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# The anti-inflammatory compound BAY-11-7082 is a potent inhibitor of protein tyrosine phosphatases

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The families of protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs) function in a coordinated manner to regulate signal transduction events that are critical for cellular homeostasis. Aberrant tyrosine phosphorylation, resulting from disruption of either PTP or PTK function, has been shown to be the cause of major human diseases, including cancer and diabetes. Consequently, the characterization of small-molecule inhibitors of these kinases and phosphatases may not only provide molecular probes with which to define the significance of particular signaling events, but also may have therapeutic implications. BAY-11-7082 is an anti-inflammatory compound that has been reported to inhibit IκB kinase activity. The compound has an α,β-unsaturated electrophilic center, which confers the property of being a Michael acceptor; this suggests that it may react with nucleophilic cysteine-containing proteins, such as PTPs. In this study, we demonstrated that BAY-11-7082 was a potent, irreversible inhibitor of PTPs. Using mass spectrometry, we have shown that BAY-11-7082 inactivated PTPs by forming a covalent adduct with the active-site cysteine. Administration of the compound caused an increase in protein tyrosine phosphorylation in RAW 264 macrophages, similar to the effects of the generic PTP inhibitor sodium orthovanadate. These data illustrate that BAY-11-7082 is an effective pan-PTP inhibitor with cell permeability, revealing its potential as a new probe for chemical biology approaches to the study of PTP function. Furthermore, the data suggest that inhibition of PTP function may contribute to the many biological effects of BAY-11-7082 that have been reported to date.

## Introduction

The protein tyrosine phosphatases (PTPs) comprise a large and structurally diverse family of proteins that includes the classical PTPs, which dephosphorylate tyrosine residues in proteins specifically, and dual specificity phosphatases (DUSPs), which recognize Ser/Thr residues as well as Tyr and may also dephosphorylate nonprotein regulators of cell signaling, including inositol phospholipids [1]. The PTPs play well-characterized roles in downregulating tyrosine phosphorylation-dependent signaling by dephosphorylating and inactivating either the protein tyrosine kinases (PTKs) themselves or the downstream targets of the PTKs [1,2]. Furthermore, it is now recognized that in certain contexts, PTPs may function positively, to promote signaling. For example, the prototypic receptor PTP, CD45, dephosphorylates an inhibitory C-terminal site

**Abbreviations**
DiFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; DUSP, dual specificity phosphatase; IKK, IκB kinase; IκB, inhibitor of κB; LPS, lipopolysaccharide; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; p-Tyr, phosphotyrosine.

 

of phosphorylation in SRC family PTKs, thereby promoting PTK activity and tyrosine phosphorylation [3,4]. In addition to its role in inhibiting signaling through the insulin and leptin receptors, PTP1B, the first PTP to be purified to homogeneity, is reported to be overexpressed in breast tumors and to play a positive role in promoting HER2-induced tumorigenesis, possibly through regulating the phosphorylation status of the scaffold protein $p62^{DOK}$ [2,5]. Finally, gain-of-function mutations in the *PTPN11* gene, which encodes SHP2, result in aberrant activation of RAS–MAP kinase signaling. This is associated with developmental disorders such as Noonan's syndrome and with an increased risk of certain childhood malignancies, such as juvenile myelomonocytic leukemia and acute myeloid leukemia [6,7]. Consequently, *PTPN11* is the first example of a PTP oncogene [8]. Although such examples illustrate the fundamental importance of PTPs as regulators of signaling in their own right, much remains to be done to characterize fully the function of the family as a whole. The advent of RNA interference (RNAi) approaches to functional analysis of gene families is revealing important new roles for specific PTPs in the regulation of cell signaling [9]; nevertheless, complementary strategies, including the development of novel small-molecule inhibitors for chemical biology approaches to functional analysis, would also be of benefit.

The PTPs are defined by a highly conserved signature motif in the active site, $HC(X)_5R$, in which the cysteine residue acts as a nucleophile and is essential for catalysis [1]. The architecture of the PTP active site is such that this cysteine is characterized by a low $pK_a$ and is stabilized in the thiolate form at neutral pH [1,10]. This property not only makes it a good nucleophile in catalysis, but also renders the PTPs highly susceptible to inactivation by covalent modification of the active-site cysteine [11,12]. In fact, the controlled production of reactive oxygen species in response to a wide variety of physiological stimuli results in the transient oxidation and inactivation of members of the PTP family and underlies a new tier of control over tyrosine phosphorylation-dependent signaling [12,13]. The reactive nature of the active-site cysteine in PTPs also suggests an experimental approach to attenuating catalytic function by irreversible covalent modification of this critical residue by small-molecule inhibitors. Although irreversible inhibitors of PTP function have been identified [14], this approach has not been exploited fully.

BAY-11-7082 is currently in widespread use as an anti-inflammatory agent, and is marketed as an inhibitor of the activation of the transcription factor NFκB [15]. In the basal state, NFκB is maintained in an inactive form in the cytosol by association with regulatory proteins, termed inhibitors of κB (IκB). In response to inflammatory cytokines or cellular stresses, IκB is phosphorylated by IκB kinase (IKK), which, together with the ubiquitination and proteolytic degradation of IκB, leads to the activation and nuclear translocation of NFκB [15,16]. It has been proposed that BAY-11-7082 exerts its effects by inhibiting the activity of IKK, thereby maintaining NFκB in its inactive complex with IκB [15]. However, the presence of the vinyl group in BAY-11-7082 suggests that it may function as a Michael acceptor, which we thought may expand the spectrum of its potential targets. It has been demonstrated that aryl vinyl sulfonates, such as phenyl vinyl sulfonate, can function as irreversible, active-site-directed inhibitors of the PTPs [14]. Considering the chemical similarity between BAY-11-7082 and phenyl vinyl sulfonate, we tested the hypothesis that some of the effects of BAY-11-7082 may be exerted through inhibition of PTP function. Here we demonstrate that BAY-11-7082 is, in fact, an active-site-directed, irreversible inhibitor of PTPs with the ability to alter the status of protein tyrosine phosphorylation in cells.

## Results

### Inactivation of PTP1B by BAY-11-7082

The enzymatic activity of PTP1B was measured *in vitro* as a function of time and the concentration of BAY-11-7082. We observed that BAY-11-7082 was a potent, time-dependent inhibitor of PTP1B (Fig. 1). The inset to Fig. 1 shows the plot of the rate of inactivation of the enzyme by increasing concentrations of BAY-11-7082. This was used to calculate the second order rate constant of 50 $M^{-1}\cdot s^{-1}$ (Fig. 1A), which is approximately fivefold higher than the rate of oxidation of the enzyme by $H_2O_2$ (10 $M^{-1}\cdot s^{-1}$) [17].

It has been reported that certain classes of compounds can generate hydrogen peroxide through oxidation of the reducing agent in the assay, which may result in indirect inactivation of the PTP through oxidation of the catalytic cysteine [18,19]. Considering the high susceptibility of the active-site cysteine of PTP1B, $Cys^{215}$, to oxidation [20], we monitored whether the presence of the potent $H_2O_2$-detoxifying enzyme catalase had an impact on the inhibitory potency of BAY-11-7082. We observed that the rate of inactivation of PTP1B by BAY-11-7082 was the same in the absence and presence of catalase (Fig. 1B), suggesting that inhibition was a direct effect of the compound.

The reactive cysteine is a feature of the catalytic mechanism of all members of the PTP family;

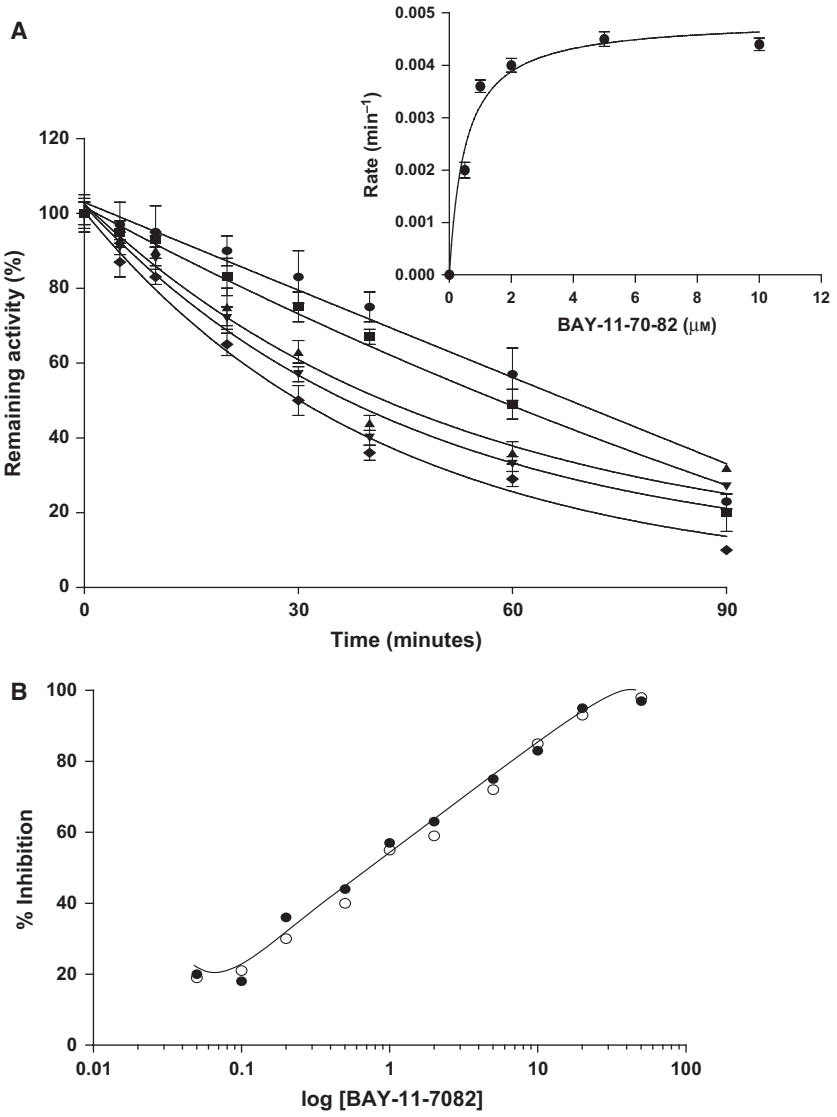


**Fig. 1.** PTP1B was inactivated by BAY-11-7082 *in vitro*. (A) The phosphatase activity of PTP1B was monitored using DiFMUP as substrate in the presence of the following concentrations of BAY-11-7082: 0.2 μM (●), 0.5 μM (■), 1 μM (▲), 2 μM (▼), 5 μM (♦) over the indicated times. The concentration dependence of the rate of inactivation was used to derive the rate of 50 $M^{-1}{\cdot}s^{-1}$ (inset). (B) PTP1B (5 nm) was assayed in Hepes buffer, pH 7.0, containing 150 mM NaCl, 0.1% BSA, 2 mM EDTA and 5 mM dithiothreitol with varying concentrations of BAY-11-7082 (0, 0.1,0.25, 0.5, 1, 2, 5, 10, 20, 50, 100 μM) in the presence and absence of catalase (0.5 $\mu g{\cdot}mL^{-1}$, equivalent to 1 unit per assay).

therefore, we tested BAY-11-7082 against a panel of classical PTPs and DUSPs. We observed that the compound was able to inhibit all of the classical PTPs that were tested with a similar potency (Table 1). Interestingly, the behavior of the DUSPs was different. The half-maximal concentration required to achieve complete inactivation of DUSPs was 100-fold higher than observed for classical PTPs. In addition, examination of PTP inhibition by the analogue compound BAY-11-7085, in which the methylphenyl group in BAY-11-7082 is substituted by a trimethylphenyl group, revealed that the presence of the bulky substitution in the aromatic ring reduced inhibitory potency towards classical PTPs. In the case of PTP1B, the $K_i$ for BAY-11-7082 was 1 μM, whereas the $K_i$ with BAY-11-7085 was 36 μM. In contrast, the DUSPs, which displayed

**Table 1.** Inhibition of a panel of PTPs by BAY-11-7082 and BAY-11-7085. The data are derived from three independent determinations.

| PTP | $K_i$ (μM) BAY-11-7082 | $K_i$ (μM) BAY-11-7085 |
|---|---|---|
| PTP1B | 1.0 | 36 |
| TC-PTP | 1.5 | 39 |
| PTP-alpha | 2.3 | 45 |
| PTP-PEST | 0.9 | 40 |
| LAR | 2.5 | 57 |
| PTEN | 100 | 200 |
| DUSP4 | 140 | 160 |
| DUSP10 | 150 | 180 |
| DUSP14 | 180 | 210 |
| DUSP16 | 125 | 205 |

higher $IC_{50}$ for both compounds than PTP1B, did not discriminate between BAY-11-7082 and BAY-11-7085 to the same extent. The classical PTPs are characterized by a deep active site, whereas the active sites of the DUSPs that we tested are shallower and, in the case of PTEN, larger so as to allow it to accommodate the inositol phospholipid head group of its substrate [21]. These data suggest that BAY-11-7082 may bind as an analogue of the substrate phosphotyrosine and that it may be possible to use such compounds to distinguish between PTPs and DUSPs.

## Mechanism of inhibition of PTP1B by BAY-11-7082

In investigating further the mechanism of PTP inhibition by BAY-11-7082, we titrated PTP1B with increasing concentrations of the substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) in the absence and presence of the inhibitor and obtained a $K_m$ of 5 μM for the substrate in both cases. Unlike the binding affinity, which was not affected by the presence of the inhibitor, the maximum rate ($V_{max}$) dropped by ~ 50% in the presence of BAY-11-7082 (Fig. 2A). Because it is known that $V_{max}$ is lowered without an effect on $K_m$ with both reversible, noncompetitive inhibitors and irreversible inhibitors, we plotted $V_{max}$ as a function of the enzyme concentration used in the assay to distinguish between these two mechanisms. In this plot (Fig. 2B), the slopes of the curves in the absence and presence of BAY-11-7082 were found to be similar; however, in the presence of inhibitor, the line did not intersect the *x*-axis at the origin, indicating that incubation with BAY-11-7082 decreased the amount of active enzyme. By contrast, a reversible, noncompetitive inhibitor would display a decreased slope compared with the control curve. The data suggest that BAY-11-7082 functioned as an irreversible inhibitor.

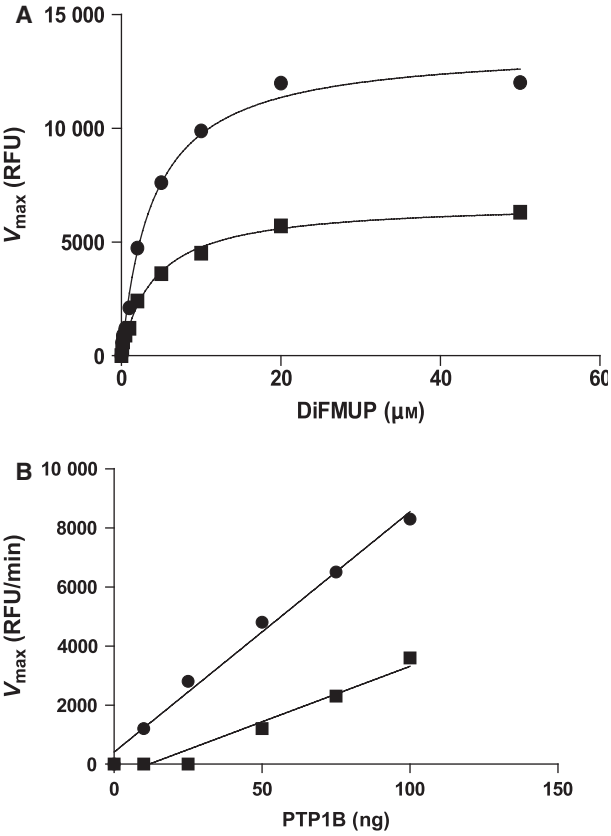


**Fig. 2.** Irreversible inhibition of PTP1B by BAY-11-7082. (A) Michaelis–Menten plot of the activity of PTP1B (5 nm) at varying concentrations of DiFMUP substrate, without (●) and with (■) BAY-11-7082 (2 μM). The $K_m$ remained unchanged in the absence and presence of the inhibitor, whereas the $V_{max}$ decreased in the presence of the inhibitor. (B) A plot of $V_{max}$ vs varying concentrations of enzyme was performed to investigate the mechanism of inhibition. BAY-11-7082 (2.5 μM) was incubated with varying concentration of PTP1B in Hepes buffer, pH 7.0, containing 150 mM NaCl, 0.1% BSA, 2 mM EDTA and 5 mM dithiothreitol for 20 min. Subsequently DiFMUP (10 μM) was added to initiate the reaction.

## Analysis of the PTP1B: BAY-11-7082 complex by MS

To define more precisely the nature of PTP1B inactivation induced by BAY-11-7082, we used a high-resolution quadrupole time of flight mass spectrometer to analyze the molecular mass of PTP1B (residues 1–405) in the absence and presence of the compound. The intact masses of untreated (Fig. 3A) and BAY-11-7082-treated (Fig. 3B) PTP1B were determined from ESI-MS spectra as 46 208 and 46 415, respectively, indicating a modification that resulted in the addition of 207 Da. Because this increase corresponds to the molecular mass of BAY-11-7082, the data suggest that BAY-11-7082 inhibited PTP1B primarily by forming a covalent adduct with the protein.

In characterizing the modification further, we subjected the samples to tryptic digestion and obtained high coverage of 75% of the PTP1B sequence from the resulting peptides. We observed a major signal corresponding to the BAY-11-7082-modified, active-site cysteine-containing peptide in the inhibitor-treated PTP1B sample. Using LC-MS/MS analysis of this peptide, we observed that the masses of b-ions prior to Cys215 were the same in the absence (Fig. 4A) and presence (Fig. 4B) of BAY-11-7082, whereas the masses for b-ions following Cys215 were increased by

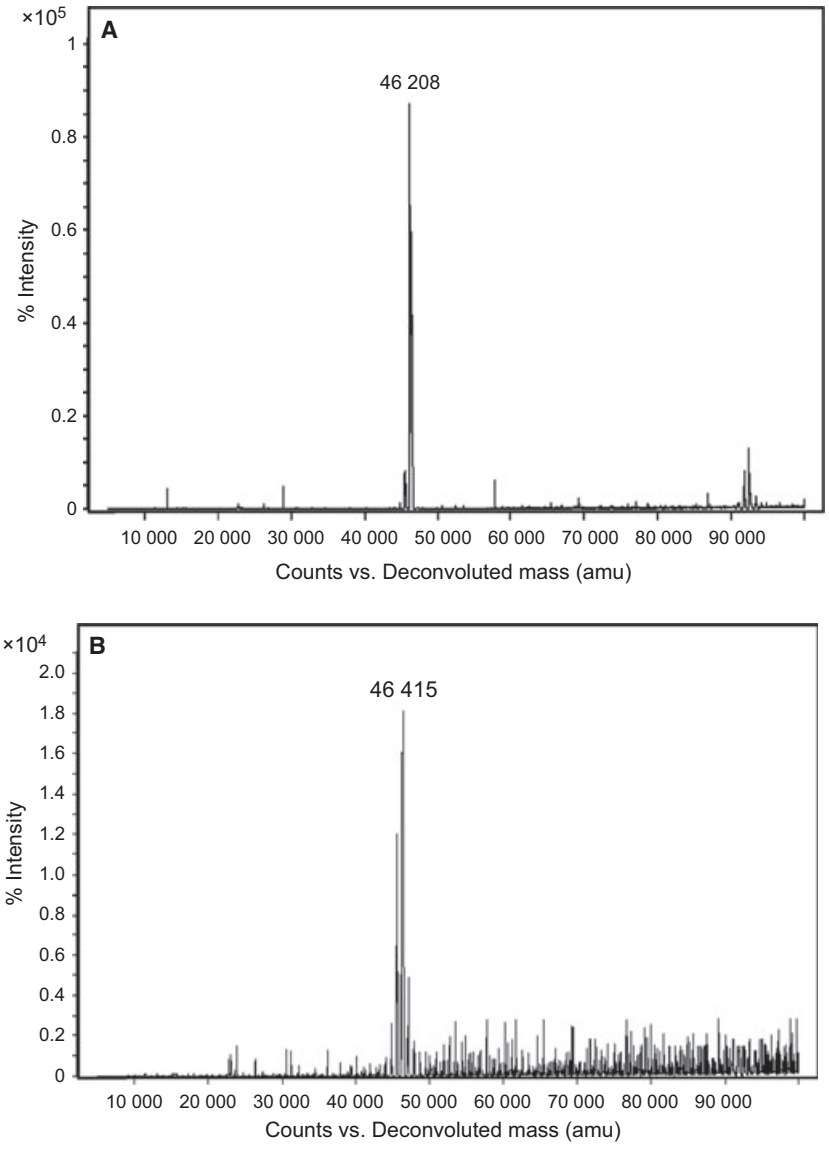


**Fig. 3.** Direct ESI-MS analysis of PTP1B before and after treatment with BAY-11-7082. PTP1B (20 μg) was incubated in the absence or presence of BAY-11-7082 (100 μM) for 10 min at 25 °C and the mass of the protein was determined by ESI-MS. (A) PTP1B (untreated) resolved predominantly into one peak corresponding to unmodified protein with an intact mass of 46 209 Da. (B) Following treatment with BAY-11-7082, the intact mass of PTP1B was measured as 46 415.17 Da, an increase of 207.5 Da.

207 only in the BAY-11-7082-treated sample (Fig. 4 and Table S1). These data suggest that BAY-11-7082 inhibited PTP1B by alkylation of the active-site cysteine, via Michael addition of the Cys215 nucleophile via the C2 position of the compound.

## Molecular basis of the interaction between BAY-11-7082 and PTP1B

To understand the molecular basis of PTP inhibition by BAY-11-7082, we used the previously solved crystal structure of the catalytic domain of the enzyme for *in silico* docking of the compound onto PTP1B. After scanning the entire surface of the open conformation of PTP1B (2HNQ) [10], the optimal site for compound docking was found to be the active site, which is consistent with the biochemical mechanism of PTP inhibition by BAY-11-7082. Our model suggests that BAY-11-7082 bound to the open conformation of the active site in a similar manner to that of the phosphotyrosine (p-Tyr) residue in a target substrate (Fig. 5A). In PTP1B, the active site is located within a cleft that is ~ 9 Å deep, at the bottom of which is the signature PTP loop containing residues critical for catalysis, including the nucleophilic cysteine that attacks the phosphate of the incoming substrate. Binding of the p-Tyr substrate is stabilized by a π–π stacking interaction with Tyr46, an invariant residue in the substrate recognition loop that defines the depth of the active-site cleft [10]. According to our docking studies, the methyl

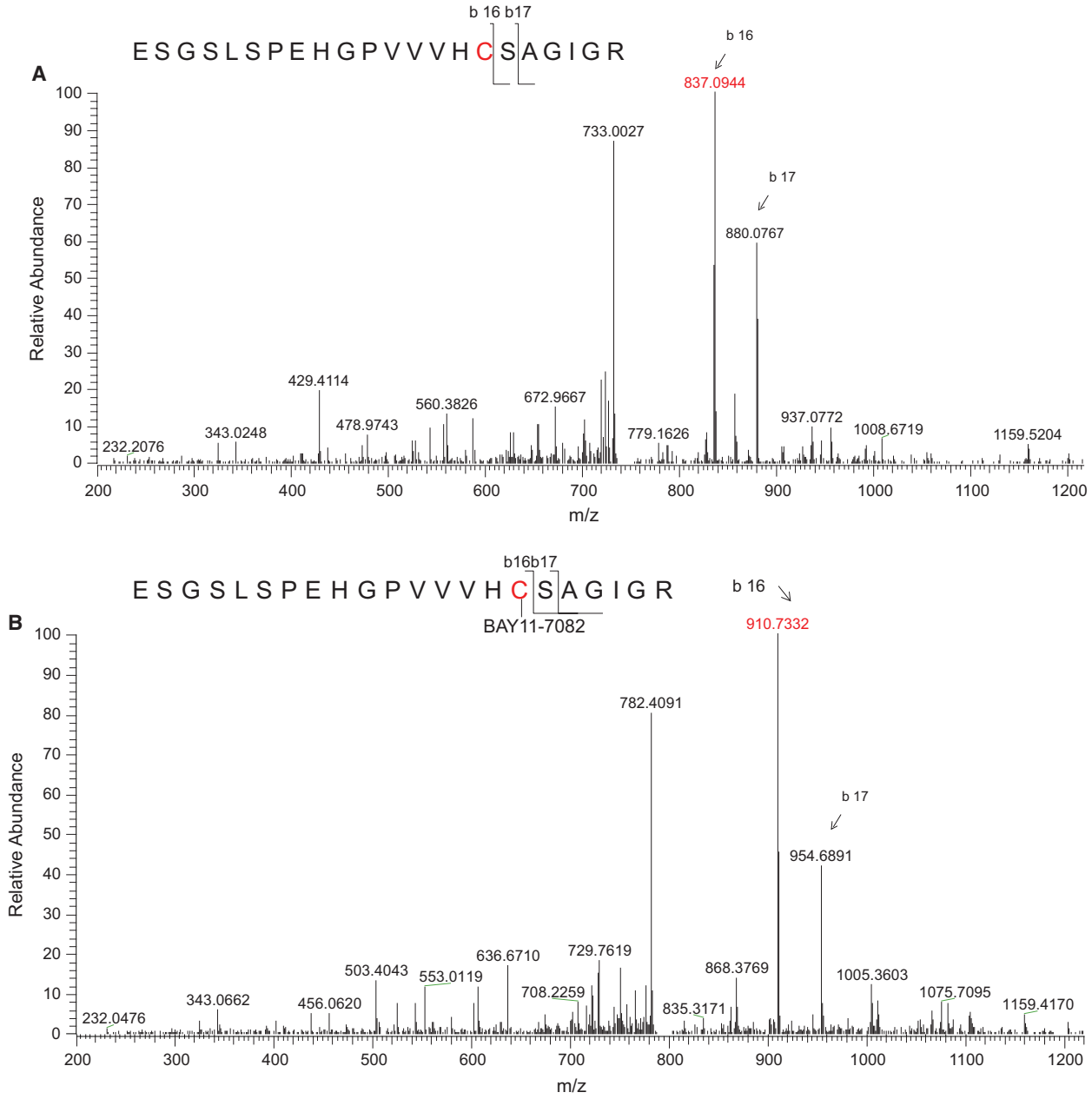


**Fig. 4.** LC-MS/MS analysis of the tryptic peptide containing $Cys^{215}$ of PTP1B. Purified PTP1B (20 μg) was incubated alone or in the presence of BAY-11-7082 (10 μM) for 10 min at 25 °C, then subjected to carbamidomethylation, trypsinized overnight and analyzed by LC-MS/MS. The spectra represent the active site signature motif peptide derived from (A) untreated PTP1B (*m/z* = 837.09) or (B) BAY-11-7082-modified PTP1B (*m/z* = 910.73).

phenyl group of BAY-11-7082 has the potential to make a similar interaction with the aromatic group of Tyr46 (Fig. 5B). Furthermore, Lys120 in the active site, which is conserved in the PTPs but not in the DUSPs, provides amino–aromatic interaction with the phenyl ring of the compound, similar to that observed with a p-Tyr substrate (Fig. 5C). The sulfonyl oxygen of the compound was found to H-bond to two critical residues in the signature PTP loop, Ser216 and Arg221 (Fig. 5C). These interactions would orient the inhibitor for attack on the C2 carbon by the Cys215 nucleophile to form a thio-ether bond (Fig. 5C). The calculated distance between Cys215 and the C2 carbon of BAY-11-7082 is ~ 5.7 Å, a distance suitable for the

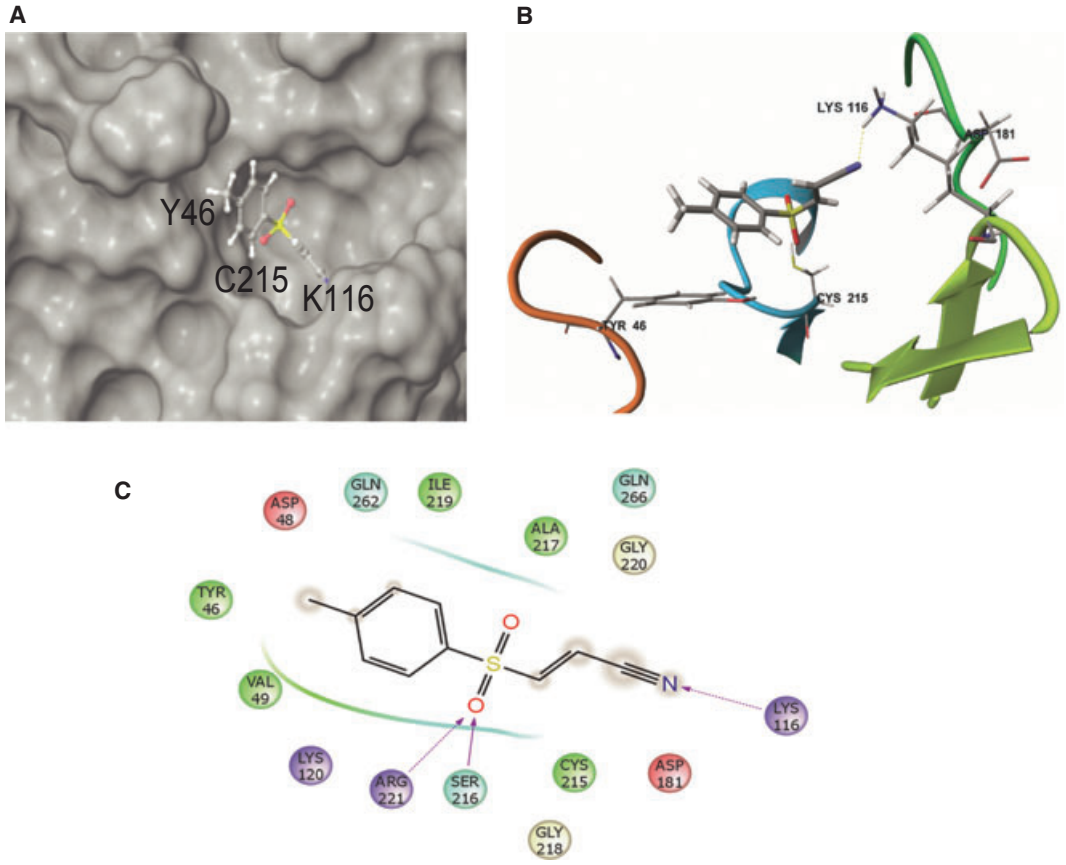


**Fig. 5.** *In silico* modeling of the interaction between BAY-11-7082 and PTP1B. (A) Surface map of the catalytic domain of PTP1B (residues 1–321) highlighting the active site cleft into which BAY-11-7082 was docked. The critical residues for inhibition of PTP1B by BAY-11-7082 are labeled. (B) Ribbon diagram showing the potential mechanism of PTP1B inhibition by BAY-11-7082. The substrate recognition loop (orange) and PTP loop (blue) stabilizes BAY-11-7082 in the active site and orient the α,β-unsaturated group of the compound such that the electrophilic C2 carbon would undergo a nucleophilic attack by Cys215 from the PTP loop (blue). The nitrile group of the compound is further stabilized by Lys116 (green). (C) Model highlighting residues in the PTP1B active site that are critical for BAY-11-7082 binding.

proposed reaction between the enzyme and the inhibitor. The model also shows the involvement of H-bonding between Lys116 and the nitrile group of BAY-11-7082, which may stabilize the open conformation of the enzyme; however, the significance of this interaction requires further investigation. Attempts to dock BAY-11-7085 into the structure of PTP1B were unsuccessful; the presence of the trimethyl group resulted in steric clashes with residues in the PTP active site, consistent with the observed impaired inhibitory potency of this compound compared with BAY-11-7082.

## Effect of BAY-11-7082 on cellular tyrosine phosphorylation

In order to test the effect of BAY-11-7082 on tyrosine phosphorylation-mediated signaling, we stimulated RAW 264 macrophages with lipopolysaccharide (LPS) and examined the level of phosphotyrosine in the absence and presence of the compound by immunoblotting with antibody to p-Tyr. We compared the effects of BAY-11-7082 with the well-studied and commonly used generic PTP inhibitor vanadate under the same conditions. Upon LPS treatment, a slight increase in tyrosine phosphorylation was observed when compared with untreated cells. However, when cells were treated with sodium orthovanadate, the extent of tyrosine phosphorylation was enhanced, both in the presence and absence of LPS (Fig. 6A). Cells treated with BAY-11-7082 displayed an increase in global tyrosine phoshorylation similar to that observed with sodium orthovanadate (Fig. 6B). The data clearly illustrate that some of the effects of BAY-11-7082 in cells may be mediated by inhibition of PTP function.

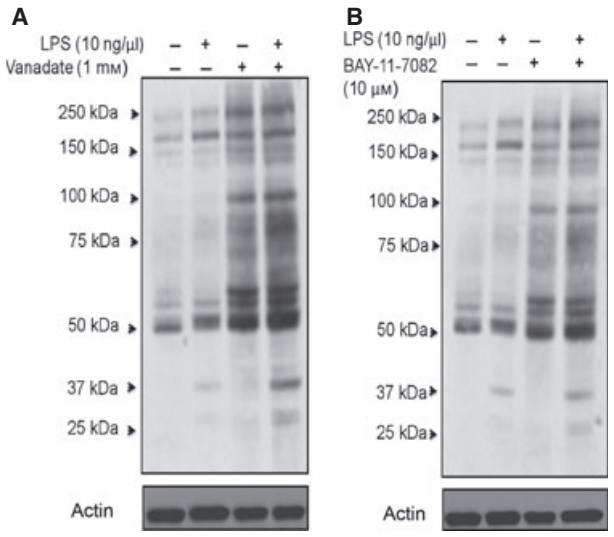


**Fig. 6.** LPS-mediated signaling in RAW 264 macrophage. (A). Immunoblot with 4G10 showing the overall change in tyrosine phosphorylation induced by LPS treatment in the absence and presence of vanadate. The two left lanes show tyrosine phosphorylation before and after LPS treatment, in the absence of vanadate; the two right lanes show tyrosine phosphorylation before and after LPS treatment, after 30 min of treatment with vanadate (1 mM). (B) The same experiment was performed in the absence and presence of BAY-11-7082. The two left-hand lanes show the effects of LPS on tyrosine phosphorylation in the absence of BAY-11-7082; the two right-hand lanes show LPS-induced tyrosine phosphorylation after 30 min of treatment with BAY-11-7082 (10 μM).

## Discussion

BAY-11-7082 has generated considerable interest as an anti-inflammatory agent with anticancer and neuroprotective properties [22]. In most cases, its effects have been interpreted in the context of inhibition of NFκB signaling, which is an important target for anti-inflammatory therapies. A large body of literature has now accumulated that presents the conclusion that BAY-11-7082 is a direct inhibitor of IKK. Specifically, it is thought that BAY-11-7082 inhibits the signal-induced nuclear translocation of NFκB through inhibiting IKK activity and the phosphorylation of IκB in response to the signal [15,23]. In fact, inhibition by BAY-11-7082 is often presented as a diagnostic test to demonstrate the involvement of IKK, and NFκB signaling, in a biological process, such as in mouse models of lung adenocarcinoma [24]. Nevertheless, the situation appears to be more complicated.

In a separate study from the Cohen lab, it has been shown that although BAY-11-7082 suppresses the activation of IKKs in response to LPS or interleukin-1 in various cells, including B-cell lymphoma and T-cell leukemia, it does not do so by inhibiting IKK directly [25]. In fact, these authors demonstrated that BAY-11-7082 does not inhibit the IKK family members *in vitro*. Instead, it prevents the conversion of IκB kinases to their active phosphorylated forms in cells by inactivating more upstream components of the MyD88 signaling network. In particular, BAY-11-7082 binds covalently to, and irreversibly inactivates E2-conjugating enzymes that are required for the formation of K63-linked and linear polyubiquitin chains. When these polyubiquitin chains are formed in response to inflammatory stimuli, they bind to NEMO, the regulatory subunit of the canonical IKK complex inducing conformational changes that lead to the activation of the IKKs [25]. Consequently, the effects of BAY-11-7082 on IKKs are indirect. In addition, there have been reports of effects of BAY-11-7082 that are independent of NFκB, including induction of cell death in Ewing's sarcoma cells [26] and the NRF2-dependent induction of antioxidant enzymes in colon cancer cells [27]. It has also been reported that in macrophages BAY-11-7082 inhibits the NLRP3 inflammasome, thereby inhibiting the production of the proinflammatory cytokines, interleukin-1β and interleukin-18, in an NFκB-independent manner [28]. Our study now extends further the biological effects of BAY-11-7082 to include its potential as an inhibitor of PTP function.

With the validation of PTP1B as a therapeutic target for the treatment of diabetes and obesity, interest has grown in exploiting the PTPs for development of small-molecule drugs [29]. Nevertheless, the reactivity of the essential active-site cysteine has posed a significant challenge to the generation of reversible active-site-directed inhibitors [30]. In the many high throughput screens of small-molecule libraries that have been conducted to date in industry and academia, it has become apparent that the presence of this potent nucleophile at the catalytic center renders the PTPs sensitive to the effects of Michael acceptors, leading to irreversible inhibition of PTP function. In this study, we have demonstrated that BAY-11-7082 is one such potent, irreversible inhibitor of PTPs that alkylates the essential cysteine residue at the active site of the enzyme (Fig. 7). Nevertheless, our data also illustrate the potential for generating specificity in the reaction between this specific thiol group in the protein and an electrophile. Such specificity may be generated from the chemical structure of the electrophilic compound, coupled with the environment of the reactive cysteine and its position in the 3D structure of the protein. In the case of BAY-11-7082, the structural features of the

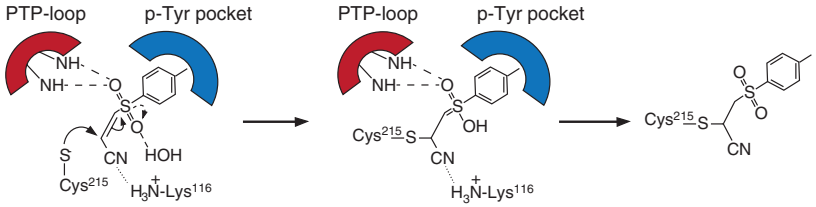


**Fig. 7.** Proposed reaction mechanism for the irreversible inactivation of PTP1B by BAY-11-7082. PTP1B was inactivated by nucleophilic attack of the active site cysteine (Cys215), present as the thiolate at neutral pH, on the C2 carbon of BAY-11-7082.

molecule suggest that it may act as a p-Tyr-mimetic, in that the phenyl and the sulfonyl group could occupy the active site and engage the same critical residues that are required for binding the p-Tyr residue in a substrate. Our modeling data are consistent with such a binding mode. Once the compound is bound to the active site, the nucleophilic cysteine can react with the C2 carbon of BAY-11-7082 to form the covalent adduct (Fig. 5). This represents a classic example of a suicide inhibitor, which is typically a substrate mimetic that irreversibly inhibits an enzyme. Interestingly, the crystal structure of the complex between the PTP YOPH and phenyl vinyl sulfonate reveals a similar interaction, leading to covalent modification of the cysteine [14]; however, in this case, the pheny vinyl sulfonate binds to the closed conformation of the active site, which is induced following substrate binding [14]. By contrast, our modeling suggests that BAY-11-7082 engages the open conformation of the active site, with the interaction between the nitrile group in the compound and Lys 116 in PTP1B helping to maintain the open, inactive state (Fig. 5).

Although the preference in industry is to focus on reversible inhibitors for drug development, there are many examples of drugs that function through covalent modification of their targets. For example, aspirin acts to acetylate a critical serine residue in the active site of the target cyclooxygenase, which irreversibly inactivates the enzyme and decreases the synthesis of prostaglandins and thromboxanes [31,32]. There are different classes of alkylating agents based on the functional group and its reactivity towards particular residues in the target protein. Reactive cysteines are notably susceptible to alkylation, and inhibitors that operate by alkylating reactive thiols in proteins have been unpopular because they may exhibit indiscriminate activity against a wide variety of proteins. Nevertheless, target-specific covalent modifiers of cysteine residues have been identified recently. For example, four irreversible inhibitors of the epidermal growth factor receptor kinase that target cysteine residues in the ATP-binding site have progressed to clinical trials [33]. Interestingly, these irreversible inhibitors acted upon the T790M epidermal growth factor receptor gatekeeper mutant, which underlies resistance to epidermal growth factor receptor therapy based upon reversible inhibitors [34]. Furthermore, PCI-32765/Ibrutinib has been identified as an irreversible inhibitor of the protein tyrosine kinase BTK for therapeutic intervention in various B-cell malignancies, including diffuse large B-cell lymphoma and chronic lymphocytic leukemia [35,36]. It blocks B-cell receptor signaling by modifying a cysteine residue at the active site of BTK. Only ~ 10 kinases in the human kinome possess such a cysteine at the active site, which contributes to the specificity of these inhibitors. Such precedents suggest that it may also be possible to generate specific covalent inhibitors of members of the PTP family, a possibility that now holds greater significance with recent indications that several members of the PTP family may function positively, to promote oncogenic signaling in various cancer [37]. Considering that PTPs are valuable therapeutic targets for a range of human diseases, and the limited drug development potential of the reversible, active site-directed inhibitors identified to date, there is a need for developing alternative strategies to target these enzymes. Perhaps BAY-11-7082, or a similar scaffold, could be used to develop more potent and specific inhibitors that target individual PTPs, by taking into account some of the structural features surrounding the active site that distinguish one PTP from another [1,38]. Even if such molecules proved unsuitable in the context of drug development, they could have a significant impact as chemical probes of PTP function.

In conclusion, we have identified BAY-11-7082 as a PTP inhibitor and defined the mechanism by which it targets an essential, active-site cysteine residue for irreversible, covalent inactivation of members of the PTP family (Fig. 7). In addition to suggesting a new strategy for generation of specific substrate analogues as inhibitors of PTP function, the data highlight the importance of reassessing some of the literature regarding the effects of BAY-11-7082. Our data indicate that the view of

BAY-11-7082 as an inhibitor of IKK activity is an oversimplification and that inhibition of PTP function, with concomitant effects on tyrosine phosphorylation-dependent signaling, must be considered when interpreting its effects on cell function.

## Experimental procedures

### Materials

BAY-11-7082 and BAY-11-7085 were purchased from Merck-Millipore (Billerica, MA, USA). LPS (*Escherichia coli* 055:B5) was obtained from Alexis Biochemicals (San Diego, CA, USA). Antibodies were purchased from Millipore (Bellerica, MA, USA) (phosphotyrosine, 4G10) and Sigma (St. Louis, MO, USA) (β-actin). Unless indicated otherwise, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### Protein expression and purification

PTP1B and PTEN were expressed as hexahistidine-tagged fusion proteins in *E. coli*-BL21 cells. The cells were lysed by sonication in lysis buffer (50 mM Hepes pH 7.0/100 mM NaCl/complete protease inhibitors; Roche, San Francisco, CA, USA) and the recombinant protein was purified from the cleared lysate using Ni-NTA Superflow (Qiagen, Valencia, CA, USA), and dialyzed against storage buffer (50 mM Hepes pH 7.0/50 mM NaCl/5 mM dithiothreitol/0.02% $NaN_3$/50% glycerol) and stored at −80 °C. DUSPs (DUSP4, DUSP10, DUSP14, DUSP16) were expressed as GST–fusion proteins in *E. coli*-BL21 cells, and purified using GST–Sepharose beads (GE, Piscataway, NJ, USA) eluted with 10 mM reduced GSH and dialyzed against storage buffer (50 mM Hepes pH 7.0/50 mM NaCl/5 mM dithiothreitol/0.02% $NaN_3$/50% glycerol) and stored at −80 °C.

### Kinetics of PTP inactivation by BAY-11-7082

The rate of inactivation of PTP1B was measured by a standard spectrofluorometric assay using DiFMUP as substrate. PTP1B was incubated with 2 mM dithiothreitol for 10 min at 4 °C, after which excess dithiothreitol was removed on a spin desalting column, centrifuging at 3000 ***g*** for 5 min at 4 °C. After quantitation by Bradford protein assay, PTP1B (1 μM) was incubated at 25 °C with varying concentrations of BAY-11-7082 (0–2 mM) in 50 mM Hepes pH 7.0, 100 mM NaCl and 0.01% Tween. Phosphatase activities were measured immediately in a continuous assay that monitors the production of DiFMU (excitation $\lambda = 358$ nm and emission $\lambda = 450$ nm). To determine the effect of catalase on BAY-11-7082-mediated enzyme inhibition, the same assay was performed in the absence and presence of catalase (1 unit per assay).

### Mass spectrometry

Recombinant PTP1B protein (~ 20 μg) was incubated with BAY-11-7082 (1 mM) for 20 min at room temperature and the mass of the protein was determined by ESI-MS analysis. For MS/MS analysis, the protein samples (without and with BAY-11-7082 treatment) were reduced and carbamidomethylated following standard procedures, after which they were digested overnight with trypsin (1 : 50 w/w) at 37 °C. Tryptic peptides were acidified by the addition of formic acid (0.1% v/v final), and aliquots of 2 μL peptide samples were analyzed on an LTQ Orbitrap XL ETD (Thermo scientific).

### Docking of BAY-11-7082 into PTP1B

The crystal structure of PTP1B (PDB ID: 2HNQ) from the Protein Data Bank was used for docking BAY-11-7082 using Schrödinger's graphical user interface MAESTRO (v. 9.2; Schrödinger, LLC, New York, NY, USA). Protein Preparation Wizard was used to remove the tungstate ion that was bound to the active site in the original crystal structure, so the active site will be free for the molecular modeling work. Following this, a subset of low-energy 3D conformations were obtained for BAY-11-7082 using the LIGPREP tool. LIGPREP explores alternate tautomers, ionization states and ring conformers to broaden the subset of input structures so all possible conformations of the ligand will be considered for docking. Molecular docking was performed using Schrodinger's Glide module, which is designed exclusively to determine binding mode and affinity between a ligand and the protein of interest. For this purpose, a grid is generated around the portion of the protein that will be used to search for potential binding sites for the ligand. In order to identify the correct binding site for BAY-11-7082 in PTP1B we constructed a grid such that it would encompass the entire protein. The docked models generated were subjected to energy minimization using MACROMODEL (v. 9.9; Schrödinger) to identify the most energetically favorable model.

### Cell culture

RAW 264 cells were obtained from ATCC (Manassas, VA, USA) and cultured according to the specified protocol [25]. For stimulation cells were treated with 100 $ng{\cdot}mL^{-1}$ LPS in the absence and presence of 10 μM BAY-11-7082.

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## Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

**Table S1.** Ions from LC-MS/MS analysis of the tryptic peptide containing $Cys^{215}$ of PTP1B in the absence (A) and presence (B) of BAY-11-7082.