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# Cellular Inhibition of Protein Tyrosine Phosphatase 1B by Uncharged Thioxothiazolidinone Derivatives

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*As important regulators of cellular signal transduction, members of the protein tyrosine phosphatase (PTP) family are considered to be promising drug targets. However, to date, the most effective in vitro PTP inhibitors have tended to be highly charged, thus limiting cellular permeability. Here, we have identified an uncharged thioxothiazolidinone derivative (compound 1), as a competitive inhibitor of a subset of PTPs. Compound 1 effectively inhibited protein tyrosine phosphatase 1B (PTP1B) in two cell-based*

*systems: it sensitized wild-type, but not PTP1B-null fibroblasts to insulin stimulation and prevented PTP1B-dependent dephosphorylation of the FLT3-ITD receptor tyrosine kinase. We have also tested a series of derivatives in vitro against PTP1B and proposed a model of the PTP1B–inhibitor interaction. These compounds should be useful in the elucidation of cellular PTP function and could represent a starting point for development of therapeutic PTP inhibitors.*

## Introduction

The control of cellular tyrosine phosphorylation levels requires the precise balance of protein tyrosine kinase (PTK) and phosphatase (PTP) activities. Similar to the expression of oncogenic PTKs,<sup>[1]</sup> nonspecific inhibition of PTPs results in a massive increase in cellular phosphotyrosine content.<sup>[2]</sup> The human PTP superfamily consists of over 100 cysteine-dependent enzymes.<sup>[3]</sup> They contain a range of noncatalytic motifs and domains that typically mediate protein–protein interactions or target PTPs to particular subcellular compartments.<sup>[3]</sup> Individual PTPs play specific roles in cellular signal transduction, including the regulation of metabolic and mitogenic signaling, cell adhesion and migration, and gene transcription. The mutation or genetic ablation of PTPs in mice causes generally adverse phenotypes, often involving immune<sup>[4,5]</sup> or neuronal development<sup>[6,7]</sup> effects. However, one notable exception is PTP1B (EC 3.1.3.48). Mice lacking this enzyme are healthy and display resistance to diet-induced diabetes and obesity,<sup>[8,9]</sup> this is likely to be due to its function as a negative modulator of insulin<sup>[10]</sup> and leptin<sup>[11]</sup> signaling. This phenotype generated interest in PTP1B as a drug target for type II diabetes. Indeed, numerous small-molecule PTP1B inhibitors with varying specificity and cell-permeability have been reported.<sup>[12,13]</sup>

The PTP catalytic mechanism involves nucleophilic attack by an anionic cysteine residue on the phosphotyrosyl phosphate group.<sup>[14]</sup> This cysteine, part of the PTP signature motif, HC(X)5R, is situated at the base of a flexible active site pocket. By crystallography, similar binding interactions have been reported for PTP1B in complex with phosphotyrosine and peptide substrates.<sup>[15–17]</sup> Specifically, the phenyl ring of the substrate phosphotyrosine forms aromatic–aromatic interactions with Y46, at one side of the pocket, and F182, which is part of

the flexible WPD loop that closes over substrates upon binding. In addition, extensive hydrogen bond interactions occur between the negatively charged phosphate group and residues within the active site pocket. Rational design and library screening projects for PTP1B inhibitors have identified several nonhydrolyzable phosphotyrosine analogs or mimetics, including aryl difluoromethylenephosphonates (DFMP)<sup>[18]</sup> and *N*-aryl oxamic acid derivatives.<sup>[19]</sup> These compounds commonly bear one or more negative charges, and additional charged moieties have been included to target a secondary phosphate binding site unique to PTP1B and the closely related TCPTP.<sup>[15]</sup> Despite achieving excellent (low nanomolar) potency in vitro,<sup>[20,21]</sup> these charged compounds have poor cell permeability and often require additional strategies, such as prodrug esterification<sup>[22,23]</sup> or fatty acid conjugation,<sup>[24,25]</sup> to improve cellular activity.

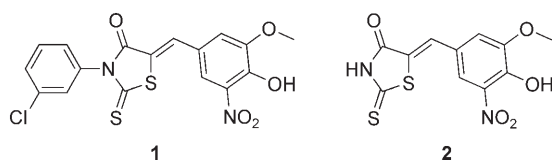
Here, we report an uncharged, bis-aryl thioxothiazolidinone compound (compound 1) that acts as a competitive PTP inhib-

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Supporting information for this article is available on the WWW under <http://www.chembiochem.org> or from the author.



itor. We have assessed its specificity *in vitro* against PTP1B and a range of other PTPs, and prepared a series of derivatives, with alterations of four functional groups, to understand the components of its structure that are critical for activity. These results are consistent with a model of the PTP1B–inhibitor complex that we have predicted by using automated docking software. Finally, we describe two new cell-based PTP inhibitor assays and show that compound 1 is effective in both systems. First, it prevents PTP1B-dependent dephosphorylation of the FLT3-ITD receptor tyrosine kinase (RTK), and second, it sensitizes wild-type (WT), but not PTP1B-null mouse fibroblasts to insulin stimulation. These experiments demonstrate that, unlike many PTP inhibitors that are effective only *in vitro*, compound 1 is active in cells, and furthermore, it has a degree of cellular specificity for PTP1B. Thus, it could be valuable as a chemical genetic tool to probe cellular PTP function, and it may also address the need for uncharged phosphotyrosine mimetics in drug development.

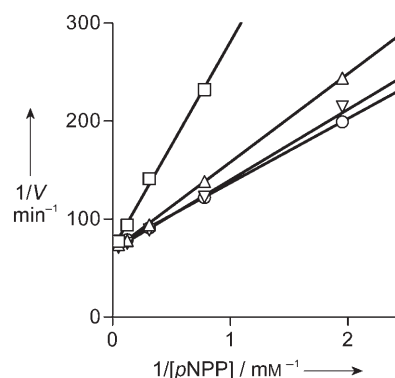
## Results

### Identification of compound 1

Compound 1 was identified as a PTP1B inhibitor by *in vitro* screening of a chemical library at Kinetek Pharmaceuticals (Vancouver, Canada). Preliminary results from our laboratory suggested that compound 1, which was unique among the molecules identified in this screen increased the insulin sensitivity of cultured cells (data not shown). We therefore undertook a detailed characterization of its *in vitro* potency and specificity, the critical components of its structure, and its cellular activity.

### Enzyme kinetic analysis

Compound 1 is soluble in organic solvents including DMSO, but it precipitates in aqueous buffers containing as little as 10 mM NaCl. It is soluble in biological buffers, including 50 mM TRIS, HEPES and PIPES. Therefore, the enzyme kinetic characterization was conducted in the absence of NaCl by using GST-tagged human PTP1B catalytic domain with *p*-nitrophenyl phosphate (*p*NPP) as a substrate. Compound 1 exhibited a competitive pattern of inhibition (Figure 1), suggesting that it binds the PTP1B active site, with a  $K_i$  of  $(1.8 \pm 0.2) \mu\text{M}$ . It is important to note that ionic strength significantly affects the measured kinetic constants of PTP inhibitors. Inhibitor assays are generally performed in the presence of NaCl, so it is not possible to directly compare the *in vitro* potency of compound 1 to other published inhibitors. Recently, a series of small-molecule inhibitors were shown to form aggregates in solution



**Figure 1.** Lineweaver–Burk plot of the competitive inhibition of PTP1B by compound 1. Reaction rates for hydrolysis of *p*NPP by PTP1B were measured at the indicated *p*NPP concentrations. (○), control, no addition of compound 1; (▽), 1.2  $\mu\text{M}$ ; (△), 3.7  $\mu\text{M}$ ; (□) 11  $\mu\text{M}$ .

that nonselectively sequester and inhibit a broad range of enzymes.<sup>[26]</sup> One of these compounds, reported as a gyrase inhibitor,<sup>[27]</sup> has a structural scaffold similar to compound 1. Thus, the  $\text{IC}_{50}$  of compound 1 was measured in the presence of 0.01% Triton X-100, which should disrupt the aggregate–enzyme interaction,<sup>[26]</sup> but no effect on activity was detected (data not shown).

### Specificity of inhibition

The PTP superfamily can be divided into two broad classes: classical PTPs act on phosphotyrosine residues, while dual-specificity PTPs may target other substrates, including phosphoserine, phosphothreonine, and phospholipids. Classical PTPs can be further classified as nontransmembrane or receptor-like enzymes.<sup>[3,28]</sup> To assess the specificity of compound 1, we purified the catalytic domains of several nontransmembrane (TCPTP, SHP1, PEP and PTP-BAS) and receptor-like PTPs (HePTP, LAR, PTP- $\mu$ , PTP- $\sigma$  and PTP- $\alpha$ ) as well as the dual-specificity PTP, MKPX as GST fusion proteins (for sequence details, see Table S1 in the Supporting Information). Individual PTPs display catalytic activity that is uniquely pH-dependent: PTPs are generally most active at pH 5–6, but only certain PTPs retain significant activity at pH 7 and higher.<sup>[29]</sup> For physiological relevance,  $\text{IC}_{50}$  values were calculated at pH 7.0 for PTPs active at this pH, and otherwise at pH 6.0. Compound 1 was similarly active against PTP1B and other nontransmembrane PTPs ( $\text{IC}_{50}$  = 3.7–5.2  $\mu\text{M}$ , Table 1). It was modestly less active against HePTP ( $\text{IC}_{50}$  = 9.3  $\mu\text{M}$ ), a cytoplasmic PTP that is classified as receptor-like, based on its homology to transmembrane PTPs.<sup>[28]</sup> For other receptor-like PTPs, compound 1 was approximately four-fold selective against PTP- $\mu$  ( $\text{IC}_{50}$  = 17  $\mu\text{M}$ ) and several-fold selective against LAR and PTP- $\sigma$ . MKPX was also poorly inhibited by compound 1 ( $\text{IC}_{50}$  > 50  $\mu\text{M}$ ). The inhibition of PTP- $\alpha$  was tested by using the fluorescent substrate DIFMUP because this enzyme was insufficiently active against *p*NPP.<sup>[30]</sup> Using this substrate, compound 1 was shown to be a weak inhibitor of PTP- $\alpha$  ( $\text{IC}_{50}$  > 100  $\mu\text{M}$ ) compared to PTP1B ( $\text{IC}_{50}$  = 11  $\mu\text{M}$ ). Thus,

Enzyme	Class <sup>[b]</sup>	IC <sub>50</sub> [μM]	
		pH 6.0	pH 7.0
PTP1B	NT	4.3 ± 0.2	4.1 ± 0.4
TCPTP	NT		5.3 ± 0.1
SHP1	NT		3.9 ± 0.6
PTP-BAS	NT		3.7 ± 0.1
HePTP	RL		9.3 ± 0.6
PEP	NT	4.5 ± 0.5	
PTP-σ	RL	NI <sup>[c]</sup>	
LAR	RL	> 50	
PTP-μ	RL	17 ± 2	
MKPX	DS	> 50	

[a] Includes enzymes tested by using pNPP. [b] NT, nontransmembrane; RL, receptor-like; DS, dual-specificity. [c] NI, no inhibition.

among the enzymes tested, compound **1** is selective for non-transmembrane PTPs over other PTP subtypes.

### Structural analysis of inhibitory activity

Previously, a series of azolidinedione derivatives were reported to have PTP1B inhibitory activity in vitro, and antihyperglycemic properties in murine models of diabetes.<sup>[31]</sup> These compounds generally consisted of a singly substituted azolidinedione ring, an aliphatic spacer, and one or more aromatic groups. However, a detailed enzyme kinetic analysis of these inhibitors was not performed. Since compound **1** contains a similar thioxothiazolidinone ring that is doubly substituted, we prepared compound **2**, which lacks the additional chlorophenyl substituent. The IC<sub>50</sub> value of compound **2** for PTP1B ((55 ± 5) μM) was more than tenfold higher than compound **1**, indicating that the chlorophenyl substituent is essential, and that the binding mode of compound **1** is likely to be distinct from the previously reported compounds. Substitution of this ring is also required for activity: removal of the chloro substituent (compound **3**, Table 2) increases the IC<sub>50</sub> to over 100 μM. How-

No.	R <sup>1</sup>	R <sup>2</sup>	IC <sub>50</sub> [μM] (PTP1B)
<b>3</b>	H	H	> 100
<b>4</b>	H	Cl	5.4 ± 0.8
<b>5</b>	F	H	12 ± 1
<b>6</b>	CH <sub>3</sub>	H	29 ± 3
<b>7</b>	OCH <sub>3</sub>	H	16 ± 1
<b>8</b>	H	OH	29 ± 1
<b>9</b>	H	CH <sub>3</sub>	8.8 ± 0.1
<b>10</b>	H	OCH <sub>3</sub>	6.3 ± 0.3
<b>11</b>	H	NO <sub>2</sub>	7 ± 1
<b>12</b>	Cl	Cl	7.0 ± 0.7

ever, the position of the substitution is not critical, since a *p*-chlorophenyl derivative (compound **4**, Table 2) was as potent as compound **1** (*m*-chlorophenyl). Furthermore, derivatives bearing substituents of different hydrophobicity and electro-negativity at these two positions retain considerable activity (compounds **5–12**, Table 2). In particular, a range of substituents were well tolerated at the *para* position: derivatives bearing methyl, methoxy, and nitro groups gave IC<sub>50</sub> values of less than 10 μM. Therefore, while a 2' or 3' substituent is required, derivatives with a variety of functional groups at these positions remain active.

The trisubstituted phenyl ring contains hydrogen bond acceptors that could mediate the active site interactions found in most PTP1B–substrate and PTP1B–inhibitor complexes. Removal of the methoxy or nitro groups from this ring (compounds **13** and **14**) reduced potency considerably (IC<sub>50</sub> > 40 μM, Table 3). Derivation of the methoxy to a hydroxyl group (com-

No.	R <sup>1</sup>	IC <sub>50</sub> [μM] (PTP1B)
<b>13</b>		68 ± 6
<b>14</b>		44 ± 6
<b>15</b>		18.1 ± 0.4
<b>16</b>		NI <sup>[a]</sup>
<b>17</b>		7.3 ± 0.5
<b>18</b>		> 100

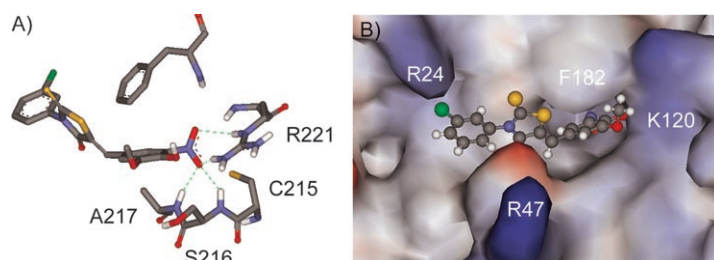
[a] NI, no inhibition.

ound **15**) resulted in only a modest loss of activity, while a dimethoxy derivative lacking the nitro group (compound **16**) did not show significant inhibition of PTP1B at 100 μM. Interestingly, the derivative in which the nitro group was replaced with a bioisosteric carboxyl group (compound **17**) had only slightly reduced activity compared to compound **1**. However, replacement with a methylene phosphonate group (compound **18**) at this position resulted in a dramatic loss of activity.

The effect of alterations in the central linker structure between the two substituted phenyl rings was also investigated (data not shown). Replacement of the extracyclic sulfur of the thioxothiazolidinone ring with oxygen had little effect. However, linkers consisting of various five-, or six-membered rings, such as imidazolidinedione and tetrahydropyridine, resulted in dramatically decreased activity (no inhibition at 50  $\mu\text{M}$ ). Thus, the thioxothiazolidinone core is also an important structural component of compound **1**.

### Computer modeling of the PTP1B–inhibitor interaction

To provide a structural explanation for the activities of the inhibitor derivatives, we used Autodock<sup>[32]</sup> to predict the binding mode of compound **1** to PTP1B. To evaluate a range of potential docking solutions, twenty-five independent docking runs were performed. The results were grouped into several clusters based on root-mean-square deviation (data not shown). The best-scoring conformations from the two lowest-energy clusters (models **A** and **B**) each had a calculated docked energy of  $-10.3 \text{ kcal mol}^{-1}$ . In both conformations, the trisubstituted phenyl ring was situated in the active site pocket with the nitro group in a position similar to the active-site phosphate group in published PTP1B–substrate complexes.<sup>[15–17]</sup> As shown in Figure 2A for model **A**, the nitro group oxygens are able to



**Figure 2.** Model of a compound **1**–PTP1B complex. Automated docking was performed using Autodock software. A) Predicted binding mode of compound **1** in the PTP1B active site pocket (model **A**). Dashed green lines indicate hydrogen bonding. B) Surface representation of the PTP1B active site in complex with compound **1**. Surface is colored by electrostatic potential (positive potentials are in blue, negative in red). The figure was generated by using DS Visualizer (Accelrys).

form hydrogen bonds to the side chain of R221, and the main-chain nitrogens of S216 and A217. The hydroxyl and methoxy groups of the trisubstituted ring form van der Waals contacts with residues of the active site pocket (Figure 2B). Outside the active site, model **A** extends to the secondary phosphate binding site, the chloro group forming favorable van der Waals interactions with R24 (Figure 2B). In model **B**, the inhibitor is in an alternative orientation with the chloro group in the vicinity of K120 and S118 (not shown). As we have shown, the inhibitory activity of compound **1** requires the chloro substituent, or another functional group at the 2' or 3' position of this ring. Thus, the contribution of this substituent to the docked energy of model **A** suggests that this is more likely to be the correct model. However, we do not dismiss model **B** as it was

recently shown that substituted phenyl groups can bind PTP1B near K120.<sup>[33]</sup> Furthermore, neither model fully explains the preference for particular substituents on the monosubstituted ring.

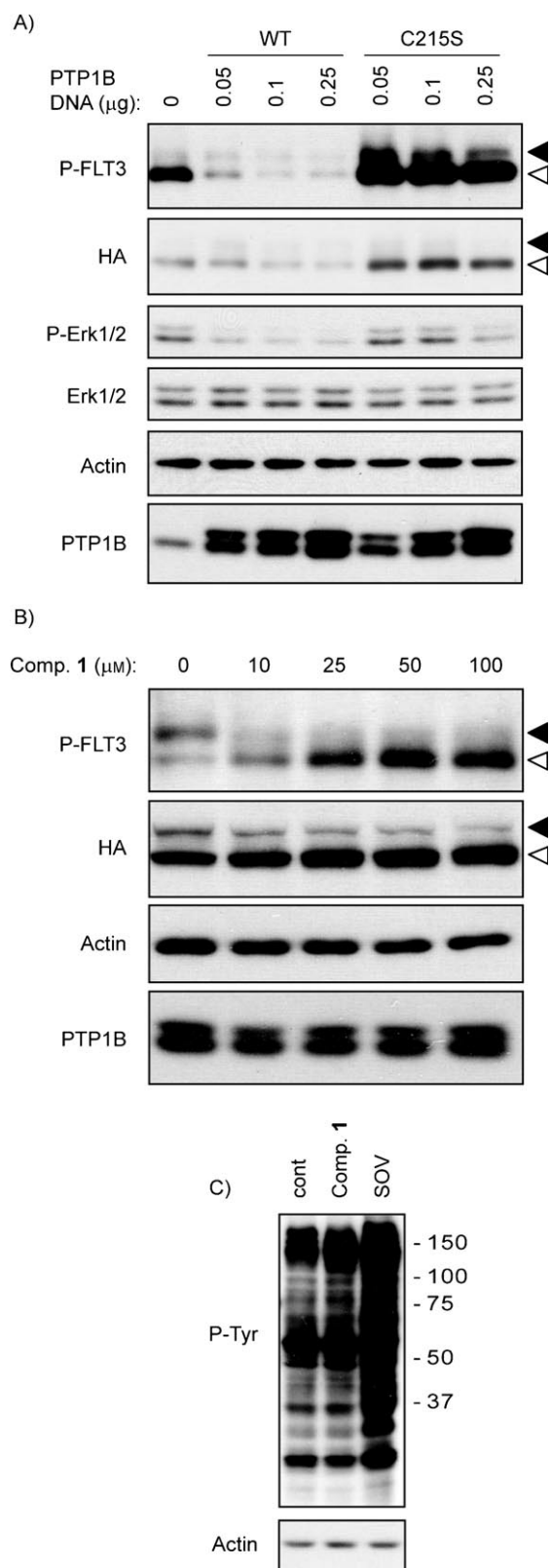
### Cellular activity of compound **1**

A recent study investigated the role of tyrosine phosphorylation in the cell-surface maturation of RTKs.<sup>[34]</sup> The processing of the FLT3 RTK was presented as a model system. This receptor is detectable as immature (small) and mature (large) glycoforms localized to the endoplasmic reticulum (ER) and plasma membrane, respectively, which can be distinguished by SDS-PAGE. A leukemogenic mutant of the receptor, FLT3-ITD, undergoes constitutive tyrosine phosphorylation and is largely retained in its immature form in the ER. Importantly, this phosphorylation is nearly completely eliminated by PTP1B overexpression. Therefore, we evaluated this system as a means to test the cellular activity of PTP1B inhibitors.

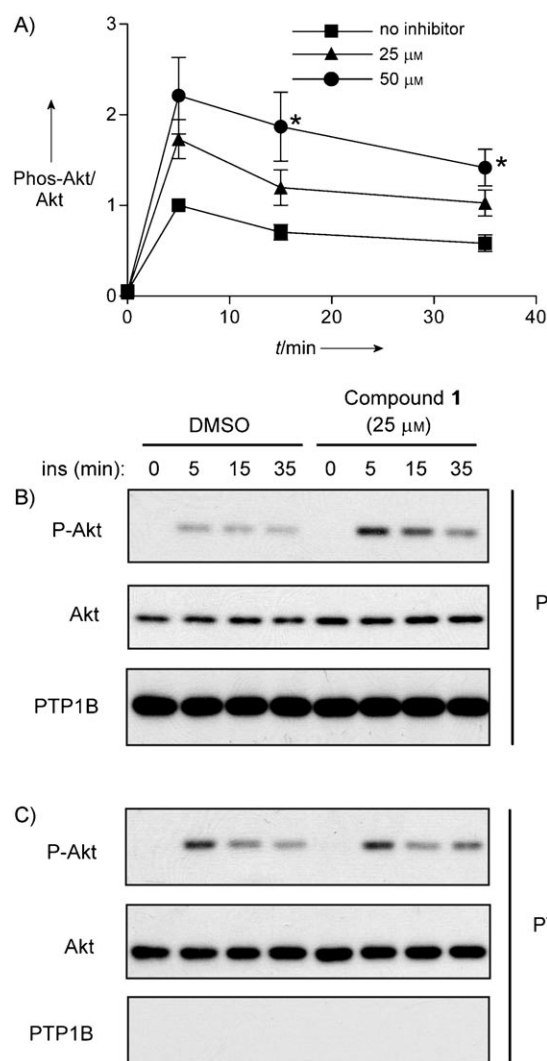
As reported, tyrosine phosphorylation of FLT3-ITD was dramatically decreased by coexpression of WT PTP1B in 293T cells (Figure 3A). To confirm that PTP1B catalytic activity is required for this effect, we also coexpressed inactive PTP1B, which was mutated at the catalytic cysteine residue (C215S). This mutant enzyme has a dominant negative effect: phosphorylation is increased compared to cells expressing the receptor alone, and as a result, the smaller, immature form accumulates. FLT3-ITD-mediated signaling was also affected: activation of Erk, a downstream effector of FLT3,<sup>[35]</sup> was decreased by overexpression of WT, but not inactive PTP1B. Overexpressed, untagged PTP1B was detected as a doublet by immunoblot, perhaps due to post-translational processing.<sup>[36]</sup>

Finally, we asked whether compound **1** could increase FLT3-ITD phosphorylation in this system. Indeed, treatment of cotransfected cells with compound **1** resulted in a clear increase in phosphorylation of the smaller glycoform, with a maximal effect at 50  $\mu\text{M}$  following a 3 h incubation (Figure 3B). Compound **1** significantly increased basal activation of Erk in nontransfected 293T cells (data not shown); therefore, its effect on FLT3-ITD-mediated Erk activation could not be assessed. As an indicator of cellular specificity, we evaluated the phosphotyrosine profiles of cotransfected cells treated with compound **1** or sodium orthovanadate (SOV), a nonspecific PTP inhibitor (Figure 3C). Compound **1** has little effect compared to SOV, which induces a pronounced increase in overall phosphotyrosine content.

We next evaluated the effect of compound **1** on the insulin sensitivity of immortalized mouse embryonic fibroblasts (MEFs). It was recently reported that PTP1B regulates insulin signaling in this cell type.<sup>[37]</sup> After pretreatment with compound **1**, cells were stimulated with insulin for the indicated times, and Akt activation was assessed by immunoblot and quantified (Figure 4A). In WT MEFs, compound **1** significantly increased the amount of phosphorylated Akt after insulin stimulation. Because of low expression levels, insulin receptor



**Figure 3.** A FLT3-ITD/PTP1B coexpression system as an assay for cell-active PTP inhibitors. A) FLT3-ITD (HA-tagged) was transiently coexpressed with either the indicated amount of WT, or with catalytically inactive (C215S) PTP1B in 293T cells. After serum deprivation, lysates were analyzed by immunoblotting with the indicated antibodies. Filled arrowheads indicate the position of the large glycoform of FLT3-ITD, while empty arrowheads point to the small glycoform. B) FLT3-ITD/WT PTP1B-coexpressing 293T cells were used to test the cellular activity of compound 1. Cells were deprived of serum and treated with the indicated final concentrations of compound 1. C) Phosphotyrosine profile of inhibitor-treated cells. Cells were transfected as above and either left untreated (cont), or treated with 50 µM compound 1, or with 50 µM SOV, followed by lysis and analysis by immunoblotting using antiphosphotyrosine antibodies.



**Figure 4.** Compound 1 sensitizes WT, but not PTP1B-null MEFs to insulin-induced Akt activation. A) WT MEFs were deprived of serum and pretreated for 3 h with 0, 25 or 50 µM compound 1 prior to stimulation with 10 nM insulin for the indicated times. Total lysates were analyzed by immunoblotting for active, phosphorylated Akt (P-Akt), and membranes were then stripped and reprobed for total Akt. Band intensity was quantified by densitometry, and for each experiment, P-Akt/Akt ratios were normalized to the value for the 5 min stimulation in the absence of inhibitor. Values correspond to mean ± standard error of the mean for three independent experiments. Statistical analysis was performed with a two-tailed, unpaired, Student's *t* test. Compared to untreated cells, \**P* < 0.05. B) As above, except WT and PTP1B-null MEFs were treated with 0 or 25 µM compound 1.

phosphorylation could not be accurately quantified. Similar experiments were then performed in parallel with PTP1B-null MEFs. At a concentration of 25 µM, compound 1 increased insulin-induced Akt activation in WT, but not PTP1B-null MEFs (Figure 4B). This result shows that the insulin-sensitizing effect of compound 1 in WT MEFs is due to inhibition of PTP1B.

## Discussion

There is compelling evidence that PTP1B directly regulates insulin signaling in vivo. First, the increased insulin sensitivity of PTP1B-null mice correlates with increased IR tyrosine phosphorylation.<sup>[8]</sup> Furthermore, re-expression in the liver of null mice<sup>[38]</sup> and overexpression in the muscle of WT mice<sup>[39]</sup> decreases both insulin sensitivity and phosphorylation of the IR and downstream signaling components. Finally, treatment of adult, diabetic mice with antisense oligonucleotides that target PTP1B in fat and liver has essentially the opposite effect.<sup>[40,41]</sup> The insulin sensitivity of cultured cells treated with PTP1B inhibitors has been used to model their potential in vivo effects. Cell types include mouse and rat myocytes as well as chinese hamster ovary (CHO) cells expressing human IR. While PTP1B overexpression in these cell lines can cause insulin resistance,<sup>[42,43]</sup> there is little evidence from knockdown studies to confirm that inhibiting endogenous PTP1B mimics the effect that is seen in vivo.

Here, we have employed a FLT3-ITD/PTP1B-coexpression system to demonstrate that a novel PTP inhibitor, compound **1** is active in cells. We believe that this approach presents advantages over existing methods; specifically, the exquisite sensitivity of FLT3-ITD phosphorylation to PTP1B catalytic activity allows a clear assessment of cellular activity. Compound **1** caused a clear increase in phosphorylation of the small glycoform of FLT3 at concentrations as low as 10  $\mu\text{M}$ . While several reported PTP1B inhibitors are more active than compound **1** in vitro, they generally display comparable, or less activity in living cells.<sup>[23,43–46]</sup> For example, Combs et al.<sup>[47]</sup> very recently described a series of benzimidazole sulfonamide PTP1B inhibitors with low nanomolar potency in vitro; however, a concentration of 80  $\mu\text{M}$  was required to induce a significant effect in cultured cells.

To confirm its cellular activity in an alternative model system, we showed that compound **1** sensitizes WT, but not PTP1B-null MEFs to insulin stimulation. The absence of an effect in cells lacking PTP1B implies that compound **1** has a degree of specificity at the cellular level. Specifically, it suggests that other PTPs that regulate IR signaling (e.g., LAR and PTP- $\alpha$ )<sup>[48]</sup> are not affected; this is in agreement with our in vitro selectivity results. This cellular specificity is further exemplified by the lack of gross changes in phosphotyrosine content in FLT3/PTP1B-expressing cells treated with compound **1**.

Our results suggest that future development of this class of compounds should be focused on optimizing the structure of the second aryl group (the chloro-substituted phenyl group of compound **1**). This group, which likely extends away from the PTP active site pocket, can be substituted with a variety of functional groups while retaining inhibitory activity. To improve potency and specificity for PTP1B, small-molecule inhibitors have previously been modified to target the secondary phosphate binding site or the nonconserved, charged residues, R47 and D48, near the active site.<sup>[20,21,49]</sup> For potential therapeutic PTP1B inhibitors, specificity against TCPTP is considered to be important because of the immune phenotype of TCPTP-null mice.<sup>[5]</sup> To this end, Asante-Appiah et al. recently demonstrated

that L119 is a selectivity determinant between PTP1B and TCPTP that can be targeted by active-site-binding compounds.<sup>[33]</sup>

## Conclusions

We have reported a novel, uncharged thioxothiazolidinone compound that acts as a competitive inhibitor of a subset of PTPs and exhibits promising potency in two cell-based assays. There has been considerable commercial interest in the development of drugs that target PTP1B for the treatment of type II diabetes. However, other PTPs, some of which are inhibited by compound **1** are also emerging as therapeutic targets.<sup>[13]</sup> We believe that this compound could be a starting point for the development of specific inhibitors that target these enzymes, or it could prove useful as a chemical genetic tool to investigate cellular PTP function.

## Experimental Section

**Chemistry:** Detailed synthetic procedures for compound **1** and its derivatives are described in the Supporting Information, which also includes <sup>1</sup>H NMR and HRMS-ESI data for all compounds.

**Antibodies and reagents.** Polyclonal antibodies against phospho-FLT3 (Y591), phospho-ERK1/2, ERK1/2, phospho-Akt (S473), and Akt were purchased from Cell Signaling Technology (Danvers, MA). Antiphosphotyrosine monoclonal (clone 4G10) and PTP1B polyclonal (for detection of mouse PTP1B) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). PTP1B monoclonal antibody (for detection of human PTP1B) was purchased from BD Transduction Laboratories (San José, CA) and actin antibody (clone C4) was purchased from MP Biochem (Solon, OH). Monoclonal antibody against hemagglutinin (HA, clone 12CA5) was purified from hybridoma supernatant by Mélanie Chagnon. Glutathione Sepharose 4B was purchased from Amersham Biosciences and Complete protease inhibitors were purchased from Roche.

**Plasmids:** Full-length human PTP1B cDNA was amplified by PCR and cloned between *Bam*HI and *Eco*RI sites of pcDNA3.1/Zeo to give pcDNA3.1/Zeo-hPTP1B WT. The pcDNA3.1/Zeo-hPTP1B C215S construct was generated by site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene). The expression construct for HA-tagged human FLT3-ITD, pcDNA3.1/Neo-hFLT3-ITD-HA, has been described previously.<sup>[34]</sup> For GST fusion constructs, PTP sequences were amplified by PCR from cDNA or IMAGE clones and inserted between the *Bam*HI and *Eco*RI sites of pGEX-2Tk (Amersham Biosciences). Insert sequences of all constructs were confirmed by DNA sequencing.

**Preparation of GST-fusion proteins:** A single colony of transformed DH-5 $\alpha$  cells was grown overnight at 37 °C in LB medium that contained ampicillin (100  $\mu\text{g mL}^{-1}$ ). This culture was then diluted by 1:100, and grown to a cell density of 0.6 OD ( $A_{600\text{ nm}}$ ) before induction with IPTG (1 mM) for 4 h at 37 °C. All purification steps were conducted at 4 °C and DTT (5 mM) was added to each purification buffer. Pelleted bacteria were resuspended in lysis buffer (20 mM HEPES/KOH pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 1X complete protease inhibitors). Samples were sonicated on ice (3  $\times$  30 s), and centrifuged at 12000g for 10 min at 4 °C. The supernatant was then incubated with glutathione Sepharose 4B beads for 1 h at 4 °C with rotation. Beads were washed 5  $\times$  5 min with wash buffer (20 mM HEPES/KOH pH 8.0, 500 mM NaCl,

0.1 mM EDTA, 0.1 % Triton X-100). Washed beads were resuspended in elution buffer (50 mM Tris/HCl pH 8.0, 10 mM reduced glutathione) and incubated with rotation for 1 h at 4 °C. Beads were pelleted, and supernatants were concentrated using Centricon filters (Millipore). Four volumes of storage buffer (25 mM HEPES/KOH pH 8.0, 62.5 % glycerol, 0.1 mM EDTA, 6 mM DTT) were added and the purified enzyme was stored at –80 °C.

**PTP assays:** Assays buffers at pH 7.0 and 6.0 were prepared using HEPES (50 mM) and PIPES (50 mM), respectively. DTT (3 mM final) and BSA (0.1 mg mL<sup>-1</sup>) were added to all buffers. Assays were conducted at 25 °C in 96-well plates (Falcon) in a volume of 100 µL. Reaction rates were determined using a Varioskan plate reader (Thermo Electron). Using *p*NPP as a substrate, absorbance was monitored at 405 nm. For assays at pH 7.0, absorbance was measured every 30 s over 10 min, and the reaction rates were calculated by linear regression. Because the *p*-nitrophenol product does not absorb strongly at pH 6.0, the reactions at this pH were stopped at 15 min by the addition of 1 M NaOH (100 µL). Using DIFMUP as a substrate (PTP- $\alpha$  only), fluorescent excitation was conducted at 358 nm and emission was measured at 455 nm every 30 s over 10 min.<sup>[30]</sup> For each enzyme,  $K_m$  values were determined from reaction rates at various substrate concentrations by nonlinear curve-fitting to the Michaelis–Menten equation using Graphpad Prism software (see Table S1 ).  $IC_{50}$  assays were conducted at substrate concentrations equal to the  $K_m$  value for each enzyme. All inhibitor assays contained 1 % DMSO (final).  $IC_{50}$  values were derived by fitting data to a sigmoidal dose–response (variable slope) curve (Prism software).  $K_i$  values were calculated essentially as described.<sup>[50]</sup> Briefly,  $K_{m(app)}$  and  $V_{max(app)}$  were calculated at different inhibitor concentrations, and the  $K_i$  value was estimated by extrapolation of a plot of  $K_{m(app)}/V_{max(app)}$  versus inhibitor concentration.  $K_i$  and  $IC_{50}$  values are mean  $\pm$  standard error of the mean for at least three independent experiments.

**Automated docking analysis:** The binding of compound 1 to PTP1B was modeled using Autodock software, version 3.0.<sup>[32]</sup> Automated docking was performed within a 60 $\times$ 60 $\times$ 60 grid (0.375 Å grid point spacing) centered on the PTP1B active site, using a published crystal structure of PTP1B (PDB ID: 1PXH).<sup>[20]</sup> Protein and ligand structures were prepared for docking with the AutoDockTools (ADT) user interface, except that the ligand was assigned Gasteiger partial charges by using DS Visualizer software (Accelrys). The ligand was kept flexible, and for each docking run, the Lamarckian genetic-algorithm–local-search method was applied for 8000 generations by using the default ADT parameters. Docked conformations from multiple runs were clustered to a threshold root-mean-square deviation of 1.5 Å with ADT.

**Cell culture, inhibitor treatment, lysate preparation, and immunoblotting:** Cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and penicillin/streptomycin (Gibco). For FLT3-ITD/PTP1B expression, 293T cells were plated at 1 $\times$ 10<sup>6</sup> cells per well in 6-well plates 20 h prior to transfection. Cells were transfected with FLT3-ITD-HA DNA (0.5 µg) and PTP1B DNA (0.25 µg) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. For dose–response experiments with PTP1B WT and C215S, the total transfected DNA was adjusted to 0.75 µg with pcDNA3.1/Zeo empty vector. Following transfection, cells were serum-starved in 0.1 % FBS DMEM overnight. For inhibitor treatment, the indicated concentration of compound 1 (1 % DMSO final) or SOV (prepared in water) was added for 3 h. Cells were lysed and analyzed by immunoblotting as described previously.<sup>[51]</sup>

For insulin time–course assays, the previously described<sup>[52]</sup> WT and PTP1B-null spontaneously immortalized MEFs were plated at 6 $\times$ 10<sup>5</sup> cells per well in 12-well plates 48 h before the experiment. Prior to insulin stimulation, cells were serum-starved for 4 h in 0.1 % FBS DMEM and pretreated with compound 1 (0, 25 or 50 µM, 1 % DMSO final) for an additional 2 h. Human insulin (Humulin R, Eli Lilly) was then added at 10 nM for the indicated time. Cell lysates were prepared and analyzed as described above. Quantification of band intensity was performed by densitometry using ImageJ software (NIH).

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- [1] B. M. Sefton, T. Hunter, K. Beemon, W. Eckhart, *Cell* **1980**, *20*, 807.
- [2] J. K. Klarlund, *Cell* **1985**, *41*, 707.
- [3] A. Alonso, J. Sasin, N. Bottini, I. Friedberg, A. Osterman, A. Godzik, T. Hunter, J. Dixon, T. Mustelin, *Cell* **2004**, *117*, 699.
- [4] L. D. Shultz, P. A. Schweitzer, T. V. Rajan, T. L. Yi, J. N. Ihle, R. J. Matthews, M. L. Thomas, D. R. Beier, *Cell* **1993**, *73*, 1445.
- [5] K. E. You-Ten, E. S. Mui, A. Itie, E. Michaliszyn, J. Wagner, S. Jothy, W. S. Lapp, M. L. Tremblay, *J. Exp. Med.* **1997**, *186*, 683.
- [6] M. Elchebly, J. Wagner, T. E. Kennedy, C. Lancot, E. Michaliszyn, A. Itie, J. Drouin, M. L. Tremblay, *Nat. Genet.* **1999**, *21*, 330.
- [7] N. Uetani, K. Kato, H. Ogura, K. Mizuno, K. Kawano, K. Mikoshiba, H. Yakura, M. Asano, Y. Iwakura, *EMBO J.* **2000**, *19*, 2775.
- [8] M. Elchebly, P. Payette, E. Michaliszyn, W. Cromlish, S. Collins, A. L. Loy, D. Normandin, A. Cheng, J. Himms-Hagen, C. C. Chan, C. Ramachandran, M. J. Gresser, M. L. Tremblay, B. P. Kennedy, *Science* **1999**, *283*, 1544.
- [9] L. D. Klamon, O. Boss, O. D. Peroni, J. K. Kim, J. L. Martino, J. M. Zabolotny, N. Moghal, M. Lubkin, Y. B. Kim, A. H. Sharpe, A. Stricker-Krongrad, G. I. Shulman, B. G. Neel, B. B. Kahn, *Mol. Cell. Biol.* **2000**, *20*, 5479.
- [10] M. F. Cicirelli, N. K. Tonks, C. D. Diltz, J. E. Weiel, E. H. Fischer, E. G. Krebs, *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 5514.
- [11] A. Cheng, N. Uetani, P. D. Simoncic, V. P. Chaubey, A. Lee-Loy, C. J. McGlade, B. P. Kennedy, M. L. Tremblay, *Dev. Cell* **2002**, *2*, 497.
- [12] S. D. Taylor, B. Hill, *Expert Opin. Invest. Drugs* **2004**, *13*, 199.
- [13] L. Bialy, H. Waldmann, *Angew. Chem.* **2005**, *44*, 3880; *Angew. Chem. Int. Ed.* **2005**, *44*, 3814.
- [14] J. M. Denu, J. E. Dixon, *Curr. Opin. Chem. Biol.* **1998**, *2*, 633.
- [15] Y. A. Puius, Y. Zhao, M. Sullivan, D. S. Lawrence, S. C. Almo, Z. Y. Zhang, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 13420.
- [16] A. Salmeen, J. N. Andersen, M. P. Myers, N. K. Tonks, D. Barford, *Mol. Cell* **2000**, *6*, 1401.
- [17] Z. Jia, D. Barford, A. J. Flint, N. K. Tonks, *Science* **1995**, *268*, 1754.
- [18] T. R. Burke, H. K. Kole, P. P. Roller, *Biochem. Biophys. Res. Commun.* **1994**, *204*, 129.
- [19] H. S. Andersen, L. F. Iversen, C. B. Jeppesen, S. Branner, K. Norris, H. B. Rasmussen, K. B. Moller, N. P. Moller, *J. Biol. Chem.* **2000**, *275*, 7101.

- [20] J. P. Sun, A. A. Fedorov, S. Y. Lee, X. L. Guo, K. Shen, D. S. Lawrence, S. C. Almo, Z. Y. Zhang, *J. Biol. Chem.* **2003**, *278*, 12406.
- [21] G. Scapin, S. B. Patel, J. W. Becker, Q. Wang, C. Desponts, D. Waddleton, K. Skorey, W. Cromlish, C. Bayly, M. Therien, J. Y. Gauthier, C. S. Li, C. K. Lau, C. Ramachandran, B. P. Kennedy, E. Asante-Appiah, *Biochemistry* **2003**, *42*, 11451.
- [22] H. S. Andersen, O. H. Olsen, L. F. Iversen, A. L. P. Sorensen, S. B. Mortensen, M. S. Christensen, S. Branner, T. K. Hansen, J. F. Lau, L. Jeppesen, E. J. Moran, J. Su, F. Bakir, L. Judge, M. Shahbaz, T. Collins, T. Vo, M. J. Newman, W. C. Ripka, N. P. H. Moller, *J. Med. Chem.* **2002**, *45*, 4443.
- [23] S. D. Larsen, T. Barf, C. Liljebris, P. D. May, D. Ogg, T. J. O'Sullivan, B. J. Palazuk, H. J. Schostarez, F. C. Stevens, J. E. Bleasdale, *J. Med. Chem.* **2002**, *45*, 598.
- [24] L. Xie, S. Y. Lee, J. N. Andersen, S. Waters, K. Shen, X. L. Guo, N. P. Moller, J. M. Olefsky, D. S. Lawrence, Z. Y. Zhang, *Biochemistry* **2003**, *42*, 12792.
- [25] A. S. Liotta, H. K. Kole, H. M. Fales, J. Roth, M. Bernier, *J. Biol. Chem.* **1994**, *269*, 22996.
- [26] S. L. McGovern, B. T. Helfand, B. Feng, B. K. Shoichet, *J. Med. Chem.* **2003**, *46*, 4265.
- [27] H. J. Boehm, M. Boehringer, D. Bur, H. Gmuender, W. Huber, W. Klaus, D. Kostrewa, H. Kuehne, T. Luebbbers, N. Meunier-Keller, F. Mueller, *J. Med. Chem.* **2000**, *43*, 2664.
- [28] J. N. Andersen, O. H. Mortensen, G. H. Peters, P. G. Drake, L. F. Iversen, O. H. Olsen, P. G. Jansen, H. S. Andersen, N. K. Tonks, N. P. Møller, *Mol. Cell. Biol.* **2001**, *21*, 7117.
- [29] G. H. Peters, S. Branner, K. B. Møller, J. N. Andersen, N. P. Møller, *Biochimie* **2003**, *85*, 527.
- [30] S. Welte, K. H. Baringhaus, W. Schmider, G. Muller, S. Petry, N. Tennagels, *Anal. Biochem.* **2005**, *338*, 32.
- [31] M. S. Malamas, J. Sredy, I. Gunawan, B. Mihan, D. R. Sawicki, L. Seestaller, D. Sullivan, B. R. Flam, *J. Med. Chem.* **2000**, *43*, 995.
- [32] G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Huey, W. E. Hart, R. K. Belew, A. J. Olson, *J. Comput. Chem.* **1998**, *19*, 1639.
- [33] E. Asante-Appiah, S. Patel, C. Desponts, J. M. Taylor, C. Lau, C. Dufresne, M. Therien, R. Friesen, J. W. Becker, Y. Leblanc, B. P. Kennedy, G. Scapin, *J. Biol. Chem.* **2006**, *281*, 8010.
- [34] D. E. Schmidt-Arras, A. Bohmer, B. Markova, C. Choudhary, H. Serve, F. D. Bohmer, *Mol. Cell. Biol.* **2005**, *25*, 3690.
- [35] F. Hayakawa, M. Towatari, H. Kiyoi, M. Tanimoto, T. Kitamura, H. Saito, T. Naoe, *Oncogene* **2000**, *19*, 624.
- [36] F. Liu, J. Chernoff, *Biochem. J.* **1997**, *327*(1), 139.
- [37] S. Galic, C. Hauser, B. B. Kahn, F. G. Haj, B. G. Neel, N. K. Tonks, T.iganis, *Mol. Cell. Biol.* **2005**, *25*, 819.
- [38] F. G. Haj, J. M. Zabolotny, Y. B. Kim, B. B. Kahn, B. G. Neel, *J. Biol. Chem.* **2005**, *280*, 15038.
- [39] J. M. Zabolotny, F. G. Haj, Y. B. Kim, H. J. Kim, G. I. Shulman, J. K. Kim, B. G. Neel, B. B. Kahn, *J. Biol. Chem.* **2004**, *279*, 24844.
- [40] B. A. Zinker, C. M. Rondinone, J. M. Trevillyan, R. J. Gum, J. E. Clampit, J. F. Waring, N. Xie, D. Wilcox, P. Jacobson, L. Frost, P. E. Kroeger, R. M. Reilly, S. Koterski, T. J. Opgenorth, R. G. Ulrich, S. Crosby, M. Butler, S. F. Murray, R. A. McKay, S. Bhanot, B. P. Monia, M. R. Jirousek, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11357.
- [41] R. J. Gum, L. L. Gaede, S. L. Koterski, M. Heindel, J. E. Clampit, B. A. Zinker, J. M. Trevillyan, R. G. Ulrich, M. R. Jirousek, C. M. Rondinone, *Diabetes* **2003**, *52*, 21.
- [42] K. Egawa, H. Maegawa, S. Shimizu, K. Morino, Y. Nishio, M. Bryer-Ash, A. T. Cheung, J. K. Kolls, R. Kikkawa, A. Kashiwagi, *J. Biol. Chem.* **2001**, *276*, 10207.
- [43] C. Wiesmann, K. J. Barr, J. Kung, J. Zhu, D. A. Erlanson, W. Shen, B. J. Fahr, M. Zhong, L. Taylor, M. Randal, R. S. McDowell, S. K. Hansen, *Nat. Struct. Mol. Biol.* **2004**, *11*, 730.
- [44] J. E. Bleasdale, D. Ogg, B. J. Palazuk, C. S. Jacob, M. L. Swanson, X. Y. Wang, D. P. Thompson, R. A. Conradi, W. R. Mathews, A. L. Laborde, C. W. Stuchly, A. Heijbel, K. Bergdahl, C. A. Bannow, C. W. Smith, C. Svensson, C. Liljebris, H. J. Schostarez, P. D. May, F. C. Stevens, S. D. Larsen, *Biochemistry* **2001**, *40*, 5642.
- [45] C. Liljebris, S. D. Larsen, D. Ogg, B. J. Palazuk, J. E. Bleasdale, *J. Med. Chem.* **2002**, *45*, 1785.
- [46] G. Liu, Z. Xin, Z. Pei, P. J. Hajduk, C. Abad-Zapatero, C. W. Hutchins, H. Zhao, T. H. Lubben, S. J. Ballaron, D. L. Haasch, W. Kaszubaska, C. M. Rondinone, J. M. Trevillyan, M. R. Jirousek, *J. Med. Chem.* **2003**, *46*, 4232.
- [47] A. P. Combs, W. Zhu, M. L. Crawley, B. Glass, P. Polam, R. B. Sparks, D. Modi, A. Takvorian, E. McLaughlin, E. W. Yue, Z. Wasserman, M. Bower, M. Wei, M. Rugar, P. J. Ala, B. M. Reid, D. Ellis, L. Gonville, T. Emm, N. Taylor, S. Yeleswaram, Y. Li, R. Wynn, T. C. Burn, G. Hollis, P. C. Liu, B. Metcalf, *J. Med. Chem.* **2006**, *49*, 3774.
- [48] A. Cheng, N. Dube, F. Gu, M. L. Tremblay, *Eur. J. Biochem.* **2002**, *269*, 1050.
- [49] L. F. Iversen, H. S. Andersen, S. Branner, S. B. Mortensen, G. H. Peters, K. Norris, O. H. Olsen, C. B. Jeppesen, B. F. Lundt, W. Ripka, K. B. Møller, N. P. Møller, *J. Biol. Chem.* **2000**, *275*, 10300.
- [50] T. Lubben, J. Clampit, M. Stashko, J. Trevillyan, M. R. Jirousek in *Current Protocols in Pharmacology*, **2001**, (Eds. S. J. Enna, M. Williams), Wiley, Chichester, pp. 3.8.1–3.8.13.
- [51] F. Gu, D. T. Nguyen, M. Stuibale, N. Dubé, M. L. Tremblay, E. Chevet, *J. Biol. Chem.* **2004**, *279*, 49689.
- [52] F. Gu, N. Dubé, J. W. Kim, A. Cheng, M. de J. Ibarra-Sanchez, M. L. Tremblay, Y. R. Boisclair, *Mol. Cell. Biol.* **2003**, *23*, 3753.

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