# Gold(I)-Mediated Inhibition of Protein Tyrosine Phosphatases: A Detailed in Vitro and Cellular Study

Divya Krishnamurthy,[†,‡] Mark R. Karver,[†,§] Edoardo Fiorillo,[‡] Valeria Orrú,[‡] Stephanie M. Stanford,[‡] Nunzio Bottini,[‡] and Amy M. Barrios*[,†]

*Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah 84112, Department of Orthopaedic Surgery and USC Institute for Genetic Medicine, Keck School of Medicine of the University of Southern California, Los Angeles, California 90033, Department of Chemistry, University of Southern California, Los Angeles, California 90089*

*Received January 31, 2008*

Gold(I) complexes containing N-heterocyclic carbene ligands were synthesized, characterized, and along with the antiarthritic drug, auranofin, tested as inhibitors of the cysteine-dependent protein tyrosine phosphatases, which are implicated in several disease states. These compounds exhibit potencies in the low micromolar range against the enzymes in vitro. At therapeutically relevant concentrations, all compounds inhibit PTP activity in Jurkat T leukemia cells with some selectivity. In addition, the gold−carbene compounds inhibit phosphatase activity in primary mouse thymocytes.

## Introduction

The protein tyrosine phosphatases (PTPs) are a family of enzymes that play integral roles in various physiological processes including regulation of signaling pathways such as T-cell signaling, cell growth, differentiation, immune response, and survival.[1,2] These enzymes serve to dephosphorylate phosphotyrosine residues of proteins and in conjunction with the balancing action of protein tyrosine kinases (PTKs), PTPs help maintain phosphorylation states in proteins.[1] Improper regulation or imbalances in PTP activity is associated with a number of diseases including obesity, cancer, and various autoimmune disorders.[2,3] For instance, PTP1B has been implicated in obesity and diabetes, while CD45 has been implicated in neurological disorders.[3,4] Recently, a single nucleotide polymorphism (C1858T) in the PTPN22 gene, which encodes a tyrosine phosphatase, LYP, [a] has been linked to various autoimmune diseases including type 1 diabetes, rheumatoid arthritis, Graves disease, lupus, and others.[5,6] The PTPs are thus emerging targets of drug design and the development of inhibitors of this family of enzymes has attracted considerable attention as potential therapeutics for these diseases.[3]

The therapeutic effects and biological activity of gold(I) compounds toward the autoimmune disease rheumatoid arthritis (RA) have been a center of focus for many years.[7] The gold-containing drugs auranofin (triethylphosphine(tetra-*O*-acetyl-1-thio-$\beta$-D-glucopyranose)gold(I), Figure 1A) solganol (sodium aurothioglucose), and myochrisin (sodium aurothiomalate) are utilized in the treatment of RA and are administered either orally or intramuscularly, but their exact mechanism of action is not clearly established.[8,9] Several pathways and biological targets have been proposed to account for the molecular mechanisms and chrysotherapeutic efficacy of the $Au^{I}$ ion. Treatment with gold is said to suppress immune responses by interfering with immunoglobulin synthesis and T lymphocyte proliferation.[10] Gold may also exert its immunomodulatory properties by altering the differentiation of monocytes into effector cells and their ability to form superoxide ions.[11] Another possible effect associated with $Au^{I}$ drugs is the inhibition of osteoclastic bone resorption through the inhibition of the cathepsins.[12,13]

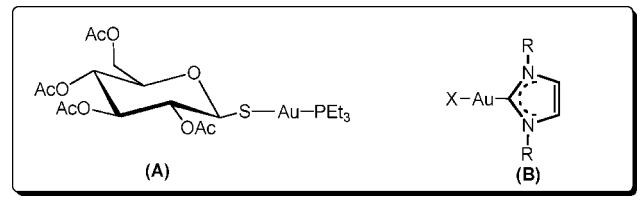



**Figure 1.** (A) Structure of auranofin. (B) Generalized structure of a gold(I) N-heterocyclic carbene complex.

The gold complexes used in clinical practice as disease modifying antirheumatic drugs are typically composed of phosphine and/or thiolate ligands in the coordination sphere of the gold(I). Of late, there has been considerable interest in N-heterocyclic carbenes (NHCs) as alternatives to phosphines as ligands for the soft $Au^{I}$ ion.[14–17] In particular, gold(I) N-heterocyclic carbene complexes (Figure 1B) are reported to exhibit antimitochondrial activity, which is of importance in developing antitumor agents.[16,18,19] The relative ease of systematic modification of the NHC substituents and the comparable donor properties of NHCs to phosphines render the NHCs attractive ligands, particularly because the groups on the ligand backbone can be systematically varied, resulting in complexes with differing sterics or lipophilicities.

While it is well established that gold(I) complexes bearing thiolate or phosphine ligands provide relief from rheumatoid arthritis through their anti-inflammatory and immunosuppressive properties, surprisingly, the corresponding effects of their NHC counterparts are yet to be investigated in detail. Given the affinity of gold(I) for soft thiol ligands,[8] the gold(I) NHCs show promise as potential inhibitors of PTPs. This is especially significant because the active site of PTPs contains a functionally important cysteine residue which acts as a nucleophile to achieve

* To whom correspondence should be addressed. Phone: (801) 581-3198. Fax: (801) 581-7087. Email: amy.barrios@utah.edu

† Department of Medicinal Chemistry, University of Utah.

‡ Department of Orthopaedic Surgery and USC Institute for Genetic Medicine, Keck School of Medicine of the University of Southern California.

§ Department of Chemistry, University of Southern California.

[a] Abbreviations: RA, rheumatoid arthritis; auranofin, triethylphosphine-(tetra-*O*-acetyl-1-thio-$\beta$-D-glucopyranose)gold(I); solganol, sodium aurothioglucose; myochrysin, sodium aurothiomalate; NHC, N-heterocyclic carbene; LYP, lymphocyte-associated protein tyrosine phosphatase (PTPN22); DTT, dithiothreitol; JTAg, Jurkat T leukemia cells expressing SV-40 large T antigen; IPTG, isopropyl-$\beta$-D-1-thiogalactopyranoside; DiFMUP, 6,8-difluoro-4-methylumbelliferone phosphate.

10.1021/jm800101w CCC: $40.75 
Published on Web 07/08/2008

phosphate hydrolysis, and the gold-containing drug, myochrisin, inhibits CD45 and PTP1B in vitro.[20] However, a detailed investigation of $Au^I$-mediated PTP inhibition in vitro and in cells is yet to be reported. In addition, the immunomodulatory effects of the gold(I) containing drugs raise interesting possibilities regarding inhibition of the PTPs involved in the immune system by gold(I) NHCs. The synthesis of gold(I) NHC complexes and their inhibition studies therefore would (a) aid in determining the potential inhibitory activity of this class of compounds toward PTPs in vitro and in cells, (b) provide insight into their mechanism of action, (c) allow a direct comparison of how efficacy of inhibition in vitro translates in vivo, and (d) ultimately facilitate the development of novel therapeutics by yielding a wealth of information on the mode of interaction, precise endogenous targets, and specificity of the inhibitor toward a particular PTP. In this study, we report the synthesis of gold(I) complexes of N-heterocyclic carbenes containing a methylimidazol-2-ylidene backbone, detailed inhibition studies of protein tyrosine phosphatases in vitro, and the cellular effects of the gold(I) inhibitors on these enzymes in Jurkat TAg cells and mouse thymocytes.

## Experimental Section

**General Considerations.** All reagents and solvents were purchased from commercial sources and used without further purification unless otherwise noted. Preparation of air or moisture-sensitive materials was carried out under an atmosphere of dry nitrogen. Thin layer chromatography (TLC) was performed using EMD silica gel 60 precoated plates (0.20 mm thickness). Column chromatography was performed using EMD silica gel (60−200 mesh). The precursor imidazolinium salts, *N*-benzyl-*N*′-methylimidazolinium bromide and *N*-methylbenzyl-*N*′-methylimidazolinium bromide, auranofin (**1**), and [$(Me_2Im)Au^ICl$] (**2**) were prepared following literature methods.[13,16,21,22] Catalytic domains of *Yersinia enterocolitica* PTP (YopH), human T-cell PTP (TCPTP), PTP1B, and CD45 were obtained from Calbiochem, New England Biolabs or Biomol in purified form and used as received. The modified pBAD plasmid encoding the catalytic domain of HePTP (aa 44−339) in frame with a noncleavable 6xHis tag was a kind gift of Lutz Tautz.[23] cDNA fragments encoding the catalytic domains of LYP (aa 2−309) and PTP-PEST (aa 2−323) were cloned between the BamH1 and the Xho1 sites of the pET28a plasmid (Novagen) in frame with a cleavable N-terminal 6xHis-tag. Recombinant proteins were purified from lysates of isopropyl-$\beta$-D-1-thiogalactopyranoside (IPTG) induced *E. coli* BL21 cells by affinity chromatography on Ni-nitrilotriacetic acid columns. 6xHisHePTP was eluted using 250 mM imidazole. Untagged LYP and PTP-PEST were eluted by incubating columns with thrombin, followed by removal of thrombin from the protein preparation by a second chromatography step on benzamidine columns. $^1$H NMR spectra were recorded on a Bruker AC 250 MHz spectrometer at ambient temperature. Chemical shifts (in ppm) were referenced to the residual solvent peak. Elemental analyses were performed by Atlantic Microlab, Norcross, GA. Fluorescence data were collected on either a Molecular Devices AD Analyst multimode plate reader with excitation and emission at 360 and 425 nm, respectively, and a dichroic mirror at 400 nm or a Molecular Devices Spectramax M5 multimode plate reader with excitation and emission at 360 and 455 nm.

**Synthesis of [(*p*-MeBzMeIm)$Au^ICl$], (3).** To a stirred solution of imidazolinium salt, *N*-methylbenzyl-*N*′-methylimidazolinium bromide (40 mg, 0.16 mmol) in $CH_2Cl_2$/methanol (1:1, 2 mL) was added tetraethylammonium chloride (29 mg, 0.16 mmol) and silver(I) oxide (20 mg, 0.08 mmol) under $N_2$ and the reaction mixture was allowed to stir overnight. To the above, dimethylsulfide gold(I) chloride (28 mg, 0.09 mmol) was added and the suspension stirred an additional 6 h under $N_2$. The resultant gray precipitate was filtered over a bed of celite, the colorless filtrate isolated, and the volatiles removed under reduced pressure to yield a colorless oil. Column chromatography on silica gel ($CH_2Cl_2$/ethyl acetate, 1:1) followed by recrystallization from $CH_2Cl_2$/pentane yielded pure **1** as a white solid. Yield: 13.7 mg, 21%. $^1$H NMR (250 MHz, $CDCl_3$, $\delta$): 2.34(s, 3H, Ph-$CH_3$), 3.84 (s, 3H, N-$CH_3$), 5.31(s, 2H, $CH_2$), 6.85 (s, 1H, CH), 6.90 (s, 1H, CH), 7.20 (m, 4H, CH). Anal. calcd for $C_{12}H_{14}N_2AuCl\cdot 0.25EtOAc$: C, 35.43; H, 3.66; N, 6.36. Found C, 35.50; H, 3.55; N, 6.42.

**Synthesis of [(BzMeIm)$Au^ICl$], (4).** A preparation method similar to that used for compound **3** resulted in the formation of **4** as a white solid. Yield: 10.7 mg, 21%. $^1$H NMR (250 MHz, $CDCl_3$, $\delta$): 3.86 (s, 3H, N-$CH_3$), 5.36(s, 2H, $CH_2$), 6.87 (s, 1H, CH), 6.92 (s, 1H, CH), 7.35 (m, 5H, CH). Anal. calcd for $C_{11}H_{12}N_2AuCl$: C, 32.65; H, 2.99; N, 6.92. Found C, 32.60; H, 3.08; N, 6.50.

**Enzyme Activation and in Vitro Inhibition Assays.** Inhibition assays were performed at room temperature in a buffer containing 50 mM Tris, pH 6.5, 100 mM NaCl, 1 mM dithiothreitol (DTT), 2 mM EDTA, and 0.01% Brij 35 for all enzymes. The following final concentrations of enzyme were used: 1.34 nM PTP1B, 5 nM LYP, 80 nM PTP-PEST, 10 nM HePTP, 0.39 nM YopH, 1.0 nM CD45, and 0.79 nM TCPTP. Stock solutions and serial dilutions of substrate and the inhibitors were made in DMSO. Aliquots of substrate and inhibitor stocks were taken such that the combined volume of DMSO was held constant and did not exceed 5% of the total reaction volume. Prior to the reaction, each enzyme was preincubated with 1 mM DTT for 30 min. Enzyme activity was measured at room temperature (21−24 °C) in 96-well plates, with fixed substrate (6,8-difluoro-4-methylumbelliferone phosphate, DiFMUP) concentration of 25 $\mu$M for all enzymes and Au(I) inhibitor concentrations ranging between 500 nM and 1.0 mM. Assays were conducted in triplicate at each concentration and the results averaged. The fluorescence increase (due to substrate hydrolysis) was measured every 60 s for 30 min. The resulting plot of inhibitor concentration versus percent enzyme activity provided the $IC_{50}$ values.

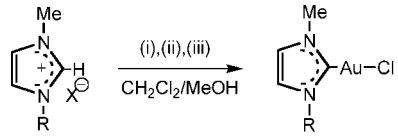
**Reversibility Assays.** The reversibility of Au(I)-mediated PTP inhibition was demonstrated in experiments in which 2.50 nM LYP was preincubated with 1 mM DTT for 30 min, followed by incubation of the activated enzyme with 25 $\mu$M complex **3** for 30 min. The incubated PTP−Au(I) mixture and a control reaction with no Au(I) inhibitor present were subjected to an additional 30 min of incubation with varying concentrations (0−6.25 mM) of L-cysteine. The substrate concentration was fixed at 25 $\mu$M, and the activity of LYP was measured in triplicate at each L-cysteine concentration.

**Lineweaver−Burk Plot Assays.** The activity of LYP was measured in the absence and presence of fixed concentrations of inhibitor at a series of substrate concentrations. Assays were conducted in triplicate for each concentration of substrate. Inhibitor concentrations were chosen such that they were approximately 4-fold higher than, 10-fold lower than, and close to the $IC_{50}$ value of the complex investigated. The reciprocal of the reaction rate was plotted as a function of the reciprocal of the substrate concentration for each concentration of inhibitor.

**Antibodies and Reagents.** The C305 hybridoma producing a monoclonal anti T cell receptor beta (TCRbeta) antibody was obtained from the American Type Culture Collection, VA. Anti-CD3 and anti-CD4 antibodies were purchased from BD Biosciences. Streptavidin was obtained from Sigma. The polyclonal anti-pTyr505-Lck and anti-pTyr416-Src antibodies were obtained from Cell Signaling Technology, Inc., while the anti-Lck (3A5) monoclonal antibody was from Santa Cruz Biotechnology. The ECL-Plus Chemiluminescence kit was obtained from GE-Amersham Biosciences.

**Cells and Cell Treatments. Jurkat T Cells.** Jurkat T leukemia cells expressing SV-40 large T Antigen (JTAg)[24] were kept at logarithmic growth in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES pH 7.3, 2.5 mg/mL D-glucose, and 100 units/mL of penicillin and 100 $\mu$g/mL of streptomycin. Fixed concentrations of Au(I)-inhibitors (100 $\mu$M of compound **1**, 200 $\mu$M of **2**, 140 $\mu$M of **3**, and 100 $\mu$M of **4**) or DMSO (control) were added to 20

**Scheme 1.** Synthesis of Gold(I) N-Heterocyclic Carbene Complexes[a]




X = $BF_4$, R = Me, **2**
X = Br, R = *p*-*Me*Bz, **3**
X = Br, R = Bz, **4**

[a] Conditions: (i) tetraethylammonium chloride; (ii) $Ag_2O$, overnight; (iii) [$(Me_2S)$AuCl], 6 h.

$\times 10^6$ cells suspended in 800 $\mu$L 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. JTAg cells preincubated with DMSO or inhibitor were divided into 400 $\mu$L aliquots containing $10 \times 10^6$ cells and stimulated with supernatants of C305 hybridomas 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 $\mu$g/mL apoprotein and leupeptin, 10 $\mu$g/mL soybean trypsin inhibitor, 1 mM $Na_3VO_4$ and 1 mM phenylmethylsulfonyl fluoride, after which lysates were clarified by centrifugation at 13200 rpm for 20 min. The total protein concentration in each cell lysate was determined by the Bradford protein assay (Bio-Rad) in order to normalize the amount of protein used in SDS-PAGE.

**Mouse Thymocytes.** Thymocytes were isolated from the homogenized thymi of 6-week old C57BL/6 mice (Taconic Farms, Inc.) after depletion of red blood cells with lysis buffer following standard procedures. Fixed concentrations of Au(I)-inhibitors (100 $\mu$M of compound **1**, 200 $\mu$M of **2**, 140 $\mu$M of **3**, and 100 $\mu$M of **4**) or DMSO (control) were added to $20 \times 10^6$ cells suspended in 800 $\mu$L 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. Thymocytes preincubated with DMSO or inhibitor were also divided into 400 $\mu$L aliquots containing $10 \times 10^6$ cells, treated with biotinylated CD3 and CD4 antibodies for 30 min, and stimulated with the cross-linker, streptavidin for 1.5 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 $\mu$g/mL apoprotein and leupeptin, 10 $\mu$g/mL soybean trypsin inhibitor, 1 mM $Na_3VO_4$ and 1 mM phenylmethylsulfonyl fluoride, after which lysates were clarified by centrifugation at 13200 rpm for 20 min. The total protein concentration in each cell lysate was determined by the Bradford protein assay (Bio-Rad) in order to normalize the amount of protein used in SDS-PAGE.

**SDS-PAGE and Immunoblots.** Aliquots of lysates were suspended in SDS sample buffer, heated at 95 °C for 5 min, and the boiled samples run on 10% SDS-polyacrylamide gels. Proteins resolved by gel electrophoresis were transferred onto nitrocellulose membranes, which were immunoblotted with the appropriate antibody (rabbit polyclonal phospho-Src family [Tyr416] or rabbit polyclonal phospho-Lck [Tyr505]) diluted as per manufacturers instructions, followed by horseradish peroxidase-conjugated secondary antibody, antirabbit IgG at 1:3000 dilution. The amounts of Lck were determined by blotting using a monoclonal anti-Lck antibody at 1:1000 dilution, followed by peroxidase-conjugated antimouse IgG at 1:3000 dilution. Blots were developed with the enhanced chemiluminescence detection system, ECL-Plus, following manufacturer's directions.

## Results and Discussion

**Synthesis of Complexes 3 and 4.** The gold(I) NHC derivatives were prepared from the appropriate imidazolinium salts following a commonly used synthetic pathway detailed in the literature (Scheme 1).[16] This route entails the formation of a silver carbene complex in situ by the treatment of the corresponding imidazolinium salt with $Ag_2O$ and subsequent transmetalation with [$(Me_2S)Au^ICl$] to generate the analogous Au(I)−NHC complex in good yields (Scheme 1). Analytically pure compounds were obtained by column chromatography and overall yields reported are based on the amount of gold(I) starting material.

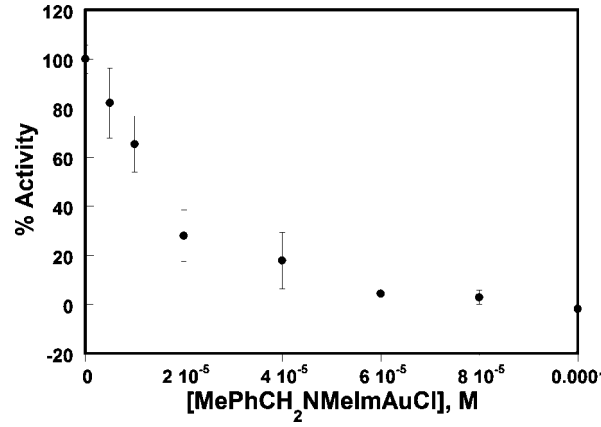



**Figure 2.** Representative $IC_{50}$ curve of PTP (LYP) inhibition by complex **3**. Conditions: 5 nM LYP, 25 $\mu$M DiFMUP, 1 mM DTT, pH 6.5.

**Table 1.** $IC_{50}$ Values (in $\mu$M) of the Gold(I) Inhibitors (**1**−**4**) with Several PTPs

| | HePTP | CD45 | TCPTP | YopH | PEST | PTP1B | LYP |
|---|---|---|---|---|---|---|---|
| **1** | 150 | >1000 | >1000 | >1000 | 9 | >400 | 200 |
| **2** | 275 | >400 | >400 | >400 | 34 | 76 | 150 |
| **3** | 22 | >150 | >150 | ~150 | 7 | 40 | 11 |
| **4** | 22 | >150 | ~150 | >150 | 9 | 26 | 10 |

**Effect of Gold(I)−NHC Complexes on Tyrosine Phosphatase Activity in Vitro.** The tyrosine phosphatase family of enzymes contain a conserved cysteine residue in their catalytic site, which acts as a nucleophile to effect hydrolysis of the phosphotyrosine substrate via a thiophosphoryl intermediate.[3,25] This active site cysteine thiol is rendered particularly acidic due to stabilization of the thiolate anion through hydrogen bonding with other residues in close proximity.[3] On the basis of the high affinity of gold(I) for thiols, particularly those with low $pK_a$ values,[13,26] it is expected that the active site cysteine is a likely target in inhibition by $Au^I$−NHCs. The ability of the gold(I) NHC complexes and the $Au^I$ drug, auranofin to inhibit PTP activity was examined.

A representative $IC_{50}$ curve for the inhibition of the phosphatase LYP by complex **3** is shown in Figure 2. Table 1 lists the gold(I) inhibitors investigated, the different PTPs assayed, and their corresponding $IC_{50}$ values. These data indicate that complex **4**, which contains a benzyl-substituted carbene ligand and the structurally similar **3**, that bears an additional methyl group on the benzyl ring of the carbene, exhibit maximum inhibitory potency for the PTPs assayed in this study. Significantly, among the carbene complexes studied herein, **3** and **4** were more potent inhibitors than complex **2,** which contains the same NHC backbone as the former but bears a methyl substituent in place of the benzyl group. This suggests some specific interaction of the benzyl side chain in **3** and **4** with the PTP active site, likely due to electronic effects or steric constraints. In all but two cases, the gold(I)−NHC complexes show enhanced tyrosine phosphatase inhibition compared to phosphine-containing auranofin. The exceptions are HePTP wherein the $IC_{50}$ value for carbene-bound **2** is approximately two times higher than that observed for auranofin, and PTP-

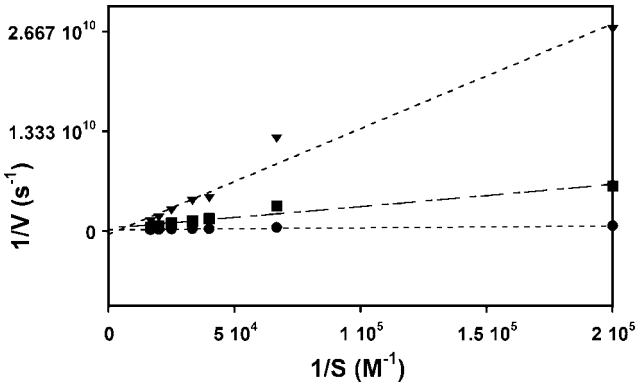



**Figure 3.** Lineweaver−Burk plot in the absence (●) and in the presence of 100 $\mu$M (▼) and 25 $\mu$M (■) complex **3**.

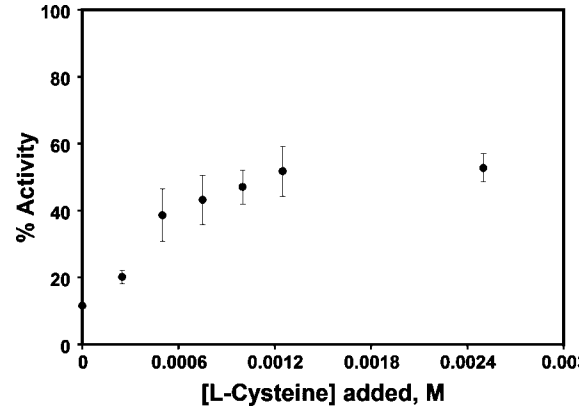



**Figure 4.** Reversibility of complex **3** mediated LYP inhibition with varying concentrations (0−6.25 mM) of L-cysteine.

PEST, where auranofin is one of the better inhibitors with a potency similar to **3** and **4**.

The different PTPs are inhibited to varying extents by the series of gold(I) compounds in a dose-dependent manner. Interestingly, all complexes exhibit significantly lower inhibition of the receptor type PTP CD45 and the nonreceptor type TCPTP, two enzymes representing distinct PTP subfamilies from LYP.[1] However, the gold compounds do inhibit PTP1B, which is sequentially homologous to TCPTP,[27] significantly better than the latter (Table 1).

The data outlined in Table 1 clearly indicate that compounds **3** and **4** are effective inhibitors of PTPs showing a slight specificity for HePTP ($IC_{50}$ = 22 $\mu$M for **3** and **4**), LYP ($IC_{50}$ = 11 $\mu$M for **3** and 10 $\mu$M for **4**) and closely related PTP-PEST ($IC_{50}$ = 7 $\mu$M for **3** and 9 $\mu$M for **4**) over the other PTPs, while compound **2** is a moderate inhibitor, exhibiting a slight preference for PTP1B ($IC_{50}$ = 76 $\mu$M) and PTP-PEST ($IC_{50}$ = 34 $\mu$M) over the rest of the phosphatases investigated. The inhibition of LYP in the low micromolar range by compounds **3** and **4** in vitro is of importance for several reasons. First, a gain-of-function LYP variant (R620W) is associated with several autoimmune diseases, underscoring the need for inhibitors of this phosphatase.[5,6] Second, barring two recent examples, there are no LYP inhibitors reported to date.[28,29] Third, compounds **3** and **4** inhibit LYP in the low micromolar range with modest selectivity for LYP over HePTP, PTP1B, CD45, TCPTP, and YopH with the exception of PTP-PEST. Finally, all of this data taken together highlights the possibility of developing novel, gold(I)-based, small-molecule inhibitors of LYP.

The observed differences in activity of the series of $Au^I$ inhibitors corroborate the hypothesis that a subtle combination of electronics and sterics in the ligand bound to the gold(I) ion leads to dramatic differences in inhibition. Molecular modeling or docking studies with individual members of the PTP family would provide further insight into the specific interaction of the inhibitors with the enzyme active site and help determine the factors at play, resulting in a more detailed representation of the structure−activity relationship. This in turn, would greatly aid in the development of inhibitors that target a specific PTP.

Because the gold(I) is expected to bind to the active site cysteine residue, it follows that the inhibition is probably competitive. To determine the mode of gold(I)-mediated inhibition, a representative example the phosphatase LYP was treated with varying substrate and inhibitor (complex **3**) concentrations and a double- reciprocal plot of rate as a function of substrate concentration was constructed. The Lineweaver−Burk plot shown in Figure 3 is indicative of competitive behavior in which the inhibitor competes with the substrate for the enzyme active site, leading to loss of enzyme activity.

The reversibility of inhibition was examined by the addition of increasing concentrations of L-cysteine to the enzyme (LYP) preincubated with inhibitor **3**. Upon addition of thiolate, the inhibited enzyme partially regained its activity, which demonstrates that gold(I) inhibitors are reversible (Figure 4). Collectively, these data point to a competitive, reversible mechanism of inhibition of PTPs by gold(I) compounds, with modest selectivity for certain PTPs.

**Cellular Studies of $Au^I$-Mediated Protein Tyrosine Phosphatase Inhibition.** In addition to numerous PTKs, nearly half of all the PTPs are expressed by T cells.[30] Many of these phosphatases and kinases carry out integral functions by regulating T cell receptor (TCR) signaling, a cascade of events triggered by engagement of the TCR by a specific antigen, which ultimately induces the proliferation and differentiation of T cells. Examples of the PTPs which modulate T cell activation include CD45, LYP, PTP-PEST, HePTP, and TCPTP.[31] These perform regulatory (negative or positive) roles by dephosphorylating TCR-associated PTKs such as the Src, Syk, and Tec families and aberrancies in their function can lead to autoimmunity.[5,6,30]

Compounds **1**−**4** show promise as PTP inhibitors in cells because in vitro results (Table 1) indicate that they are reasonably potent inhibitors of some of the phosphatases involved in TCR signaling, particularly PTP-PEST, HePTP, and LYP, and moreover show moderate specificity for these enzymes over CD45 and TCPTP. To evaluate the ability of the gold(I) compounds to inhibit PTPs in T cells, complexes **1**−**4** were tested on the Jurkat T Antigen (JTAg) human T cell line. As a read-out of PTP activity, we analyzed the phosphorylation of Lck, a PTK involved in the initial stages of TCR signaling whose activity is positively regulated by tyrosine phosphorylation at Tyr394 and negatively regulated by phosphorylation at Tyr505. Lck is a well-known physiological substrate of the PTPs CD45 and LYP through its two major phosphorylation sites at Tyr505 and Tyr394, respectively.[30,32] Tyr394 of Lck is also dephosphorylated by PTP-PEST in T cells (Y. Arimura and T. Mustelin, personal communication).

Following incubation in the absence (0 $\mu$M, control) or presence of compounds (100 $\mu$M of compound **1**, 200 $\mu$M of **2,** 140 $\mu$M of **3**, and 100 $\mu$M of **4**) for 1 h, cells were left untreated or stimulated with C305 for 2 min at 37 °C and subsequently lysed. Lysates were probed with anti-pSrc(Tyr416), an antibody which selectively recognizes the phosphorylated Tyr416 in Src, and in T cell lysates cross-reacts with the equivalent pTyr394 site of Lck. Anti-pSrc(Tyr416) immunoblots

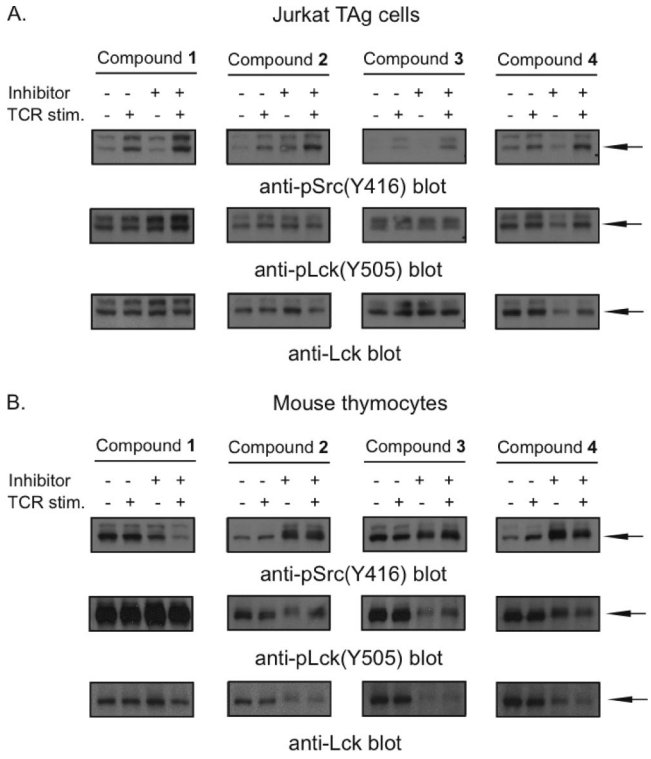



**Figure 5.** (A), Top panels, anti-pSrc(Tyr416) immunoblots of lysates of JTAg cells treated with compounds **1**−**4** (lanes 3 and 4 in each panel) or untreated (lanes 1 and 2 in each panel) and either left untreated (lanes 1 and 3 in each panel) or stimulated (lanes 2 and 4 in each panel) with C305 for 2 min. Middle panels, anti-pLck(Tyr505) of same filters. Bottom panels, anti-Lck blot of same samples. (B) Top panels, anti-pSrc(Tyr416) immunoblots of lysates of mouse thymocytes treated with compounds **1**−**4** (lanes 3 and 4 in each panel) or untreated (lanes 1 and 2 in each panel) and either left untreated (lanes 1 and 3 in each panel) or stimulated (lanes 2 and 4 in each panel) with biotinylated anti-CD3 and anti-CD4, followed by cross-linking with streptavidin for 1.5 min. Middle panels, anti-pLck(Tyr505) of same filters. Bottom panels, anti-Lck blot of same samples.

of the lysates demonstrated increased phosphorylation at 56 kDa, (Figure 5A, top panel) relative to the respective controls, corresponding to higher phosphorylation of Lck at Tyr394. Thus, treating cells with the gold(I) inhibitors **1**−**4**, increased Tyr394 phosphorylation of Lck suggesting that the compounds inhibited the PTP(s) involved in regulating this site in T cells, namely LYP and PTP-PEST. To assess whether the compounds caused any parallel increase in phosphorylation of Lck at the Tyr505 residue, the blots were probed with anti-pLck(Tyr505), an antibody that is specific for the Tyr505 phosphorylation site of Lck. Lck phosphorylation at Tyr505 was unaffected (Figure 5A, middle panel), indicating that CD45 is not significantly inhibited by the gold compounds. As a loading control, the blots were probed with anti-Lck antibody to determine the total amount of Lck present in the lysates (Figure 5A, bottom panel). The data obtained in JTAg cells are in excellent agreement with the pattern of PTP inhibition observed in vitro, where the compounds were the least potent in inhibiting CD45 and better inhibitors of LYP and PTP-PEST (for example, compounds **1** and **4** were 5 and 15 times more active toward LYP, respectively, while **2** and **3** were 3 and 15 times more potent compared to CD45). Our preliminary data suggest that the compounds are able to inhibit LYP and PTP-PEST activity in cultured cells, resulting in increased activation of Src family kinases in T cells. However, the effect of the compounds on overall TCR signaling is difficult to predict at this stage because nonspecific inhibition of other PTPs (for example HePTP) and/or other proteins involved in the signaling by the gold complexes cannot be ruled out.

We then tested whether the compounds would show similar effects in primary T cells. Freshly isolated mouse thymocytes were incubated with different amounts of gold(I) inhibitors (100 $\mu$M of compound **1**, 200 $\mu$M of **2**, 140 $\mu$M of **3**, 100 $\mu$M of **4**) for 1 h, stimulated with anti-CD3 and anti CD4 antibodies, plus a secondary cross-linking antibody for 1.5 min at 37 °C, lysed and blotted with anti-pSrc(Tyr416). Although only low levels of TCR stimulation could be achieved in thymocytes, the gold(I)-carbene compounds **2**, **3**, and **4** caused increased phosphorylation of Lck on Tyr394 (Figure 5B, top panel), which is consistent with inhibition of PEP (mouse homologue of LYP) and PTP-PEST. We noticed that compound **1** did not significantly affect phosphorylation of Lck on Tyr394 in thymocytes. When blots were reprobed with the anti-pLck(Tyr505) antibody, we found that similar to JTAg cells, phosphorylation of Lck at Tyr505 was not affected in mouse thymocytes (Figure 5B, middle panel, anti-Lck control blots are shown in bottom panels) by compounds **2**, **3**, and **4**, thus excluding a substantial inhibition of CD45 in these cells. However, compound **1** appeared to enhance phosphorylation of Lck at Tyr505 in JTAg cells, which could be due to the nonspecific action of auranofin elsewhere in a pathway that controls CD45 and/or the corresponding kinase, Csk. A possible explanation for the observed differences in the effect of compound **1** on JTAg cells and thymocytes is differential uptake of the inhibitors by mouse thymocytes relative to JTAg cells. Alternatively, considering that auranofin is not expected to significantly inhibit LYP at the concentrations used in our cellular experiments and that LYP and PEP are highly expressed in both cell types (V. Orru, E. Fiorillo, and N. Bottini; unpublished observation), the observed data would be compatible with a differential pattern of PTP expression in the two cell types, with thymocytes expressing less PTP-PEST than JTAg cells.

## Conclusions

A series of gold(I) compounds (**1**−**4**) were tested for their activity against protein tyrosine phosphatases in vitro and in cells and each was capable of inhibiting PTPs in vitro, with differing potencies. Among the complexes investigated, the gold(I) N-heterocyclic carbene derivatives **3** and **4** exhibited higher activity in vitro, while all complexes were poor inhibitors of CD45 and TCPTP compared to the other PTPs assayed. The affinity of $Au^{I}$ for thiolate ligands suggests that inhibition occurs through binding of the complex to the active site cysteine residue and the competitive, reversible inhibition scheme observed here supports this mechanism. Protein phosphorylation data obtained on a human T cell line and primary mouse thymocytes incubated with the compounds are compatible with intracellular inhibition of LYP and PTP-PEST but not of CD45, which correlates well with the results obtained in vitro. The data suggest that with comprehensive structure−activity relationship studies, therapeutically relevant, membrane-permeable, and PTP-selective small molecule gold(I) inhibitors can eventually be developed.

**Supporting Information Available:** Table S1 listing elemental analysis data for compounds **3** and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Alonso, A.; Sasin, J.; Bottini, N.; Friedberg, I.; Osterman, A.; Godzik, A.; Hunter, T.; Dixon, J.; Mustelin, T. Protein tyrosine phosphatases in the human genome. *Cell* **2004**, *117*, 699–711.

(2) Zhang, Z. Y. Protein tyrosine phosphatases: structure and function, substrate specificity, and inhibitor development. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 209–234.
(3) Bialy, L.; Waldmann, H. Inhibitors of protein tyrosine phosphatases: next-generation drugs. *Angew. Chem., Int. Ed.* **2005**, *44*, 3814–3839.
(4) Elchebly, M.; Payette, P.; Michaliszyn, E.; Cromlish, W.; Collins, S.; Loy, A. L.; Normandin, D.; Cheng, A.; Himms-Hagen, J.; Chan, C. C.; Ramachandran, C.; Gresser, M. J.; Tremblay, M. L.; Kennedy, B. P. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* **1999**, *283*, 1544–1548.
(5) Vang, T.; Congia, M.; Macis, M. D.; Musumeci, L.; Orru, V.; Zavattari, P.; Nika, K.; Tautz, L.; Tasken, K.; Cucca, F.; Mustelin, T.; Bottini, N. Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat. Genet.* **2005**, *37*, 1317–1319.
(6) Bottini, N.; Vang, T.; Cucca, F.; Mustelin, T. Role of PTPN22 in type 1 diabetes and other autoimmune diseases. *Semin. Immunol.* **2006**, *18*, 207–213.
(7) Forestier, J. Rheumatoid arthritis and its treatment by gold salts. *J. Lab. Clin. Med.* **1935**, *20*, 827–840.
(8) Shaw, C. F. Gold-based therapeutic agents. *Chem. Rev.* **1999**, *99*, 2589–2600.
(9) Brown, D. H.; Smith, W. E. The Chemistry of the Gold Drugs Used in the Treatment of Rheumatoid Arthritis. *Chem. Soc. Rev.* **1980**, *9*, 217–240.
(10) Lorber, A.; Simon, T.; Leeb, J.; Peter, A.; Wilcox, S. Chrysotherapy. Suppression of immunoglobulin synthesis. *Arthritis Rheum.* **1978**, *21*, 785–791.
(11) Hashimoto, K.; Whitehurst, C. E.; Matsubara, T.; Hirohata, K.; Lipsky, P. E. Immunomodulatory effects of therapeutic gold compounds. Gold sodium thiomalate inhibits the activity of T cell protein kinase C. *J. Clin. Invest.* **1992**, *89*, 1839–1848.
(12) Chircorian, A.; Barrios, A. M. Inhibition of lysosomal cysteine proteases by chrysotherapeutic compounds: a possible mechanism for the antiarthritic activity of Au(I). *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5113–5116.
(13) Gunatilleke, S. S.; Barrios, A. M. Inhibition of lysosomal cysteine proteases by a series of Au(I) complexes: A detailed mechanistic investigation. *J. Med. Chem.* **2006**, *49*, 3933–3937.
(14) de Fremont, P.; Scott, N. M.; Stevens, E. D.; Nolan, S. P. Synthesis and structural characterization of N-heterocyclic carbene gold(I) complexes. *Organometallics* **2005**, *24*, 2411–2418.
(15) Baker, M. V.; Barnard, P. J.; Brayshaw, S. K.; Hickey, J. L.; Skelton, B. W.; White, A. H. Synthetic, structural and spectroscopic studies of (pseudo)halo(1,3-di-*tert*-butylimidazol-2-ylidine)gold complexes. *J. Chem. Soc., Dalton Trans.* **2005**, 37–43.
(16) Baker, M. V.; Barnard, P. J.; Berners-Price, S. J.; Brayshaw, S. K.; Hickey, J. L.; Skelton, B. W.; White, A. H. Cationic, linear Au(I) N-heterocyclic carbene complexes: synthesis, structure and anti-mitochondrial activity. *J. Chem. Soc., Dalton Trans.* **2006**, 3708–3715.
(17) Wang, H. M. J.; Chen, C. Y. L.; Lin, I. J. B. Synthesis, structure, and spectroscopic properties of gold(I)-carbene complexes. *Organometallics* **1999**, *18*, 1216–1223.
(18) Barnard, P. J.; Baker, M. V.; Berners-Price, S. J.; Day, D. A. Mitochondrial permeability transition induced by dinuclear gold(I)−carbene complexes: potential new antimitochondrial antitumour agents. *J. Inorg. Biochem.* **2004**, *98*, 1642–1647.
(19) Barnard, P. J.; Ho, A. Y. Y.; Baker, M. V.; Day, D. A.; Berners-Price, S. J. Gold−Phosphine and Gold−Carbene complexes as potential mitochontrial targeting antitumor agents. *Proc. Gold* **2003**, 1–5.
(20) Wang, Q.; Janzen, N.; Ramachandran, C.; Jirik, F. Mechanism of inhibition of protein-tyrosine phosphatases by disodium aurothiomalate. *Biochem. Pharmacol.* **1997**, *54*, 703–711.
(21) Haider, J.; Kunz, K.; Scholz, U. Highly selective copper-catalyzed monoarylation of aniline. *Adv. Synth. Catal.* **2004**, *346*, 717–722.
(22) Sutton, B. M.; Walz, D. T.; Mcgusty, E.; Dimartino, M. J. Oral Gold: Antiarthritic Properties of Alkylphosphinegold Coordination Complexes. *J. Med. Chem.* **1972**, *15*, 1095–1098.
(23) Mustelin, T.; Tautz, L.; Page, R. Structure of the hematopoietic tyrosine phosphatase (HePTP) catalytic domain: structure of a KIM phosphatase with phosphate bound at the active site. *J. Mol. Biol.* **2005**, *354*, 150–163.
(24) Clipstone, N. A.; Crabtree, G. R. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature* **1992**, *357*, 695–697.
(25) Denu, J. M.; Dixon, J. E. Protein tyrosine phosphatases: mechanisms of catalysis and regulation. *Curr. Opin. Chem. Biol.* **1998**, *2*, 633–641.
(26) Fricker, S. P. Medicinal uses of gold compounds: past, present, and future. *Gold Bull.* **1996**, *29*, 53–60.
(27) Galic, S.; Hauser, C.; Kahn, B. B.; Haj, F. G.; Neel, B. G.; Tonks, N. K.; Tiganis, T. Coordinated regulation of insulin signaling by the protein tyrosine phosphatases PTP1B and TCPTP. *Mol. Cell. Biol.* **2005**, *25*, 819–829.
(28) Yu, X.; Sun, J. P.; He, Y.; Guo, X.; Liu, S.; Zhou, B.; Hudmon, A.; Zhang, Z. Y. Structure, inhibitor and regulatory mechanism of Lyp, a lymphoid-specific tyrosine phosphatase implicated in autoimmune diseases. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 19767–19772.
(29) Xie, Y.; Liu, Y.; Gong, G.; Rinderspacher, A.; Deng, S. X.; Smith, D. H.; Toebben, U.; Tzilianos, E.; Branden, L.; Vidovic, D.; Chung, C.; Schurer, S.; Tautz, L.; Landry, D. W. Discovery of a novel submicromolar inhibitor of the lymphoid specific tyrosine phosphatase. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2840–2844.
(30) Mustelin, T.; Alonso, A.; Bottini, N.; Huynh, H.; Rahmouni, S.; Nika, K.; Louis-dit-Sully, C.; Tautz, L.; Togo, S. H.; Bruckner, S.; Mena-Duran, A. V.; al-Khouri, A. M. Protein tyrosine phosphatases in T cell physiology. *Mol. Immunol.* **2004**, *41*, 687–700.
(31) Mustelin, T.; Rahmouni, S.; Bottini, N.; Alonso, A. Role of protein tyrosine phosphatases in T cell activation. *Immunol. Rev.* **2003**, *191*, 139–147.
(32) Wu, J.; Katrekar, A.; Honigberg, L. A.; Smith, A. M.; Conn, M. T.; Tang, J.; Jeffery, D.; Mortara, K.; Sampang, J.; Williams, S. R.; Buggy, J.; Clark, J. M. Identification of substrates of human protein-tyrosine phosphatase PTPN22. *J. Biol. Chem.* **2006**, *281*, 11002–11010.

JM800101W