action and potentially higher selectivity (Hardy and Wells, 2004) if the allosteric docking site is a structure unique for CD45. We sought to develop selective inhibitors of CD45 that bind to an allosteric intracellular site.

D1 is the catalytically active domain, whereas D2 is catalytically inactive but contributes to the overall secondary structure of intracellular CD45 (Streuli et al., 1990; Glover and Tracey, 2000). The D2 domain regulates the activity of the D1 domain positively and negatively (Felberg and Johnson, 1998), and can affect the signals downstream of the TCR (Kashio et al., 1998; Wang et al., 1999; Greer et al., 2001). D1 and D2 are connected by a "linker" domain that is unstructured but stabilizes a D1–D2 interface that forms a network of hydrogen bonds, hydrophobic interactions, and salt bridges (Nam et al., 2005).

We hypothesized that a molecule docking near the linker or D1–D2 interface could act as an allosteric CD45 phosphatase modulator. Here, we report the design, optimization, and characterization of selective allosteric inhibitors of CD45 that are active in vitro, ex vivo, and in vivo. We propose that the approach may be expanded to develop inhibitors for other receptor PTPs.

Materials and Methods

CD45 Target Structure for Docking Studies. Structure manipulation and visualization were done using SYBYL 7.3 (Tripos, Inc., St. Louis, MO). The crystal structure of domains D1 and D2 of CD45 bound to phosphopeptide \( \gamma \)-immunoreceptor tyrosine-based activation motif-1 was used (PDB ID 1YGR). The bound phosphopeptide was removed and all selenium methionines were replaced by methionine. The crystallographic water molecules and other heteroatoms were removed from the file. The Reduce program (version 3.1; Word et al., 1999) was used to add hydrogen atoms to the target atoms and to optimize the orientation of the polar hydrogens. Both N and C termini of the protein were modeled in the ionized state. All histidine residues were protonated and the catalytic cysteine was modeled as a thiolate.

Database for Screening. The three-dimensional structures of National Cancer Institute (NCI) database (release 3; NCI, Bethesda, MD) compounds were generated using the CONCORD module of SYBYL. Generalized AMBER force field atom types and parameters were assigned to the first 120,000 compounds using the antechamber module of AMBER (Case et al., 2005). Partial charges of the compounds were calculated using the AM1-BCC method (Jakalian et al., 2002).

Docking of NCI Compounds. The NCI compound library was docked using OMEGA version 2.2 and FRED version 2.0 (OpenEye Scientific Software, Inc., Santa Fe, NM). OMEGA generated multiple low-energy conformers of each compound, which were then docked by FRED into the allosteric cavity present in CD45 phosphatase. One hundred poses for each conformer were kept. These poses were then rescored by using the solvated interaction energy function (Naim et al., 2007) as shown in eq. 1:

\[
\Delta G_{\text{bind}} = \alpha | E_c + \Delta G_{\text{vdw}} + E_{\text{polar}} + \gamma \Delta MSA | + C
\]

\( \Delta G_{\text{bind}} \) is the change in the reaction free energy between the bound and free states, calculated by solving the Poisson equation with the BRI BEM boundary element method program (Purisima, 1998) using a molecular surface generated with a variable-radius solvent probe (Bhat and Purisima, 2006). The \( \Delta MSA \) term is the change in molecular surface area upon binding. Default values of the parameters were used (Cui et al., 2008). Further docking of the top-scoring poses was performed with the WILMA in-house docking program (Naim et al., 2007).

Final selections of compounds for experimental testing were based not only on solvated interaction energy top scores, but also on visual inspection of the binding mode (shape complementarity between the target and ligand), electrostatic interaction, and the chemical nature of the compounds.

Two-Dimensional Similarity Search. For two of the experimentally validated hits, we carried out a two-dimensional similarity search of a drug-like subset of the ZINC database (Irwin and Shoichet, 2005) to find molecules that are structurally similar to query compounds. The UNITY program (version 7.3.5; Tripos, Inc.) was used for this search, with Tanimoto coefficients of 0.70 and 0.75 for compounds 34932 (28p) and 45739 (37p), respectively. A total of 34 analogs of compound 34932 (28p) and 36 analogs of compound 45739 (37p) were procured for testing in biologic assays.

Compounds. Initial "hit" compounds, including 28p, 37p, and 57p, were obtained from the NCI. Compounds 210 and 211 were purchased from Chembridge (San Diego, CA), whereas compounds 214, 215, and 216 were purchased from InterBioScreen (Moscow, Russia). Stocks of 4 mM were prepared in dimethylsulfoxide, with dilutions made in Hanks’ balanced salt solution. Dimethylsulfoxide vehicle control was used in each assay. Competitive CD45 inhibitor RWJ-60475 ([2-(4-bromophenoxy)-5-nitrophenoxy]hydroxymethyl phosphonic acid) (Enzo Life Sciences, Inc., Farmingdale, NY) was a positive control. Quality control and purity of compounds were verified by high-performance liquid chromatography and mass spectrometry, and also by nuclear magnetic resonance where indicated.

In Vitro Phosphatase Assay. Enzymatic activity of CD45 was assayed using a modified version of the Malachite Green Assay (30), using the CD45 Drug Discovery Kit (Enzo Life Sciences, Inc.). CD45 phosphatase was preincubated with test compounds or controls for 10 minutes in the wells of a 96-well plate before the addition of 1 mM phosphorylated substrate, the negative regulatory position of pp60-Src. The reaction proceeded for 20 minutes, before the addition of Biomol Green reagent and spectrophotometric quantification of phosphate content at 620 nm in a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA) with blanks subtracted. For assays with DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate) substrate, phosphatase activity was quantified by measuring the fluorescence of its reaction product at 485 nm and 535 nm. The signal to noise ratios were always >5-fold, and the assays were in the linear range.

To determine the mode of CD45 inhibition, we tested various concentrations of the inhibitor with three different amounts of peptide substrate. The inhibitor and substrate were added simultaneously, with dilutions made in Hanks balanced salt solution. Dimethylsulfoxide vehicle control was used in each assay. Competitive CD45 inhibitor RWJ-60475 ([2-(4-bromophenoxy)-5-nitrophenoxy]hydroxymethyl phosphonic acid) (Enzo Life Sciences, Inc., Farmingdale, NY) was a positive control. Quality control and purity of compounds were verified by high-performance liquid chromatography and mass spectrometry, and also by nuclear magnetic resonance where indicated.

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To determine the mode of CD45 inhibition, we tested various concentrations of the inhibitor with three different amounts of peptide substrate. The inhibitor and substrate were added simultaneously, and the reaction was stopped after 20 minutes. A double-reciprocal plot of 1/Vo versus 1/So revealed the mode of inhibition.

To determine the reversibility of CD45 inhibition, absorbance was monitored for 120 minutes, and data collected at various time points were plotted for each peptide substrate with and without the CD45 inhibitor.

Control Phosphatascs Used in Counterassays. Protein tyrosine phosphatase-1B (PTP-1B; accession number NM_002827, amino acids 1–321), Src-homology 2 domain (SH2)–containing Protein-1
In Vivo Immunosuppression. All animal protocols were approved by our institutional animal care and use committee. Six-week-old female C57BL/6 mice (Harlan Laboratory, Montreal, QC, Canada) were used. To induce the delayed-type hypersensitivity (DTH) reaction, mice were first "primed" by 1 × intraperitoneal (100 µg) and 1 × subcutaneous (10 µg) injections of ovalbumin (OVA) antigen in saline. Twelve days later, the mice were challenged in the right footpad with 1 µg OVA in saline, whereas the left footpad of each mouse was unchallenged and was used as the internal control. Mice were randomized and divided into four groups (n = 4 each; groups A–D) that received drug or control treatments by 1 × intraperitoneal injection. Group A was the vehicle control. Group B received 3 mg/kg compound 211 <1 hour after footpad antigen challenge (day 0). Group C received 3 mg/kg compound 211 3 days after footpad antigen challenge (day 3), after initial signs of inflammation were observed. Group D remained an untreated control group until day 6, at the time of maximal footpad swelling; at this time, the mice received 3 mg/kg compound 211. Right and left footpad thickness was measured at days 6–9 after footpad antigen challenge, using an electronic caliper, with the mice under mild isoflurane anesthesia. For quantification, the difference in right/left footpad thickness was calculated in each mouse, and the individual differences were pooled for each group ± S.E.M. (n = 4 mice per group).

Mouse Footpad Section Preparation and Staining. Mice were euthanized on day 9 postchallenge and feet were removed and stored at 4°C in 4% paraformaldehyde/phosphate-buffered saline solution. Samples were then fixed in 10% neutral buffered formalin for 48 hours, decalcified in 6% HCl for 48 hours, and embedded in paraffin. Sections (4 µm) on SuperFrost/Plus slides (Fisher Scientific, Rockford, IL) were dried and deparaffinized in xylene and rehydrated through graded alcohols to water. Sections were stained with

(SHP-1; NM_002828, amino acids 1–354), MEK-kinase Phosphatase (MKP; a dual-specificity phosphatase (DSP); AF165519, full-length amino acids 1–184), leukocyte common antigen-related (LAR) receptor protein tyrosine phosphatases D1–D2 domains (LAR D1–D2; ENST00000359847, amino acids 1301–1907), protein tyrosine phosphatase-sigma D1–D2 domains (σ-D1–D2; BC014812, amino acids 883–1501), PEST-enriched phosphatase (PEP; NM_015967, amino acids 1–323), and protein tyrosine phosphatase proline-glutamic acid-serine-threonine rich (PEST; BC051980, amino acids 1–454) were cloned in vector pGEX with a glutathione S-transferase (GST) tag, expressed in E. coli DH5α strain and purified in house. Control Lyphoid phosphatase (LYP, also protein tyrosine phosphatase, nonreceptor type 22 [PTPN22]; AF165519, full-length protein tyrosine phosphatase D1-D2 domains. The pGEX-2TK plasmid expressing the CD45 gene and encoding ampicillin resistance was used as a template for mutagenesis. Primers overlapping the desired mutation region with a single or dual base pair mismatch were purchased from Integrated DNA Technologies (Coralville, IA). The mutagenic oligodideoxynucleotides were as follows: 5′-CTT TTA TAA TGA GCT ACT GGA TAC CTG AAG TGA TGG-3′ (K10031), 5′-CTT TTA TAA TGA GCT ACT GGG CAC CTG AAG TG-3′ (K1003A), 5′-GOT AGT AGA TAT TTT CTA AGG GAT AAA AGC TCT AC-3′ (V1175G), and 5′-GOT AGT AGA TAT TTT CTA AGG GAT AAA AGC TCT AC-3′ (V1175TA). Mutations were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). DNA was purified (EZNA Mini-Prep Kit; Omega Bio-Tek, Norcross, GA) from ampicillin-resistant colonies and was sequenced (McGill University and Genome Quebec Innovation Centre, Montreal, QC, Canada). The desired colonies were grown, harvested, and lysed in 20 mM HEPES-KOH pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), and 1 mg/ml protease inhibitors. After sonication in Triton X-100 buffer, CD45-GST proteins were purified using glutathione-Sepharose 4B beads and eluted in 50 mM Tris-HCl pH 8.0, 5 mM DTT, and 10 mM reduced glutathione.

Circular Dichroism. The circular dichroism spectra of cytoplasmic CD45 D1–D2 (Enzo Life Sciences, Inc.) and LAR D1–D2 (purified in house, GST-tagged) were studied. LAR D1–D2 is the specificity control, because the compounds do not inhibit LAR activity in vitro. CD45 or LAR proteins were at a final concentration of 0.04 mg/ml in 10 mM Tris buffer, pH 7.0 (adjusted with H2PO4). Compounds 211 and 57p were added at a concentration of 0.01 mg/ml (an approximate molar PTP/compound stoichiometry of 1:40). Phosphatases and compounds were allowed to interact in solution for approximately 30 minutes before measuring the circular dichroism spectra in a Chirascan spectropolarimeter (Applied Photophysics, Surrey, UK). The circular dichroism spectra were recorded at 0.5-nm intervals from 180 to 280 nm at 25°C. The baseline spectrum of buffer was autosubtracted from all samples, and the spectrum of the 211 or 57p compound alone was subtracted from the respective sample CD45 plus compound. The spectra shown are representative of four independent assays. Intentional denaturation of CD45, by incubation of the same samples at 70°C for 15 minutes, was done as a control.

In Vivo Immunosuppression. All animal protocols were approved by our institutional animal care and use committee. Six-week-old female C57BL/6 mice (Harlan Laboratory, Montreal, QC, Canada) were used. To induce the delayed-type hypersensitivity (DTH) reaction, mice were first "primed" by 1 × intraperitoneal (100 µg) and 1 × subcutaneous (10 µg) injections of ovalbumin (OVA) antigen in saline. Twelve days later, the mice were challenged in the right footpad with 1 µg OVA in saline, whereas the left footpad of each mouse was unchallenged and was used as the internal control. Mice were randomized and divided into four groups (n = 4 each; groups A–D) that received drug or control treatments by 1 × intraperitoneal injection. Group A was the vehicle control. Group B received 3 mg/kg compound 211 <1 hour after footpad antigen challenge (day 0). Group C received 3 mg/kg compound 211 3 days after footpad antigen challenge (day 3), after initial signs of inflammation were observed. Group D remained an untreated control group until day 6, at the time of maximal footpad swelling; at this time, the mice received 3 mg/kg compound 211. Right and left footpad thickness was measured at days 6–9 after footpad antigen challenge, using an electronic caliper, with the mice under mild isoflurane anesthesia. For quantification, the difference in right/left footpad thickness was calculated in each mouse, and the individual differences were pooled for each group ± S.E.M. (n = 4 mice per group).

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Cell Lines and Cell Culture. Human leukemia cell lines Jurkat and J45.01 were kindly provided by Dr. Pauline Johnson (University of British Columbia, Vancouver, BC, Canada). J45.01 is a CD45-negative variant of Jurkat cells, and exhibits impaired TCR signaling (Koretzky et al., 1991). The KB line is a human nasopharyngeal cell. EL4 cells are a mouse thymoma. HEK293 are human kidney cells.

Cellular Activation, Treatment, Lysis, and Immunoblotting. Splenocytes prepared from young adult female C57BL/6 mice were semi-purified as described (Thomson et al., 1987) and 5 × 10⁶ cells per well were cultured in six-well plates containing 0.5 µM immobilized (plastic-bound) 2C11 monoclonal antibody (eBioscience, San Diego, CA).

Stimulation with or without treatment with test compounds was simultaneous and consisted of a 48-hour incubation with 0.5 µM of the CD45 allosteric inhibitors. Cells were collected and whole cell content was quantified by the addition of 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 0.75% SDS. After 2 minutes, the concentration of thionitrobenzoic acid, as a measure of total thiol, was determined spectrophotometrically at 412 nm (Riddle et al., 1983).

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hematoxylin and eosin to visualize the infiltration of immune cells in the footpad region (Gill method; Fisher Scientific). Sections were analyzed by conventional light microscopy, and pictures were taken at ×10 magnification.

Results

Identification and In Vitro Validation of Hit CD45 Inhibitors. The D1 and D2 domains of CD45 are joined by a flexible four–amino acid linker, and several residues from each domain contribute to a stable interaction between the two. The interface forms a pocket comprising D1 and D2 as the walls and the linker as the base (Fig. 1A). We hypothesized that a small molecule might interact with a binding groove at this crevice. We screened a 120,000-compound subset of the NCI database (release 3) by in silico virtual docking of the compounds within the region defined by the box enclosing the D1–D2 interface (Fig. 1B).

The screen identified approximately 200 potential binders having a predicted binding free energy of −7.5 kcal/mol or better, and these were selected for further evaluation by visual inspection of the binding mode. From these, we selected 84 compounds interacting with both the D1 and D2 domains of CD45, and whose chemical compositions satisfied the Lipinski rule of five for drug-like properties. Compounds were procured and tested against CD45 in an in vitro phosphatase assay.

Compounds coded as 28p and 37p (Supplemental Fig. 1A) inhibited, in a dose-dependent manner, the ability of CD45 to dephosphorylate pp60-Src peptide. Compounds 28p and 37p have IC50 values of 4.6 and 1.1 μM, respectively (Fig. 2A). Eighty-two other compounds did not inhibit CD45 at concentrations >50 μM and were not pursued.

These hits were docked at a pocket of CD45 formed by the interface of D1-D2 and the linker that joins these domains. Residues from D1 and D2 form hydrogen bonds and hydrophobic contacts with compounds 28p or 37p. For both inhibitors, the D2 domain contributes the majority of predicted contacts (Supplemental Fig. 1, B and C).

CD45 Selectivity of the Inhibitors. The hit CD45 inhibitors 28p and 37p were tested against a panel of PTPs that include LAR, PTP1B, PTP-σ, SHP-1, and MKPX. These PTPs were chosen because they bear structural similarity to CD45, and the tandem phosphatase domains D1 and D2 are highly conserved among the receptor-like proteins CD45, LAR, and PTP-σ. The IC50 data are summarized in Table 1.

28p was a broad inhibitor of all PTPs tested and in fact was a more potent inhibitor of PTP1B and SHP-1 than of CD45. Inhibitor 37p was highly selective for CD45 phosphatase. Among the PTPs tested, the only other effect detected was on PTP1B, but with a significant 15-fold difference in IC50 value. As a control, we show that 57p was also predicted to dock near the CD45 D1–D2 interface by the in silico screen, but did not inhibit any of the PTPs on the panel. Supplemental Figure 2 shows the inhibition profiles of 28p versus a PTP panel and of 37p versus PTP1B.

The different binding modes predicted for 37p and 28p provide a possible explanation for selectivity by 37p and the lack of selectivity by 28p. Strong 28p interactions with the D1 domain may be sufficient to cause inhibition of PTP1B and SHP-1, which lack a D2 domain, making 28p nonselective. By contrast, 37p causes poor inhibition of PTPs lacking a D2 domain (e.g., constructs of PTPs LAR and σ expressing both domains were tested in counterassays).

For further validation, the activity of all control PTPs was tested using two different substrates [pNPP (data shown in Table 1; see the Supplemental Material for details) and DIFMUP (Supplemental Fig. 2C)], yielding similar results. In addition, the activity of control LAR was also tested using an insulin receptor–derived phosphopeptide, also yielding similar results (data not shown).

Because pNPP and DIFMUP are not good substrates in vitro for CD45, they were not used for this enzyme. Instead, CD45 inhibition was tested independently against two different phosphopeptides representing the cognate Src substrates at positions pY-527 (data shown in Table 1) and pY-416 (Fig. 3).

On the basis of CD45 selectivity, we chose 37p for developing second-generation agents.

Hit-to-Lead Generation. The 37p molecule was cut at two locations; by rational design, we determined that the remaining central phenyl ring should be derivatized with substituents at the ortho-, meta- and para- positions. Twenty-nine analogs of 37p were thus purchased and tested in the in vitro phosphatase assays. Five analogs are shown, coded 210, 211, 214, 215, and 216 (Fig. 2B). They are potent and selective CD45 inhibitors, with improved inhibition of CD45 by 2- to 4-fold over the parent compound 37p (Fig. 2C), reaching nanomolar IC50. With regard to selectivity, these five analogs retained selectivity against CD45 (Table 1) and did not significantly inhibit other PTPs at 50 μM (the highest dose tested). Thus, compounds 210, 211, 214, 215, and 216 are smaller and more potent than the parent, and are highly selective for CD45.

Analog 211 was selected as a lead compound because of its high potency, selectivity, stability, and membrane permeability predicted in silico. Because compound 211 was considered a lead, additional selectivity controls were completed against PTPs PTPN22 (the catalytic domain PEP and the full-length PTPN22), which plays a role in T-cell signaling, as well as its family member PEST. Compound 211 had no effect on the in vitro phosphatase activity of these enzymes (Supplemental Table 2).

Allosteric Binding Site of 211 on CD45 Phosphatase. Fig. 2D shows the in silico model of the binding of compound 211 on the CD45 pocket. The compound is anchored at one end by a hydrogen bond between its acetyl carbonyl and the Ser1163 side chain hydroxyl group. The acetyl methyl packs against a hydrophobic surface formed by the side chains of Val1175 and Leu1160. At the opposite end, one naphthoquinone oxygen forms a hydrogen bond with the ammonium on the side chain of Lys1003. The increased potency of 211 over 37p may be partly...
because it has three times fewer rotatable bonds, resulting in a lower entropic cost upon binding. To validate the predicted binding model, we generated mutant CD45 proteins with substitutions affecting two of the predicted binding sites. Lys1003 was predicted to form a tight hydrogen bond with the carbonyl group of the core structure of 211 (Fig. 2D), whereas Val1175 contributes to hydrophobic interactions with the side chain aromatic ring of 211. If a mutant CD45 retains phosphatase in vitro catalytic activity, but 211 cannot inhibit it, this can be interpreted as a failure of 211 to bind at the mutant pocket. Thus, wild-type Lys1003 was mutated to Ile or Ala, and wild-type Val1175 was mutated to Gly or Ala, for a total of four point mutants.

The wild-type and mutant CD45 intracellular D1–D2 domain proteins were purified and used in phosphatase in vitro assays with and without compound 211 (Fig. 2E). We first established that mutant forms of CD45 have the same level of phosphatase activity as the wild-type enzyme (see Supplemental Fig. 3 for phosphatase activity). Compound 211 did not inhibit CD45 mutants K1003A and V1175A, but inhibited wild-type CD45, at the two concentrations tested. In addition, compound 211 inhibited mutants K1003I and V1175G at a significantly reduced rate. As a control, we observed strong inhibition of both wild-type and mutant CD45 when using competitive CD45 inhibitor RWJ-60475 (Beers...
et al., 1997). This confirms that mutated CD45 behaves similarly to wild-type CD45 and, taken together, validates the region encompassing the K1003 and V1175 residues as the orthosteric binding site for 211.

We postulated that an allosteric inhibitor binding at that site could influence CD45 phosphatase activity through conformational changes. We measured the circular dichroism spectra of CD45 with and without 211 (Supplemental Fig. 4A). When compound 211 is coincubated with CD45, it appears that there is a dramatic loss of secondary structure, akin to an unfolding of the protein. This effect was not observed in the presence of noninhibitory compound 57p; when LAR phosphatase was coincubated with compound 211, there was no change in secondary structure in this control protein (Supplemental Fig. 4B).

Together, the data in Fig. 2 validate that functional inhibition by compound 211 requires a structure near the CD45 D1–D2 linker region, which is outside the orthosteric site. The circular dichroism data suggest a conformational change as a possible cause for reduced CD45 activity.

Noncompetitive and Irreversible Mechanism of Inhibition.

Docking, modeling, and site-directed mutagenesis data suggest an allosteric pocket far from the orthosteric substrate binding and catalytic site. To address the mechanism of action, we studied the inhibition of CD45 by compound 211 at three different concentrations of phosphopeptide substrate.

The inhibitor and substrate were added simultaneously to the CD45 enzyme. CD45 activity was measured after 20-minute incubation of the indicated four different concentrations of compound 211 and the indicated three different concentrations of the pp60-src substrate. A double-reciprocal Lineweaver-Burk analysis of reaction velocity (1/Vo) versus substrate concentration (1/So) showed that all lines intersect at a common $K_m$ value. These data indicate a noncompetitive mode of inhibition (Fig. 3A).

To further characterize the mechanism of inhibition, potential reversibility of the inhibition was studied in vitro versus two src-derived phosphopeptide substrates. The inhibition of CD45 was plotted over time, up to 120 minutes (reaction plateaus after 20 minutes when substrate is depleted). If the inhibition were reversible, one would expect the phosphatase reaction to proceed slowly over time. However, the compound 211–treated CD45 shows an early plateau indicating an irreversible mechanism of inhibition (Fig. 3, B and C). In a permutation of this assay, the addition of more substrate to the reaction after 20 minutes showed that although untreated CD45 remains active, the compound 211–treated CD45 is not active.

It is worth noting that compound 211 is very effective at preventing CD45 dephosphorylation of the pY-527-peptide substrate (approximately 80% inhibition at $V_{max}$; Fig. 3B) and it is less effective at preventing CD45 dephosphorylation of the pY-416-peptide substrate (approximately 35% inhibition at $V_{max}$; Fig. 3C). This observation further supports an allosteric mechanism of action, because poisoning the catalytic site of CD45 prevents activity equally at any substrate.

Fig. 3. Binding mode and reversibility of CD45 inhibitor compound 211. (A) Lineweaver-Burk analysis of compound 211 inhibition on CD45 phosphatase. CD45 activity was measured at varying substrate concentrations as described in Materials and Methods, at the indicated concentrations of compound 211. The data are representative of three independent experiments. (B and C) The reversibility of 211 inhibition of CD45 was assayed by allowing the assay to proceed for 120 minutes. Substrates tested include the inhibitory C-terminal pY-527 and the autacatalytic Src phosphosubstrate pY-416. (D) Compound 211 does not oxidize free cysteines in CD45 or in PTP1b. A chemically related compound 1,2-NQ known to oxidize PTP1b is used as a positive control.
These data allow us to make predictions about the cellular consequences of CD45 inhibition. CD45 dephosphorylates both the regulatory C-terminal tyrosine (Y505) and the autocatalytic tyrosine (position Y394 in human Lck and position Y393 in mouse Lck). Functional CD45 thus creates a state of equilibrium for Lck molecules that allows for a primed Lck response when the TCR is engaged. Preferential inhibition of the removal of the inhibitory tyrosine, by compound 211, can shift the pool of Lck molecules toward the inactive state. The phosphorylated C-terminal Y505 of Lck interacts intramolecurally with its own sulfhydryl (SH)-2 domain, thus sequestering Y393. In this conformation, trans-phosphorylation of Y393 is not possible and Lck remains inactive. Thus, we can predict that the reduced pool of resting state Lck would result in diminished activation of the T cell signaling pathway in the presence of CD45 inhibitor compound 211.

**CD45 Inhibition by Compound 211 Does Not Involve Oxidative Mechanisms.** Irreversible inhibition by compound 211 could suggest a mechanism of oxidation of one or more cysteine residues in CD45. There is a precedent in 1,2-NQ that inhibits CD45 through oxidation in a substrate-reversible manner (Urbanek et al., 2001). However, our data indicate that compound 211 inhibits CD45 noncompetitively with respect to substrate (Fig. 3A). 1,2-NQ is also an irreversible inhibitor of PTP1b through covalent modification of cysteines (Iwamoto et al., 2007).

Hence, we studied whether compound 211 could modify free cysteines of CD45 or PTP1b, and 1,2-NQ was used as a positive control. Assays utilizing 5,5'-dithiobis-(2-nitrobenzoic acid) substrate quantified the free SH groups in the proteins with and without compound treatment. Treatment conditions, times of incubation, and protein-compound stoichiometry replicated the conditions used in the PTP functional assays, except that DTT was omitted because it prevents or reverses oxidative mechanisms (Urbanek et al., 2001; Iwamoto et al., 2007).

Compound 211 did not diminish free –SH in CD45 or in PTP1b (Fig. 3D). The positive control, 1,2-NQ, diminished free –SH in PTP1b by approximately 85% and did not detectably affect free –SH in CD45 (Fig. 3D). Note that under these assay conditions, compound 211 inhibits the activity of CD45 selectively by approximately 80% (even in the presence of freshly added reducing agent 2 mM DTT), whereas 1,2-NQ inhibits both CD45 and PTP1b (and its efficacy on PTP1b is affected by the presence of DTT).

Together, the data indicate that compound 211 inhibits CD45 selectively over several other PTPs, has an allosteric site that requires residues at the linker domain of D1-D2, alters the structure of intracellular CD45, is irreversible, is noncompetitive, and does not inhibit by way of free –SH chemical modification.

**Cellular CD45 Activity Is Inhibited by Compound 211.** To study whether cellular CD45 is a target of compound 211, we studied changes in the phosphorylation of Lck because it is a substrate of CD45. We studied Jurkat T cells and used the CD45-negative variant of Jurkat, called J45.01, as a control. J45.01 cells exhibit impaired TCR signaling and are hyper-phosphorylated at the Tyr505 position of Lck (Koretzky et al., 1991). Each of these two cell lines were treated for 24 hours with vehicle control or compound 211 at 0.5 μM, and lysates were probed by Western blot for various forms of Lck (Fig. 4) and quantified by densitometry using total Zap-70 as the control.

In Jurkat cells, treatment with compound 211 caused an approximately 50% increase in Lck pY394 compared with untreated cells (P < 0.05; see Fig. 4D for quantification). However, in J45.01 cells, treatment with compound 211 caused no change in Lck pY394 (Fig. 4A). In both Jurkat and J45.01 cells, treatment with compound 211 caused no change in baseline Lck pY-505 (Fig. 4B) or in total Lck (Fig. 4C).

Because CD45 normally dephosphorylates the Lck pY-394 position, it is expected that a CD45 inhibitor would increase Lck pY-394 phosphorylation above baseline levels. Thus, quantifying the phosphorylation of Lck as a surrogate assay for CD45 activity, we conclude that compound 211 inhibits the function of CD45. On the basis of data from J45.01 cells (which express many PTPs), we infer that CD45 expression is required to detect changes in Lck pY-394.

Nonspecific toxicity by CD45 inhibitors was excluded by assays measuring metabolic activity in lymphoid cells (Jurkat and J45.01 cells) as well as nonlymphoid cells that do not express CD45 (KB and HEK293 cells). After 24 hours of culture with 0.5 μM CD45 inhibitors (the concentration used for biochemical assays), the percentage of cell viability was not affected (Supplemental Table 1).

**CD45 Inhibitors Diminish TCR Signaling in Activated Splenocytes.** Studies of TCR signaling were extended in biochemical and biologic assays using semipurified primary T cells. Freshly isolated mouse T cells were stimulated by cross-linking anti-CD3 antibody 2C11 for 48 hours with or without CD45 inhibitor 37p and its analog 211, each at 0.5 μM. Whole cell lysates were processed in Western blots probed with antibodies against Lck pY393, Zap-70 pY-319, and p-mitogen-activated protein kinase (MAPK).
These proteins are phosphorylated after a signal from the TCR.

The TCR-stimulated cultures had a significant induction of Lck pY-393, Zap-70 pY-319, and p-MAPK (Fig. 5A) compared with the control nonstimulated culture. Treatment with compound 211 causes a reduction of 40–50% in TCR-mediated activation (Fig. 5B). Thus, these compounds affect signaling through the TCR, likely because they interfere with CD45 function. As a further readout, we measured IL-2 protein released in the supernatants. As measured by quantitative ELISA tests, IL-2 levels were significantly reduced in the drug-treated groups compared with the vehicle-treated group (Fig. 5C). In baseline controls, cells not activated by TCR cross-linking did not produce detectable IL-2, indicating that this is a reliable readout of TCR (and CD45)-mediated activation. Inhibition of Lck pY-393 and IL-2 production by compound 211 are consistent with each other. However, inhibition by 37p was less pronounced than anticipated from the in vitro assays, likely because it is not stable in aqueous solutions for longer than 12 hours (data not shown).

To rule out a direct cytotoxic effect, cell viability (trypan blue exclusion) and metabolic activity (MTT assays) were tested in primary splenocytes treated with CD45 inhibitors. These assays were done using the same culture and activation conditions used above for the biochemical and bioassays. At the relevant concentration of 0.5 μM compound 211, the trypan blue exclusion method revealed no changes in the ratio of live to dead cells after treatment compared with the untreated control (Fig. 5E). However, the MTT assays revealed that there was approximately 40% less metabolic activity in the compound 211–treated, TCR-stimulated splenocytes (Fig. 5D). Thus, it appears that CD45 inhibitors do not compromise cell integrity, yet they prevent TCR-mediated activation and therefore prevent an increase of the cellular metabolic rate. A reduced metabolic rate correlates with decreased IL-2 production and inhibition of T-cell signaling pathways.

![Figure 5](link-to-image)
We also investigated the ability of CD45 inhibitor compound 211 to influence Lck phosphorylation in resting splenocytes. Cells were treated with 0.5 μM compound for 48 hours, but without TCR cross-linking. Lck pY-393 and pY-505 did not change after treatment with compound 211 (Supplemental Fig. 5). This was expected because there is no TCR signal driving the activation and proliferation of these cells, hence CD45 function is not absolutely required and CD45 inhibition by 211 should not affect the signaling pathway.

Taken together, the data show that a potentially allosteric CD45 inhibitor causes a chemical knockdown of CD45 function, and diminishes TCR-dependent activation and signaling.

A CD45 Inhibitor Is Immunosuppressive In Vivo.

Compound 211 was selected for studies in vivo, in wild-type C57/BL6 mice. A dose-escalation study of a single intraperitoneal injection for compound 211 up to the maximum dose tested of 12 mg/kg showed that it was well tolerated, with the only undesirable side effect being approximately 25% neutropenia after 24 hours.

A dose of 3 mg/kg caused an approximately 50% decrease in lymphocytes without significant neutropenia, no observable adverse side effects in behavior, and no change in creatinine or amylase levels or in the numbers of red blood cells and platelets.

We tested compound 211 at a dose of 3 mg/kg in a footpad model of DTH in wild-type C57/BL6 mice. In this model, we detected signs of increased footpad thickness, redness, and inflammation at day 3 after challenge, with maximal footpad thickness at day 6 postchallenge. Compound 211 (3 mg/kg single dose) was injected intraperitoneally within 1 hour of footpad challenge (day 0, group B) or at day 3 after footpad challenge (day 3, group C) (Fig. 6A).

In group B mice, injected within an hour of challenge, there was only slight inflammation in one of four mice at day 3, and no progression to full DTH in any of the mice at day 6 postchallenge (Fig. 6B). These data indicate that systemic inhibition of CD45 at the time of footpad insult results in a block of DTH induction and that the efficacy of inhibition of CD45 can be long lasting.

In group C mice, injected with 211 at day 3 postchallenge, inhibition of DTH was achieved although the drug was delivered after early onset of inflammation. Quantification done at day 6 postchallenge showed that this group had an almost complete normalization of footpad thickness compared with control and untreated mice (groups A and D, respectively) (Fig. 6B). These data indicate that the CD45 inhibitor is effective at preventing DTH when administered systemically at the onset of inflammation.

When compound 211 was injected at day 6 after challenge (group D), a time when inflammation is maximal, there was no significant reduction of footpad thickness over the next 3 days (e.g., until day 9 postchallenge). Thus, although the CD45 inhibitor might prevent T cell–mediated inflammation, it does not clear the existing edema in the footpad.

Staining of paraffin-embedded footpad sections with hematoxylin and eosin confirmed the presence of greater infiltration of cells in the vehicle-treated control (group A) compared with groups B or C, which received 3 mg/kg compound 211 at days 0 and 3, respectively (Supplemental Fig. 6). A further comparison of the right foot (challenged, untreated) versus the left foot (naive, untreated) of a mouse from group A shows the degree of cellular infiltration. The infiltrating cells are presumed to be lymphoid based on morphology. Thus, the inflammation after OVA challenge was likely mediated by an immune response, which is suppressed by CD45 inhibitor compound 211.

**Discussion**

This work provides evidence of an allosteric site in CD45, in which a noncompetitive small molecule can bind and cause inhibition of the CD45 PTP activity. The inhibitors were effective immunomodulators ex vivo and in vivo; therefore, they may be interesting lead compounds for therapeutic programs. Importantly, similar approaches can be used to target the D1-linker–D2 interface of other PTPs.

**Binding at a Nonorthosteric Pocket, and Allosteric Mechanisms.** Molecular docking and site-directed mutagenesis suggests that the CD45 inhibitors bind in a pocket near the interface between the D1 and D2 domains, adjacent to the linker region. Hit compounds 28p and 37p were predicted to dock at this allosteric site. 37p exhibits selectivity toward CD45 over similarly structured PTPs such as LAR, and is smaller and more compact than the nonselective 28p. A compound derived from 37p, compound 211, was also modeled to bind in the D1–D2 pocket.
A key interaction for compound 211 was predicted to be at CD45 Lys1003 through a hydrogen bond with the “core” naphthoquinone moiety of compounds 211 and 37p. Furthermore, the “noncore” portion of compound 211, an anilino-phenyl ring with an acetyl side group, was also predicted to interact with CD45 through hydrophobic interactions with Leu1160 and Val1175, as well as a second hydrogen bond with Ser1163. These predictions are consistent with the site-directed mutagenesis data. Mutation of Lys1003 and Val1175 did not compromise CD45 phosphatase activity but abolished the capacity of compound 211 to inhibit CD45 activity.

Site-directed mutagenesis strongly indicates an allosteric mechanism of action. Mutated serine and valine residues prevented compound 211, but not competitive inhibitor RWJ, from inhibiting CD45 phosphatase activity. Interestingly, the in vitro phosphatase activity of the mutant CD45 enzymes was equivalent to that of wild-type CD45, suggesting that these residues are not critical to CD45 activity. However, the CD45 residues that were mutated are required for compound 211-induced inhibition.

It is likely that these residues allow compound 211 to interact with and reposition the D2 domain, which in turn affects the D1 domain that contains the catalytic region. This is supported by the change in conformation noted in the circular dichroism experiments, in which compound 211 caused significant changes to the α-helicity of the CD45 intracellular domain but did not affect the overall structure of the intracellular domain of LAR or LAR activity in vitro.

Together, the data indicate that the inhibitor inflicts a conformational change upon CD45, potentially unfolding the protein. There are precedents in which an allosteric inhibitor irreversibly destabilizes an enzyme via a nonoxidative mechanism (Piver et al., 2001; Ucar et al., 2005; Domínguez et al., 2012).

High Potency and Selectivity at Inhibiting CD45 Signaling in T Cells. It is encouraging that the in vitro data correlate with the cell-based biochemical and biologic data. Compound 211 inhibition of CD45 caused a differential effect upon the two CD45 substrates. There was increased Lck pY-393, an indication that CD45 function is inhibited. On the other hand, there were no changes in the steady state Lck pY-505, which is also dephosphorylated by CD45.

This was a surprising result because we expected both pY-393 and pY-505 to increase in CD45-inhibited T cells. However, our observation is consistent with reports that only high CD45 activity can dephosphorylate Lck pY-393, whereas low CD45 activity is sufficient to dephosphorylate Lck pY-505 (McNeill et al., 2007). Thus, it is possible that after pharmacologic inhibition of CD45, the pY-505 remains in a steady state but pY-393 levels are increased.

In resting primary T cells, there are low to undetectable Lck pY-393 but there are significant levels of Lck pY-505. Stimulation with TCR cross-linking results in a time-dependent increase in Lck pY-393 and no detectable changes in the steady state of Lck pY-505. Thus, inhibition of CD45 during stimulation via TCR results in the prevention of the time-dependent increase in Lck pY-39, without significant alterations to the steady state of Lck pY-505. These events result in the inactivation of Lck, as demonstrated by downstream readouts (e.g., p-Zap-70, p-MAPK, and IL-2 production are reduced by CD45 inhibitors).

These data are consistent with the view that CD45 functions as a rheostat to keep Lck in a primed state, ready for activation (Hermiston et al., 2003). The allosteric CD45 inhibitors reduce CD45 activity and the pool of Lck that is capable of becoming activated. Under TCR-mediated stimulation, if removal of p-Y505 is inefficient, the Lck molecule is commonly referred to as inactive because Y393 is inaccessible for autophosphorylation (Amrein et al., 1993). This results in hypophosphorylation of Y393 with minimal effects detected on the steady state phosphorylation of Y505. Poor Lck activation extends to inefficient recruitment of Zap-70 to the CD3 z-chain and poor Zap-70 pY-319. Downstream of this, CD45 inhibitors reduce the amount of p-Erk, indicating effects upon cellular proliferation (Crawley et al., 1997), decrease IL-2 production, and reduce metabolic activity in the T cells.

Substrate Specificity. Compound 211 is a selective inhibitor of CD45 and does not affect several related PTPs. Notably, compound 211 also has some degree of substrate specificity when two pp60-Src phosphopeptides were tested. The dephosphorylation of the negative regulatory pp60-Src pTyr is inhibited by approximately 80%. The dephosphorylation of the autocatalytic activating pp60-Src pTyr is inhibited by approximately 35%. To our knowledge, this is the first report of a differential inhibition of these substrates.

Immunosuppression In Vivo. The DTH reaction to OVA was inhibited in footpad-challenged mice by treating them with a single dose of 3 mg/kg compound 211. This was accomplished in a therapeutic paradigm administering compound 211 after antigen challenge, and after slight inflammation had already set in. However, there was no suppression of the signs of edema when the inflammatory response was well established, likely because T-cell activation is not relevant at this stage of pathology (Askenase and Van Loveren, 1983).

DTH prevention is likely due to the inhibition of T-cell activation during antigen challenge, and not due to the apoptosis of T cells. The dose of 3 mg/kg did reduce circulating lymphocytes by approximately 50%; however, given that the in vivo data were collected 6 days after the single injection of compound 211, circulating lymphocytes had ample time to recover from depletion. At day 6, inhibition of footpad inflammation was near 100%, which we interpret as the complete blockage of T-cell activation.

In this work, we have identified and validated a novel binding site for inhibitors on CD45 phosphatase intracellular domain, identified inhibitors in vitro, and improved upon their potency for CD45 inhibition via chemical modification. A lead allosteric CD45 inhibitor suppresses splenocyte activation and inhibits the immune response in vivo.

Together, these findings bode well for potential therapeutic applications of allosteric CD45 inhibitors. In particular, CD45 inhibitors may be used as immunosuppressive agents relevant in the treatment of T cell– or B cell–mediated autoimmune diseases, preventing organ transplant rejection, and in certain forms of leukemia or lymphoma. The targeting of an allosteric site on CD45 may be expanded to target similar domains of other PTPs.

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Authorship Contributions

 Participated in research design: Perron, Saragovi.
 Conducted experiments: Perron, Chowdhury, Aubry, Saragovi.
 Contributed new reagents or analytic tools: Purisima.
 Performed data analysis: Perron, Chowdhury, Aubert, Tremblay, Saragovi.
 Wrote or contributed to the writing of the manuscript: Perron, Chowdhury, Tremblay, Saragovi.

References


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Selective Inhibitors of CD45 PTP 563


MOLECULAR PHARMACOLOGY

Allosteric non-competitive small molecule selective inhibitors of CD45 tyrosine phosphatase suppress T-cell receptor signals and inflammation in vivo

Michael Perron, Shafinaz Chowdhury, Isabelle Aubry, Enrico Purisima, Michel L. Tremblay, H. Uri Saragovi

Supplemental Figures and Tables

Docking of first generation compounds onto CD45

The “hits” 28p or 37p were docked at a pocket of CD45 formed by the interface of D1-D2 and the linker that joins these domains. Residues from D1 and D2 form hydrogen bonds and hydrophobic contacts with compounds 28p or 37p. For both inhibitors, the D2 domain contributes the majority of predicted contacts (Figure S-1).

Docking predicts that the sulfonic acid groups of 28p form hydrogen bonds with the D2 domain backbone carbonyl of Tyr957 and Phe997, as well as Lys1136 and Lys1181 side-chain amino groups, and the Ser1163 side-chain hydroxyl group. A hydroxyl moiety of 28p contacts the D1 domain at the Asp766 side chain via hydrogen bonding. One central phenyl group in the inhibitor is sandwiched between the methylene groups of Glu801 of the D1 domain and Met999 of the D2 domain. Tyr1001 in D2 packs against a naphthyl group.

As for 37p, docking predicts that its carbonyl groups make tight hydrogen bonds with the D2 domain amino groups of Lys1003 and Asn1159. The phenyl ring of the compound is sandwiched between Leu805 of the D1 domain and the side chain methylene groups of Gln1174 of the D2 domain. One of the naphthoquinone rings of 37p packs against a hydrophobic wall composed of the side chains of Met999, Phe997 and Val1006 in the D2 domain.
Figure S-1. Docking of compounds at a pocket region near the CD45 D1 D2 domain interface.

(A) 2D structures of hit compounds 28p and 37p

(B, C) 28p and 37p docked at the D1-D2-linker interface of CD45, the putative allosteric binding site. D1 domain is shown in green, and D2 domain in yellow. Panel B shows the detailed interactions with the binding pocket. Panel C shows the binding pocket as a solvent-excluded surface. Hydrogen bond interactions are shown as dashed lines.
Figure S-2. Specificity counter-assays for different PTPs and different substrates

(A) Protein tyrosine phosphatases SHP-1, MKPX, and PTP-Sigma were incubated with 28p, and PTP1B was tested against 37p, using a generic pNPP substrate. The curves are generated from activity rates at different concentrations of inhibitor. Data are shown as a percent of untreated control. (B) The inhibition by 28p on LAR and PTP1B are shown. (C) DIFMUP substrate was used on CD45 and PTPN22, and assayed for fluorescence as a readout of phosphatase activity after 20 minutes incubation with the indicated concentrations of 211. Data are shown as a percent of control untreated enzyme.
Figure S-3. Quantification of wild type and mutant CD45 phosphatase activity

The basal phosphatase activity of wild type and mutant CD45 was assayed in a modified Malachite Green assay. Free phosphate liberated from a phospho-peptide substrate was quantified by measurement of $\text{OD}_{620}$. All CD45 proteins show similar phosphatase enzymatic activity, equivalent to wild type. This assay controls for intact enzymatic activity of the mutants and aids in the interpretation of the inability of 211 to inhibit the mutant proteins.
Figure S-4. Inhibitors alter the conformation of CD45

Far-UV range circular dichroism spectra of CD45 D1-D2 in 10 mM Tris-H$_3$PO$_4$ buffer, pH 7.0. Measurements were made in a 0.5 mm cuvette

(A) 0.04 mg/mL CD45 alone, or CD45 co-incubated for 30 minutes with 0.01 mg/mL compound 211, or 0.01 mg/mL compound 57p which is an inactive compound used as control. Heat-denatured CD45 is also shown as control. Removal of compound 211 (by centrifugation) did not result in the renaturation of CD45 (data not shown) after 30 min.

(B) 0.04 mg/mL LAR alone, or LAR co-incubated for 30 minutes with 0.01 mg/mL compound 211. Compound 211 alone is shown as control.
Figure S-5. Effect of 211 treatment on Lck phosphorylation in unstimulated cells
EL4 cells were treated with vehicle or 0.5 µM 211 for 24 hours ± stimulation of the TCR with plate-bound anti-CD3 antibody. Cells were lysed and samples were blotted for p-Lck Y393 as a measure of T cell signaling activation. 211 inhibited phosphorylation in activated splenocytes, but had no effect on p-Lck Y393 levels in unstimulated cells. Actin protein is shown as a loading control.
Figure S-6. Histological staining of sections obtained from mouse foot-pad.
Tissue sections were stained with H&E and viewed under 10x magnification. Cell infiltration is apparent in the right footpad of antigen challenged group A, but it is diminished in antigen challenged drug-treated groups B and C, and absent in the unchallenged left footpad.
Table S-1. Cell viability with 0.5 µM CD45 inhibitor treatment for 24 hours

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<th>J45.01</th>
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<td>37p</td>
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<td>95 ± 0.7</td>
<td>94 ± 4.5</td>
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<tr>
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<td>95 ± 1.4</td>
<td>94 ± 1.0</td>
<td>94 ± 0.5</td>
<td>110 ± 3.8</td>
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<tr>
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<td>91 ± 3.0</td>
<td>94 ± 3.9</td>
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<tr>
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Table S-2. Additional 211 selectivity controls. The inhibitory capacity of compound 211 was tested against additional PTPases related to CD45, using pNPP substrate. The proteins had PTP enzymatic activity, but 211 at the highest concentration tested (40 or 50 μM) did not significantly inhibit these enzymes.

<table>
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<tr>
<th>Compound</th>
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