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Thiuram Disulfides as Pseudo-irreversible Inhibitors of the Lymphoid Tyrosine Phosphatase

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Abstract

We have screened a small library of thiuram disulfides for inhibition of lymphoid tyrosine phosphatase (LYP) activity. The parent thiuram disulfide, disulfiram, inhibited LYP activity *in vitro* and in Jurkat T cells whereas diethyldithiocarbamate failed to inhibit LYP at the concentrations tested. Compound **13**, an N-(2-thioxothiazolidin-4-one) analog, was the most potent LYP inhibitor in this series, with an IC₅₀ of 3 μ M. Compound **13** was found to inhibit LYP pseudo-irreversibly, as evident by the time-dependence of inhibition with a K_i of 1.1 μ M and a k_{inact} of 0.004 s⁻¹. The inhibition of LYP by compound **13** could not be reversed significantly by incubation with glutathione or by prolonged dialysis, but could be partially reversed by incubation with dithiothreitol. Compound **13** also inhibited LYP activity in Jurkat T cells.

Keywords

Disulfiram; thiuram disulfides; PTP; screening; inhibitors

The improper regulation of tyrosine phosphorylation is implicated in multiple human diseases including diabetes, cancer, hypertension and autoimmunity.^[1] As a consequence, there is significant interest in identifying chemical inhibitors of the enzymes responsible for regulating tyrosine phosphorylation, namely the tyrosine kinases and protein tyrosine phosphatases (PTPs). One enzyme of particular interest in autoimmunity is the lymphoid tyrosine phosphatase (LYP, PTPN22), a negative regulator of early T cell receptor (TCR) signaling.^[2] A single nucleotide polymorphism (SNP) C1858T in the *PTPN22* gene results in a gain of function variant of LYP that has been associated with numerous autoimmune disorders including type I diabetes, rheumatoid arthritis, systemic lupus erythematosus, Graves' disease, and myasthenia gravis.^[3, 4] On the other hand, another SNP in the same *PTPN22* gene, G788A, leads to a putative loss of function variant that is found to be protective against systemic lupus erythematosus.^[5] LYP is also a positive regulator of anaphylaxis and inhibition of LYP helps to mitigate anaphylaxis in mice.^[6] Taken together, these findings reinforce the potential of LYP inhibition for the treatment of immune system disorders.

The catalytic domains of PTPs are highly homologous and all contain the PTP signature motif, (H/V)C(X)₅R(S/T), in the conserved active site.^[7] The local environment in the PTP active site lowers the pK_a of the active site cysteine residue, making it highly reactive and susceptible to oxidation.^[8] Therefore, several PTPs including LYP are inactivated by reversible or irreversible oxidation of the catalytic cysteine residue upon exposure to oxidizing agents.^[8–11] Our laboratory has previously shown that nitrosating agents such as sodium nitroprusside and the active site-directed NO-donating peptide, Ac-ARLIEDNE-(HCyNO)-TAREG-NH₂, cause reversible, competitive and covalent oxidative inactivation

of LYP.^[12] Thiophilic heavy metals ions including zinc(II), gold(I) and mercury(II) readily inhibit LYP activity.^[13–16] In addition, S-thiolation can inhibit PTP activity. For example, PTP1B is inhibited by hydrogen sulfide-mediated S-thiolation during ischemia and reperfusion.^[17] Furthermore, in the presence of excess of H₂O₂ *in vitro*, the catalytic cysteine residue of LYP forms a disulfide bond with C129 to reversibly inactivate the enzyme.^[18] These examples illustrate the diverse mechanisms by which PTP activity can be regulated by virtue of the reactivity of the catalytic cysteine residue.

Thiuram disulfides are a chemically distinct class of disulfides that can inhibit enzymes containing low pK_a thiols by forming mixed disulfides.^[19] A prototype of this class of compounds, disulfiram (Figure 1, trade name Antabuse), is used clinically for the treatment of alcohol dependence.^[20] Disulfiram inhibits aldehyde dehydrogenase, an enzyme involved in alcohol metabolism, through S-thiolation, leading to acetaldehyde build-up and unpleasant symptoms following the ingestion of alcohol.^[21] Disulfiram, along with another thiuram disulfide, thiram (Figure 1), have also been shown to possess anticancer activity.^[22–24] The anticancer activity of disulfiram has been attributed to S-thiolation of several biological targets including protein kinase C (PKC),^[19] glutathione-S-transferase (GST),^[25] nuclear factor-κB^[26] and the caspases.^[27] Based on the known reactivity of disulfiram towards biological thiolates and the knowledge that LYP is inactivated by cysteine oxidation and disulfide bond formation, we hypothesized that disulfiram and other thiuram disulfides can inhibit LYP by S-thiolation.

Results and Discussion

To test this hypothesis, we first investigated the ability of disulfiram to inhibit the activity of LYP and two other closely related PTPs. Disulfiram was a moderately potent inhibitor of LYP activity (IC₅₀ = 47 ± 2 μM), but surprisingly did not inhibit the activity of PTP-PEST or CD45 at concentrations up to 500 μM. The product of disulfiram reduction and known metabolite of the drug, diethyldithiocarbamate, had no activity against LYP, PTP-PEST or CD45. While the selectivity of disulfiram for LYP over PTP-PEST and CD45 may seem counterintuitive based on the propensity of disulfiram to react with thiol-dependent enzymes, there is precedence for selective inhibition of LYP by thiophilic reagents.^[14, 28] In order to determine whether the selectivity seen *in vitro* is operative in cells, we investigated the ability of disulfiram and diethyldithiocarbamate to inhibit LYP activity in T cells. LYP is a negative regulator of early T cell receptor signaling, inactivating the tyrosine kinase Lck by dephosphorylating the phospho-tyrosine 394 position.^[29] On the other hand, CD45 is capable of dephosphorylating both phospho-tyrosine 394 and phospho-tyrosine 505 of Lck.^[30] Therefore, a LYP-selective inhibitor with cellular activity would be expected to induce an increase in phosphorylation at Y394 only, while a CD45 inhibitor would induce an increase in phosphorylation at both Y394 and Y505. The results of an in-cell LYP and CD45 inhibition study are shown in Figure 2. The T cell receptor stimulated cells show an increase in phosphorylation at Y394 in the presence of disulfiram, but no increase in phosphorylation at Y505, indicating that disulfiram shows some selectivity for LYP over CD45 in cells. The disulfiram metabolite, diethyldithiocarbamate, shows no inhibition of either enzyme, consistent with the *in vitro* data. Furthermore, disulfiram shows a dose-dependent inhibition of LYP activity in T cells.

Based on the dose-dependent, biologically relevant inhibition of LYP by disulfiram, we were interested both in identifying analogs with greater potency and also further investigating the mechanism of inhibition. To this end, we screened a library of 16 commercially available thiuram disulfides (Figure 3) for inhibition of LYP. In our screen we included the sodium salt of the disulfiram metabolite diethyldithiocarbamate (Figure 3, compound **17**) as a negative control and epigallocatechin-3,5-digallate (EGCDG)

(compound **18**, not shown), the most potent inhibitor of LYP reported so far as a positive control.^[16] Each compound in the library was screened in triplicate at 10 μ M and 50 μ M concentrations, except the positive control, EGCDG, which was screened at 100 nM and 500 nM concentrations. The results of this screening exercise are summarized in Figures S1 and 4, respectively. Compounds that showed more potent inhibition of LYP than the prototype Disulfiram (Compound **1**) were carried forward for further validation. The IC₅₀ values of these hits are summarized in Table 1.

Tetraethylthiuram disulfide (disulfiram, **1**) was a superior inhibitor of LYP compared to tetramethyl (thiram, **2**) and tetrabutyl (**4**) analogs. However, the increased steric bulk around the thiuram nitrogens with the tetraisopropyl derivative (**3**) lead to an increase in LYP inhibitory potency. Replacing one or both of the alkyl substituents on each thiuram nitrogen with an aryl substituent was generally not well tolerated, with compounds **5**, **6**, **8** and **9** showing little to no inhibition of LYP. However, among the aromatic substituted compounds, compound **7**, containing a strongly electron withdrawing aryl ring, showed the best activity against LYP. Cyclizing the substituents on the thiuram nitrogens to form a piperidine (**10**) or morpholine (**11**) ring lead to more potent inhibition. Monosubstitution of the thiuram nitrogens is also well tolerated with compounds **12**, **13** and **15** showing potent inhibition of LYP. However, aromatic monosubstitution (**14**) provided only moderate inhibition of LYP. Monosubstitution with a sulfolane moiety (**15**) provided greater inhibition of LYP than disubstitution with one sulfolane moiety and a methyl group (**16**). The most potent thiuram disulfide analog in this series was compound **13**, in which both of the thiuram nitrogen atoms are monosubstituted with N-(2-thioxothiazolidin-4-one). This is not surprising as a class of thiazolidine-2,4-diones have previously been reported to inhibit LYP at submicromolar concentrations.^[31]

Disulfiram inhibits several of its enzyme targets by forming mixed disulfides with low pK_a cysteine residues; this covalent inhibition can usually be reversed by adding thiol reducing agents such as DTT or 2-mercaptoethanol.^[19, 21, 25] To determine whether our top hit, compound **13**, is a pseudo-irreversible inhibitor of LYP activity, we first investigated time-dependent inhibition of LYP by compound **13**. Compound **13** inhibited LYP in a time-dependent manner with K_i of 1.1 $\pm$ 0.4 μ M and k_{inact} of 0.0040 $\pm$ 0.0007 s⁻¹ (Figure 5A and 5B). Activity could not be restored by prolonged dialysis even after 3 changes of the dialysis buffer to give a total of 10⁹-fold dilution (Figure 5C). Inhibition of LYP by compound **13** was partially reversed by post-incubation with DTT (Figure 5D), but could not be reversed significantly by glutathione, the physiological reducing agent (Figure S2A). All of these findings indicate that compound **13** inhibits LYP covalently by S-thiolation, and although this inhibition can be partially reversed by DTT, it is likely to be irreversible under physiological conditions.

When LYP was co-incubated with compound **13** and a reducing agent, either DTT or glutathione, the ability of compound **13** to inhibit LYP was diminished significantly, indicating that the corresponding dithiocarbamate formed may have much lower LYP inhibitory potency (Figure 5E and S2B). We have already demonstrated that this is the case for diethyldithiocarbamate (**17**). To confirm this result with compound **13**, we pre-incubated compound **13** with the reducing agents for 1 h to ensure reduction to the corresponding dithiocarbamate. The ability of compound **13** to inhibit LYP activity is significantly compromised after incubation with a reducing agent (Figures 5F and S2C), consistent with our hypothesis that a thiol-disulfide exchange is essential for LYP inhibition.

With the aim of validating the biological relevance of our best hit, we then tested compound **13** for in-cell LYP inhibition in the Jurkat T Antigen (JTAG) human T cell line. LYP negatively regulates early stage T cell receptor (TCR) signaling by dephosphorylating Lck at

position Y394.^[29] Phosphorylation of Lck at Y394 activates the kinase, which in turn phosphorylates ZAP-70 Y319. Therefore, by monitoring the phosphorylation levels of Y394 of Lck or Y319 of ZAP-70, we obtained a sensitive read-out for LYP activity in Jurkat T cells.^[16] Upon treatment of Jurkat T cells with compound **13**, phosphorylation levels of ZAP-70 Y319 increased in a dose-dependent manner in both unstimulated and stimulated cells, as measured by phospho-flow cytometry. These results indicate that compound **13** inhibits LYP activity in Jurkat T cells, promoting T cell activation (Figure 6).

In order to gain further insight into the structure activity relationship for LYP inhibition by the thiuram disulfides, we performed an atom-based 3D Quantitative-Structure-Activity-Relationship (QSAR) study.^[32, 33] By iteratively using 12 out of the 17 (70%)^[34] compounds as the training set, 3D QSAR model was generated to predict the remaining activity for the 30% of the compounds in the test set. Figure 7 displays the correlation between predicted vs. experimental remaining activity (R^2 value of 0.8). The individual contribution of each parameter used to build the QSAR model is summarized in Table 2. This data indicate the physicochemical properties of the ligands contributing to the predicted QSAR. Overall, the presence of hydrophobic/non-polar moieties and electron-withdrawing groups on the thiuram nitrogen were the most important factors contributing to low micromolar LYP inhibition, accounting for a total of 77% of the overall contribution (see also Figure S3). We suggest the following mechanistic interpretation based on the integration of our experimental and computational data. Substitution of thiuram nitrogen with electron withdrawing groups might aid in activating the nucleophilic attack by the catalytic cysteine residue of LYP onto the thiuram disulfide; whereas substitution with hydrophobic groups might provide favorable van der Waals interactions with the active site of LYP.

Conclusions

In the present study, we have screened a library of thiuram disulfides against LYP to identify several low micromolar, covalent, pseudo-irreversible LYP inhibitors. Disulfiram inhibited LYP preferentially over CD45 both *in vitro* and in Jurkat T-cells. However, the reduced metabolite of disulfiram, diethyldithiocarbamate, did not inhibit LYP activity *in vitro* or in cells indicating the importance of thiol-disulfide exchange reaction for the potency of thiuram disulfides. Computational modeling and Quantative-Structure-Activity-Relationship indicates that the presence of hydrophobic/non-polar moiety and electron-withdrawing groups on the thiuram nitrogen were the most important factors contributing to the LYP inhibition. Compound **13**, an N-(2-thioxothiazolidin-4-one) analog, was the most potent LYP inhibitor in this library with IC_{50} of 3 μ M and K_i of 1.1 μ M. Compound **13** showed covalent inhibition of LYP that cannot be reversed by dialysis but can be partially reversed by treatment with DTT. The reduced product of compound **13**, the corresponding dithiocarbamate, only weakly inhibited LYP. Compound **13** was further shown to inhibit LYP in Jurkat T-cells in a dose-dependent manner to activate T-cell signaling.

Experimental Section

General considerations

All the reagents were purchased from commercial sources and used without further purification. The library of thiuram disulfides was purchased from Aldrich Market Select. Stock solutions and serial dilutions of the substrates were made in DMSO. The cDNA fragment encoding the catalytic domain of LYP (aa 2-309) was cloned between the BamH1 and the Xho1 sites of pET28a plasmid (Novagen) in frame with a thrombin-cleavable N-terminal 6xHis-tag. Recombinant protein was purified from lysates of IPTG-induced *Escherichia coli* BL21 cells by affinity chromatography on a Ni-nitrilotriacetic acid column.

Untagged LYP was eluted by incubating the column with thrombin, followed by removal of thrombin from the protein preparation by a second chromatography step on a benzamidine column. The purified catalytic domain of human recombinant CD45 was obtained from Enzo Life Sciences. All of the enzyme activity assays were performed at ambient temperature using a buffer containing 50 mM Bis-Tris, pH 6.5, 100 mM NaCl, 1 mM Tris (2-carboxyethyl)phosphine hydrochloride (TCEP), 2 mM EDTA, and 0.01% Brij 35. Fluorescence data were collected on a Molecular Devices Spectramax M5 multimode plate reader with excitation and emission at 360 and 455 nm, respectively. The increase in the fluorescence due to the hydrolysis of the substrates was measured every 60 s for 30 min and the fluorescence values in the linear range of the Michaelis-Menten kinetics curve were used for the data analysis.

In Vitro Assay for Library Screening

The library of thiuram disulfides was created in a source plate with 1 mM DMSO solution of compounds 1–17 and 10 μ M DMSO solution of the compound 18 (epigallocatechin-3,5-digallate). A 5 μ L aliquot or a 1 μ L aliquot of each of these compounds was transferred to corresponding position in the assay plate for screening at inhibitor concentration of 50 μ M or 10 μ M, respectively. A 4 μ L aliquot of DMSO was transferred to each well containing inhibitor of the assay plate when screening at 10 μ M inhibitor concentration but not when screening at 50 μ M inhibitor concentration, to ensure a constant concentration of DMSO in each well. A 5 μ L aliquot of DMSO was transferred to each of the control wells. A 1 μ L aliquot of a DMSO solution of DiFMUP (150 μ M stock) was also transferred to the wells of the assay plates. The total amount of DMSO in each reaction well was held constant at 6% of the total reaction volume (100 μ L). A 50 nM solution of enzyme in activity buffer was preincubated with 1 mM TCEP for 30 min to ensure complete activation. The preactivated enzyme was further diluted with the activity buffer just prior to addition to the assay plate, so that addition of a 94 μ L of the diluted enzyme to each well would result in a final enzyme concentration of 5 nM. The final TCEP concentration in each reaction well was 100 μ M. The final concentration of DiFMUP was 1.5 μ M. Each compound in the library was screened in triplicate and the results were averaged. The percent relative activity for each compound was determined by factoring the measured fluorescence values for the compound treated wells over the control wells treated with DMSO only.

In Vitro Inhibition Assays

Stock solutions and serial dilutions of the compounds for determination of IC_{50} were made in DMSO. All the hits were screened at inhibitor concentrations ranging between 500 nM to 500 μ M. The total DMSO concentration in each reaction was held constant at 6% of the total reaction volume (100 μ L). The concentrations of enzymes (both LYP and PTP-PEST), TCEP and substrates were the same as those used in the library screening assays. The percent relative activity at different concentrations was determined by factoring the measured fluorescence values for the compound treated wells over the control wells treated with DMSO only. The resulting plot of inhibitor concentration versus percent enzyme activity provided the IC_{50} values.

For determination of the dissociation constant, K_i , the final concentration of LYP used was 2 nM and the concentration of DiFMUP used was 5 μ M. The remaining conditions were the same as those used in the library screening assay. The increase in the fluorescence due to the hydrolysis of the substrates was measured in 30 s intervals for 30 min and the fluorescence values in the linear range of the Michaelis-Menten kinetics curve were used for the data analysis. The dissociation constant (K_i) and the first order rate constant for covalent bond formation (k_{inact}) were calculated as described previously.^[35] In short, for each inhibitor concentration, an observed rate constant (k_{obs}) was calculated using the equation $P = (v_i/$

$k_{\text{obs}}(1 - e^{-k_{\text{obs}}t})$, where P = product formation, v_i = initial velocity, and t = time. The apparent dissociation constant (K_{app}) for the reversible enzyme inhibitor complex and the first order rate constant for covalent bond formation (k_{inact}) were obtained by a non-linear regression analysis of the derived k_{obs} values at different inhibitor concentrations with equation $k_{\text{obs}} = k_{\text{inact}}[I]/(K_{\text{app}} + [I])$. The dissociation constant (K_i) was calculated using the formula $K_i = (K_{\text{app}}/(1 + [\text{DiFMUP}]/K_{\text{mDiFMUP}}))$ where K_{mDiFMUP} is the K_{m} of DiFMUP and was determined experimentally.

Reversibility by dialysis

A 25 μM solution of LYP was preincubated with 1 mM TCEP for 1 h to ensure complete activation. After this, 500 μM of compound **13** or DMSO control was added to the enzyme and aliquots of these reactions were used to determine the activity of LYP at $t=0$ and after 90 min incubation using the conditions similar to those used in library screening assay. The reaction mixture was then dialyzed using Pierce Slide-a-Lyzer MINI 20k dialysis tubes against the activity buffer described in the general considerations for 3 exchanges of prolonged dialysis at 4°C to achieve 10^9 -fold dilution. LYP activity was determined post-dialysis and was normalized using the activity obtained with the DMSO control treated LYP at $t=0$.

Reversibility with reducing agents

A 5 nM solution of LYP was preincubated with 1 mM TCEP for 1 h to ensure complete activation. For studies involving post-incubation with reducing agent, we then incubated the activated LYP with either 5 μM of compound **13** or DMSO for 30 min. After 30 min, different concentrations of DTT or glutathione were added to the reactions and incubated for 1 h, following which the LYP activity was measured as described in library screening assays, with 5 μM DiFMUP as the substrate. For co-incubation studies, activated LYP was incubated simultaneously with different concentrations of DTT or glutathione and 5 μM compound **13** for 1 h, following which the LYP activity was measured as described above. For pre-incubation studies, 5 μM compound **13** was preincubated with different concentrations of DTT or glutathione for 1 h and then this preincubated mixture was added to the preactivated LYP and incubated 30 min further, after which the LYP activity was measured as described above. For all of these reactions, the LYP activity remaining was normalized using the activity obtained with the DMSO control treated LYP that was not treated with DTT or glutathione.

Antibodies

The polyclonal anti-pSrc(Tyr416) (cross-reactive with pLck(Tyr394)) and anti-pLck(Tyr505) antibodies were obtained from Cell Signaling Technology, Inc. The monoclonal anti-Lck antibody was obtained from Santa Cruz Biotechnology. The ECL-Plus chemiluminescence kit was obtained from GE-Amersham Biosciences.

Cell culture

Jurkat T cells expressing the SV-40 large T Antigen (JTA_g)^[36] were kept at logarithmic growth in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA), 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES pH 7.3, 2.5 mg/ml D-glucose, 100 units/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Life Technologies, Carlsbad, CA).

Immunoblotting

The indicated concentrations of inhibitors or DMSO (control) were added to cells suspended in RPMI 1640, and incubated for 1 h at room temperature. The volume of DMSO added was

held constant at less than 2% of the total volume. JTA_g cells preincubated with DMSO or inhibitor stimulated with supernatant of C305 hybridoma^[37] for 2 min or left untreated. Cells were lysed in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA pH 8.0 containing 1% NP-40, 10 µg/mL aprotinin and leupeptin, 10 µg/mL soybean trypsin inhibitor, 1 mM Na₃VO₄, and 1 mM phenylmethylsulfonyl fluoride, after which lysates were clarified by centrifugation. The protein concentration in each cell lysate was determined by Bradford protein assay (Bio-Rad) in order to normalize the amount of protein used in SDS-PAGE. Aliquots of lysates were suspended in SDS sample buffer, heated at 95 °C for 5 minutes and the boiled samples run on SDS-polyacrylamide gels. Proteins resolved by gel electrophoresis were transferred onto nitrocellulose membranes (Hybond ECL, GE Healthcare). Immunoblotting was performed using appropriate dilutions per manufacturer's instructions of unconjugated primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (purchased from GE Healthcare). Blots were developed with the enhanced chemiluminescence detection system, ECL-Plus, following manufacturer's directions.

Phospho-flow cytometry

JTA_g cells were pretreated with the indicated concentrations of compound **13** or DMSO for 30 minutes in RPMI 1640 at 37°C. Cells were then stimulated with supernatants of C305 hybridoma^[37] for 2 min at 37°C or left unstimulated. Cells were fixed immediately with BD Cytofix buffer, permeabilized using BD Phosflow Perm Buffer III and stained with AlexaFluor-488-conjugated anti-pZAP-70 (Y319) antibody according to the manufacturer's protocols (BD Biosciences, San Jose, CA). Cell fluorescence was analyzed by FACS using a BD LSR II (BD Biosciences). Data was analyzed using FlowJo software (TreeStar, Ashland, OR). The percentage of positive cells in each sample was calculated relative to the DMSO-treated controls by using the Overton cumulative histogram subtraction method.^[38] This algorithm calculates the percentage of positive cells in a test sample histogram when compared to a control sample histogram. The Overton subtraction was calculated using FlowJo software (TreeStar, Inc.).

3D QSAR modeling

Quantitative structure activity relationship (QSAR) studies were performed on the selected 17 compounds.^[32, 33] Relative percentage activity remaining upon treatment with 50 µM inhibitor was used as the desired experimental activity, as IC₅₀s were not available for all the compounds and the activity remaining upon treatment with 50 µM inhibitor correlated well with the IC₅₀s of most of the compounds. Three-dimensional conformations of all the compounds were generated using LigPrep module of Schrodinger's program.^[39] Only the lowest energy conformations were retained for the analysis. These compounds were optimally aligned using atom-based flexible alignment method. To perform the atom-based 3D-QSAR, PHASE module of the Schrodinger's program was used^[40]. This approach considers various properties of the molecule in space by constructing a potential grid around the aligned molecules. Each grid point in the 3D space is scored according to one or more of the following atomic properties: propensity for the site to be a H-bond donor, hydrophobic or non-polar, negative or positive charge centers, electron withdrawing group (such as non nitrogen and oxygen) and other atom-types. Using a grid spacing of 1 Å and partial least square (PLS) fitting model, QSAR model was generated. PLS factor of one was considered for the model generation and increasing the factor did not increase the predictive ability of the model. The model generated was visualized to understand the atomic properties and its role in predicting the binding activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Chemical Structures of disulfiram and thiram.

Figure 2.

a) Intracellular inhibition of LYP and CD45 activity by disulfiram and diethyldithiocarbamate. Top panels: anti-pLck (Y394) immunoblots of lysates of Jurkat Tag cells treated with either DMSO (lanes 1 & 2) or 75 μ M of disulfiram and diethyldithiocarbamate (lanes 3 & 4) and unstimulated (lanes 1 & 3) or stimulated (lanes 2 & 4) with C305 supernatant for 2 min. Middle panels: anti-pLck(Y505) blots of the same samples. Bottom panels: anti-Lck blots of the same samples as a loading control. b) Dose-dependent inhibition of LYP by disulfiram in T cells. JTag cells treated either with DMSO (lanes 1 & 2) or increasing concentrations of disulfiram (lanes 3–5) and either unstimulated (lane 1) or stimulated for 2 min with C305 supernatant (lanes 2–5). Upper panel shows the anti-pLck(Y394) immunoblot and lower panel shows anti-Lck loading control of the same samples.

Figure 3.
Structures of the compounds screened as potential inhibitors of LYP activity.

Figure 4.

Results of the initial screen to identify LYP inhibitors. Compounds 1–17 were screened at 50 μ M, while epigallocatechin-3,5-digallate was screened at 500 nM.

Figure 5.

Compound **13** is a covalent inhibitor of LYP. a) Compound **13** inhibits LYP in a time-dependent manner. Activity of 2 nM LYP treated with various concentrations of compound **13** was measured at 30 s intervals for 1200 s, and the observed rate constant (k_{obs}) for each reaction was calculated and described in the experimental section. b) A plot of k_{obs} vs concentration of compound **13** gave K_{app} and K_{inact} as described. c) A 25 μM solution of LYP was incubated with 500 μM of compound **13** for 90 min and then dialyzed for 3 exchanges of 1000-fold dialysis buffer to a final dilution of 10^9 -fold. The activity of LYP was normalized to a DMSO-treated sample at t=0. d) A 5 nM solution of LYP was incubated with 5 μM compound **13** for 30 min followed by post-incubation with various concentrations of DTT for 1 h. e) A 5 nM solution of LYP was co-incubated simultaneously with 5 μM compound **13** and various concentrations of DTT for 1 h. f) A 5 nM solution of LYP was incubated for 30 min with 5 μM compound **13** that had been pretreated with various concentrations of DTT for 1 h. In panels C, D, E and F, the activity of a treated sample is represented by the black bar, while the activity of an untreated control is shown in grey.

Figure 6.

Compound **13** increases activation of ZAP-70 in human T cells. JTA9 cells were pre-incubated with 25 μ M (green graphs), 50 μ M (purple graphs), or 100 μ M (red graphs) compound **13** or with DMSO alone (blue graphs) for 30 min at 37°C, followed by stimulation with C305 supernatant (solid graphs) or left unstimulated (dashed graphs) for 2 min at 37°C. Graphs show cell fluorescence after staining with an AlexaFluor-488-conjugated anti-phospho-ZAP-70 (Y319) antibody. Median fluorescence intensity (MFI) of each sample is shown. The % of positive cells was calculated for each sample by Overton subtraction. The % positive cells for the compound-treated TCR-stimulated samples compared to the DMSO-treated TCR-stimulated sample were 12.5%, 20.1%, and 26.0% for 25 μ M, 50 μ M, and 100 μ M compound **13**, respectively. The % positive cells for the compound-treated unstimulated samples compared to the DMSO-treated unstimulated sample were 8.0%, 28.6%, and 46.8% for 25 μ M, 50 μ M, and 100 μ M compound **13**, respectively.

Figure 7.

Atom-based QSAR study. QSAR model scatter plot with experimental vs predicted LYP activity remaining in the presence of 50 μ M inhibitor based on atom-based 3D-QSAR predictions (values are in %).

Table 1

Hit validation of selected hits. IC₅₀ values are in μ M.

Compound #	LYP
1	47 $\pm$ 2
3	7.0 $\pm$ 0.7
10	15.0 $\pm$ 0.5
11	23 $\pm$ 3
12	10 $\pm$ 1
13	3.0 $\pm$ 0.3
15	13 $\pm$ 1
16	34 $\pm$ 3

Table 2

Percentage contribution of each atomic property in predicting the activity of a thiuram disulfide based on the atom-based QSAR model.

Atomic property	Percentage contribution
H-bond donor	0.16
Hydrophobic or non-polar	64
Negative ionic	3.4
Positive ionic	0.61
Electron-withdrawing	13
Miscellaneous	19