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A Phosphoramidate Strategy Enables Membrane Permeability of a Non-nucleotide Inhibitor of the Prolyl Isomerase Pin1

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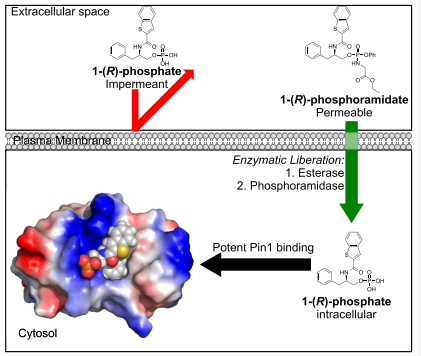
ABSTRACT: The membrane permeability of nucleotide-based drugs, such as sofosbuvir (Sovaldi), requires installation of phosphate-caging groups. One strategy, termed "ProTide", masks the anionic phosphate through an N-linked amino ester and an O-linked aromatic phospho-ester, such that release of the active drug requires consecutive enzymatic liberation by an esterase and then a phosphoramidase, such as Hint1. Because Hint1 is known to be selective for nucleotides, it was not clear if the ProTide approach could be deployed for non-nucleotides. Here, we demonstrate that caging of a phosphate-containing inhibitor of the prolyl isomerase Pin1 increases its permeability. Moreover, this compound was processed by both esterase and phosphoramidase activity, releasing the active molecule to bind and inhibit Pin1 in cells. Thus, Hint1 appears to recognize a broader set of substrates than previously appreciated. It seems possible that other potent, but impermeable, phosphate-containing inhibitors might likewise benefit from this approach.



KEYWORDS: *ProTide, Hint1, pro-drug, phosphate-containing, PPIase, oncology*

Phosphate groups are important in molecular recognition throughout biology. However, inhibitors that incorporate phosphates or phospho-mimetics are often too anionic to be passively permeable to biological membranes.[1−3] Accordingly, many prodrug strategies have been developed to mask phosphates,[4−6] allowing better balance between potency, selectivity, and permeability. One of the most successful of these prodrug approaches is the phosphoramidate-based "ProTide" technology (Figure 1).[7] This caging group relies on the consecutive action of esterase activity on the *O*-carboxy ester, followed by liberation of the phospho-ester by intramolecular nucleophilic attack and then hydrolysis of the *N*-linked amino ester by a intracellular phosphoramidase, often the histidine triad nucleotide binding protein 1 (Hint1).[8] Together, these activities deliver the active, phosphate-bearing molecule to the cytosol. ProTide approaches have proven especially successful in enhancing the cellular delivery of nucleotide-based antivirals, including blockbuster drugs (i.e., sofosbuvir[9,10]), clinical candidates (i.e., NUC-1031[11]), and tool molecules (i.e., 4Ei-10[12]). However, the applicability of ProTide technology to non-nucleotides is nascent; while phosphoramidates have been shown to improve the plasma lifetime and clogP values for a handful of non-nucleotides,[13−16] it is not yet clear whether they can be enzymatically liberated in cells. Indeed, Hint1 is a selective, metabolic enzyme, which might not be considered likely to accept non-nucleotides that are structurally distinct from its natural substrates. Crystal structures of substrate-bound Hint1 have supported this idea, revealing a restrictive substrate envelope that is dominated by polar interactions around the phosphate (Supporting Information Figure 1).[8] However, we noted that an adjacent, hydrophobic pocket, which is normally involved in accommodating the nucleobase, was potentially more amenable to alternative substrates (Supporting Information Figure 1). A search of appropriate, phosphate-containing inhibitors in the literature turned our attention to inhibitors of the peptidyl-prolyl isomerase, Pin1.

Pin1 is considered an attractive cancer target, owing to its high expression in breast and prostate tumors and the strong antitumor effects of Pin1 knockdown.[17] Pin1 is a two-domain protein composed of a catalytic peptidyl-prolyl isomerase domain and noncatalytic WW domain. Both of these domains bind selectively to prolines that are adjacent to phosphorylated Ser/Thr (*e.g.* the pS/T-Pro motif). Only the catalytic domain, however, can isomerize this bond, facilitating interconversion between *cis* and *trans* peptidyl−prolyl bonds.[18] Potent inhibitors of Pin1's catalytic activity, such as 1-(*R*)-phosphate, were described by Pfizer and the phosphate was found to impart significant affinity, but these molecules were too polar to be

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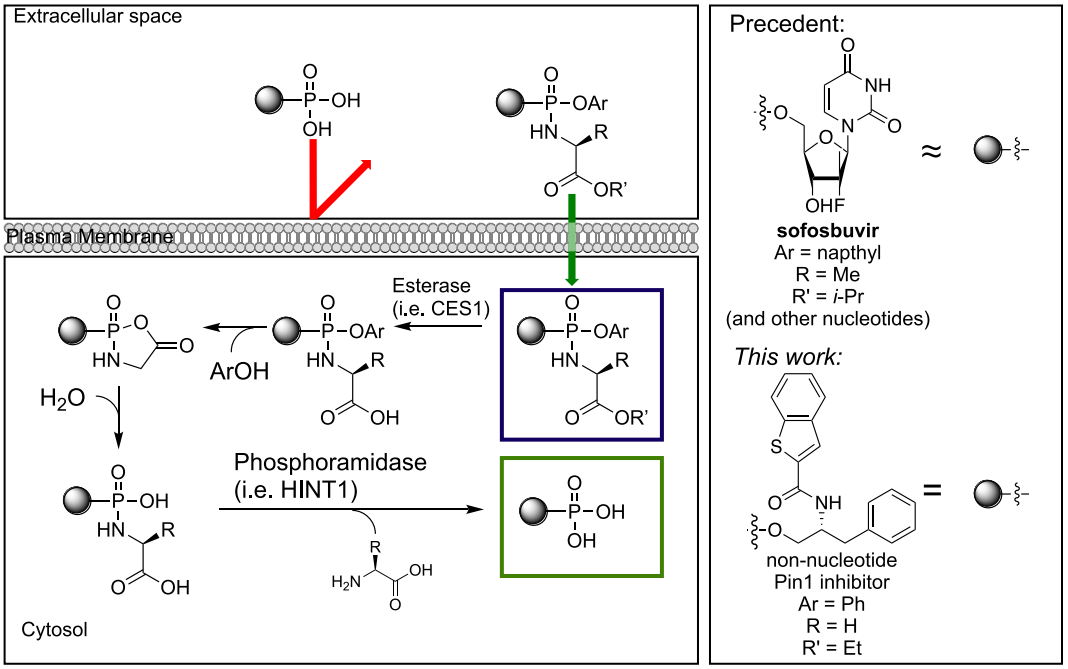
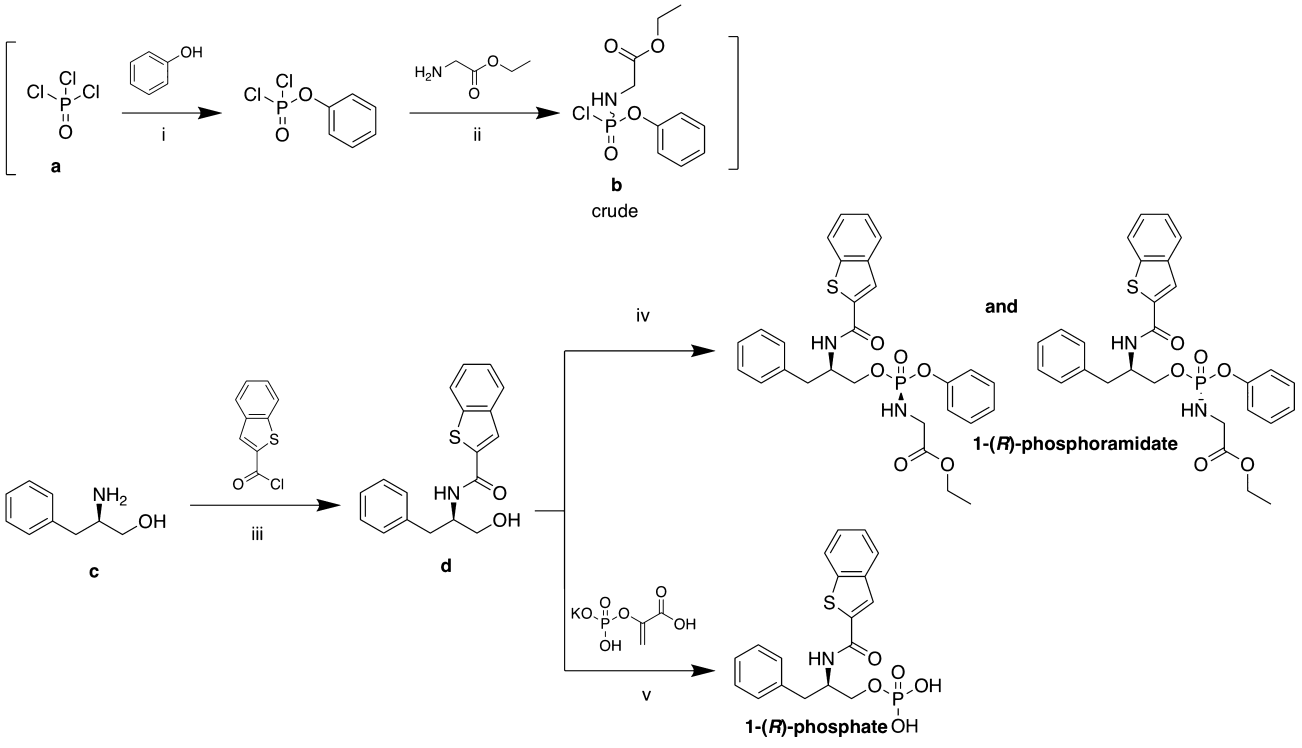


Figure 1. Schematic of phosphoramidate liberation in the cytosol. Phosphoramidates, such as sofosbuvir, are enzymatically liberated by a carboxylesterase (i.e., CES1) and subsequent phosphoramidase activity (i.e., Hint1) to reveal the free phosphate form. This mechanism has been exploited in multiple nucleotide-based drugs but has not yet been demonstrated to work for non-nucleotides.

Scheme 1. Synthesis of the Pro-drug 1-(R)-Phosphoramidate and Its Free Phosphate Form 1-(R)-Phosphate[a]



[a]Reaction conditions: (i) DIPEA, DCM, −78 °C to rt, 2 h; (ii) DIPEA, DCM, −78 °C to rt, 2 h;[29] (iii) DIPEA, THF, rt, 18 h; (iv) (1) t-BuMgCl, THF, rt, 1 h; (2) **b**, 18 h; (v) $NBuHSO_4$, DMF, 80°C, 6 h.[28].

membrane permeable.[19] Attempts to improve these compounds focused on replacing the phosphate and optimizing nonpolar contacts.[19−23] Alternatively, previous work has overcome the poor permeability of phosphate-bearing Pin1 inhibitors using bis-POM masking groups.[24] Likewise, cyclic peptides[25] and covalent inhibitors lacking the phosphate have been explored.[26,27] While these efforts yielded important insights into the potential of Pin1 as a drug target, we envisioned a complementary approach, in which ProTide technology might

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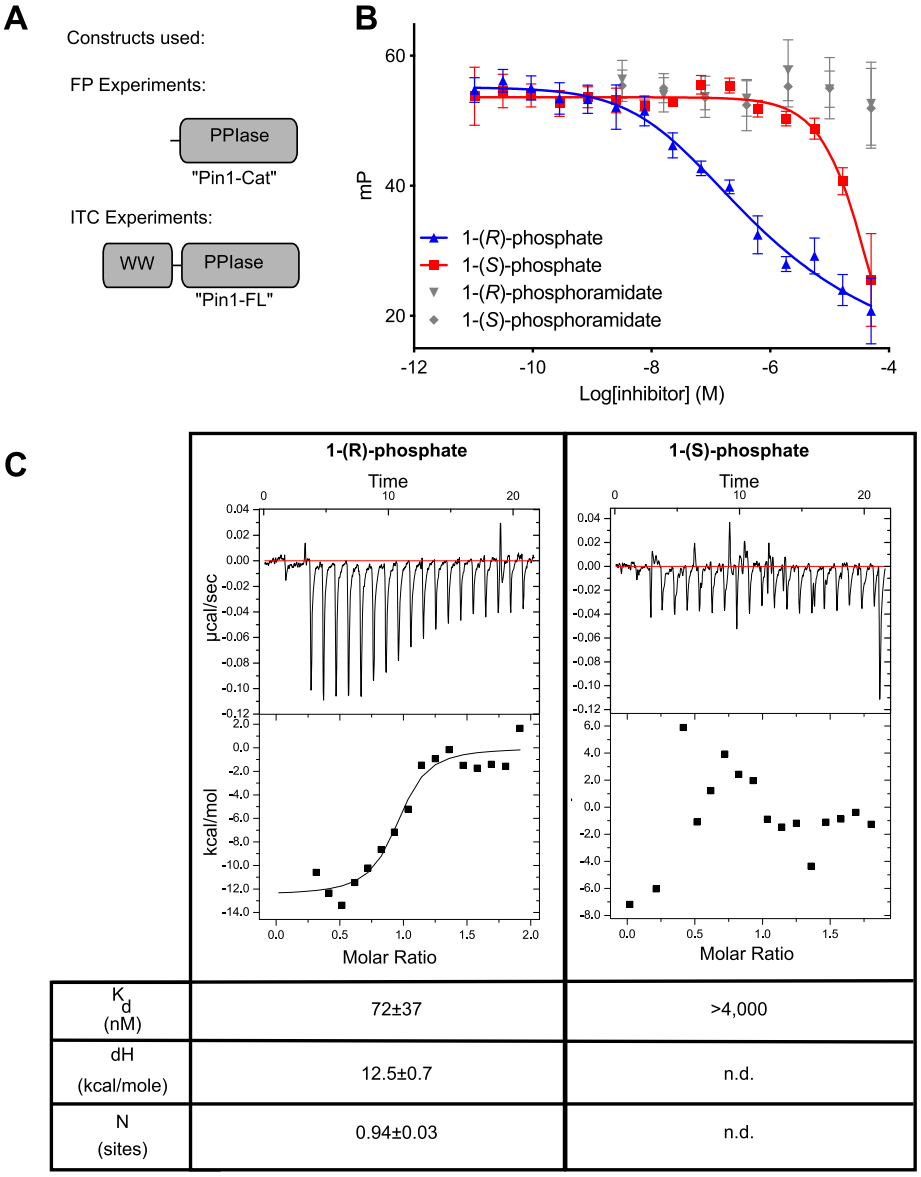

	1-(R)-phosphate	1-(S)-phosphate
K_d (nM)	72±37	>4,000
dH (kcal/mole)	12.5±0.7	n.d.
N (sites)	0.94±0.03	n.d.

Figure 2. 1-(R)-Phosphate, but not the control, binds to Pin1's catalytic domain in vitro. (A) Schematic of the constructs used in this study: a truncated Pin1 lacking the WW domain (Pin1-Cat) and full length Pin1 (Pin1-FL). (B) 1-(R)-Phosphate, but not 1-(S)-phosphate, competed with a labeled tracer (FITC-WFYpSPFLE; Pintide) for binding to Pin1-Cat, as measured by FP. Importantly, neither 1-(R) or 1-(S)-phosphoramidates bind Pin1. Results are the average of triplicates and the error bars represent SD. (C) ITC experiment confirming that 1-(R)-phosphate binds Pin1-FL, and with a stoichiometry ~1, consistent with preferential binding to the catalytic domain. Importantly, 1-(S)-phosphate did not have detectable binding.

be used to increase the permeability of Pfizer's molecule, 1-(*R*)-phosphate (Figure 1).

RESULTS AND DISCUSSION

To probe this possibility, we first synthesized 1-(*R*)-phosphoramidate and its enantiomer, 1-(*S*)-phosphoramidate, as well as the free phosphates: 1-(*R*)-phosphate and 1-(*S*)-phosphate (Scheme 1), guided by reported routes.[19,28,29] It is important to note that the phosphoramidates contain an additional stereocenter about the phosphorus atom and, in these proof-of-concept studies, these compounds were used as a ~1:1 mixture of diastereomers (Supporting Information Figure 5). Based on cocrystal structures, we anticipated that 1-(*R*)-phosphate would bind Pin1, while 1-(*S*)-phosphate would be an important, inactive control (Supporting Information Figure 2). To test this idea, we measured binding to the purified catalytic domain of human Pin1 (Pin1-Cat; residues 45−163) by fluorescence polarization (FP). In these experiments, we estimated inhibition constant (IC_{50}) values, based on competition with a fluorescent peptide FITC-WFYpSPFLE (PinTide) that is known to bind and inhibit the Pin1 catalytic site. As expected, we found that the 1-(*R*)-phosphate (IC_{50} < 300 nM), but not 1-(*S*)-phosphate (IC_{50} > 10,000 nM), bound to Pin1-Cat (Figure 2B). Also, we confirmed that neither of the pro-drugs, 1-(*R*)-phosphoramidate or 1-(*S*)-phosphoramidate, were able to bind Pin1-Cat (IC_{50} > 10,000 nM). Having confirmed that 1-(*R*)-phosphate binds the catalytic site, we turned to studying the full-length protein (Pin1-FL). As mentioned above, Pin1-FL also contains a noncatalytic WW domain, which has been shown to bind phosphorylated peptides containing a *trans*-proline.[30] We expected that 1-(*R*)-phosphate might be selective for the catalytic site over the WW domain, because it mimics the twisted-amide transition state that is only preferred by that site.[19,31] Indeed, using isothermal

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ACS Med. Chem. Lett. 2020, 11, 1704−1710

titration calorimetry (ITC), we found that 1-(*R*)-phosphate bound Pin1-FL with a stoichiometry ~1 (N = 0.94 ± 0.03), suggesting that it primarily interacts with the catalytic site (Figure 2C). We also noticed that a dissociation constant (K_d) of 1-(*R*)-phosphate for Pin1-FL (72 ± 37 nM) was enhanced over the value measured for binding the truncated Pin1-Cat (see above); an improvement that was expected because similar effects have been previously observed for Pin1 substrates.[32] Together, these binding studies showed that 1-(*R*)-phosphate binds Pin1 with the expected affinity and domain preference *in vitro*.

Next, we measured the relative hydrophobicity and permeability of 1-(*R*)-phosphoramidate and 1-(*R*)-phosphate. Using octanol−water partitioning, the phosphoramidate was calculated to be significantly more hydrophobic than the phosphate (Table 1). Consistent with this difference, the phosphoramidate was also more permeable (Table 1). Encouraged by this result, we then explored whether 1-(*R*)-phosphoramidate might be enzymatically liberated to 1-(*R*)-phosphate in cells. To ask this question, K562 cells were treated for 5 h under serum-free conditions, followed by extensive washing, centrifugation, ethyl acetate extraction, and measurement of the reaction products by ultrahigh-performance liquid chromatography mass spectrometry (UPLC-MS). Satisfyingly, both the intermediate product of esterase activity and the 1-(*R*)-phosphate product were detected in the treated K562 cell lysate (Figure 3A). In addition, a small amount of the dephosphorylated metabolite was also present and its identity confirmed with an authentic standard. The free phosphate peak increased with time, consistent with enzymatic turnover (Figure 3B). To ensure that 1-(*R*)-phosphate was indeed being processed by intracellular enzymes, we repeated the extractions in media lacking cells. In these controls, neither the intermediate nor 1-(*R*)-phosphate were identified, confirming that enzymatic activity was required. Together, these results suggest that 1-(*R*)-phosphoramidate is cell-permeable and that it is converted to its active form in cells.

Table 1. Installation of a Phosphoramidate Dramatically Improves Hydrophobicity and Permeability[a]

Compound	logP	logPe (cm/s)
1-(*R*)-phosphoramidate	0.27	−4.6
1-(*R*)-phosphate	<−4.6	<−6.3

[a]Partitioning coefficient (logP) values were determined by equilibrium octanol water partitioning and permeability constant (logPe) by PAMPA. The levels of 1-(*R*)-phosphate were near limit of detection (LOD).

The next question is whether the liberated 1-(*R*)-phosphate might engage Pin1. Due to the low permeability of 1-(*R*)-phosphate itself (see Table 1), this question could not previously be addressed. To test it, we performed a cellular thermal shift assay (CETSA). Specifically, K562 cells were treated with 1-(*R*)-phosphoramidate (25 μM) or solvent alone (0.25% DMSO) for 5 h to allow for liberation of the active molecule. Then, cells were heated on a temperature gradient, lysed, and the soluble fraction assayed for Pin1 abundance by Western blot. We found that Pin1 was partially protected by the compound treatment (Figure 4A), consistent with binding of 1-(*R*)-phosphate to Pin1. This result was also repeated in MDA-MB-231 cells, a model of metastatic breast cancer. This experiment was important because Pin1 has been specifically implicated in both prostate and breast cancers.[17] In these experiments, we leveraged the findings from the K562 studies and performed the CETSA near the most sensitive, half-maximal temperature (48 °C). As in the K562 cells, Pin1 was stabilized (Figure 4B). Together, these results suggest that 1-(*R*)-phosphate is released from 1-(*R*)-phosphoramidate and that it binds Pin1 in two cancer cell types.

To explore whether 1-(*R*)-phosphoramidate was converted by the known, enzyme-based mechanism in the cytosol, we employed an inhibitor of Hint1, TrpGc.[33−35] In early experiments, we had noticed that, at time points longer than 24 h, treatment of MDA-MB-231 cells with 1-(*R*)-phosphoramidate led to a dose-responsive increase in Pin1 levels, even at normal temperatures (Figure 5A). Using this biomarker, we found that cotreatment with TrpGc (100 μM) blocked the cellular activity of 1-(*R*)-phosphoramidate (Figure 5B), supporting an essential role for Hint1.

Finally, we wanted to ask whether pharmacological inhibition of Pin1 would replicate the effects seen in knockdown studies. Because Pin1 has both catalytic and WW domains that bind to pS/T-Pro motifs, it is not clear from the knockdown experiments which subset of its cellular roles might be mediated

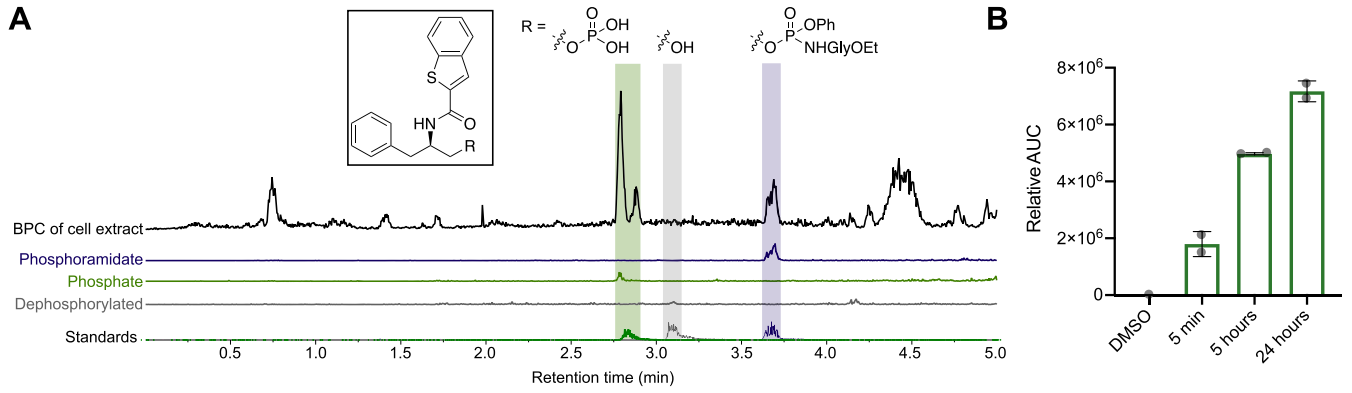

Figure 3. Cellular liberation of 1-(R)-phosphate by cytoplasmic enzymes. K562 cells treated with 1-(R)-phosphoramidate were washed, pelleted, and extracted with EtOAc. (A) The extract was analyzed by UPLC-MS to yield the base peak chromatogram (black). Then, the peaks corresponding to the mass of the phosphoramidate (purple), the phosphate (green), and the dephosphorylated metabolite (gray) were identified in the treated sample and compared to the approximate elution window of the authentic standards (bottom). (B) To understand release of the phosphate product over time, a time course experiment was conducted and the peak area quantified. A solvent control (DMSO) was used to subtract the background. The averages of duplicate experiments are shown, with the full range.

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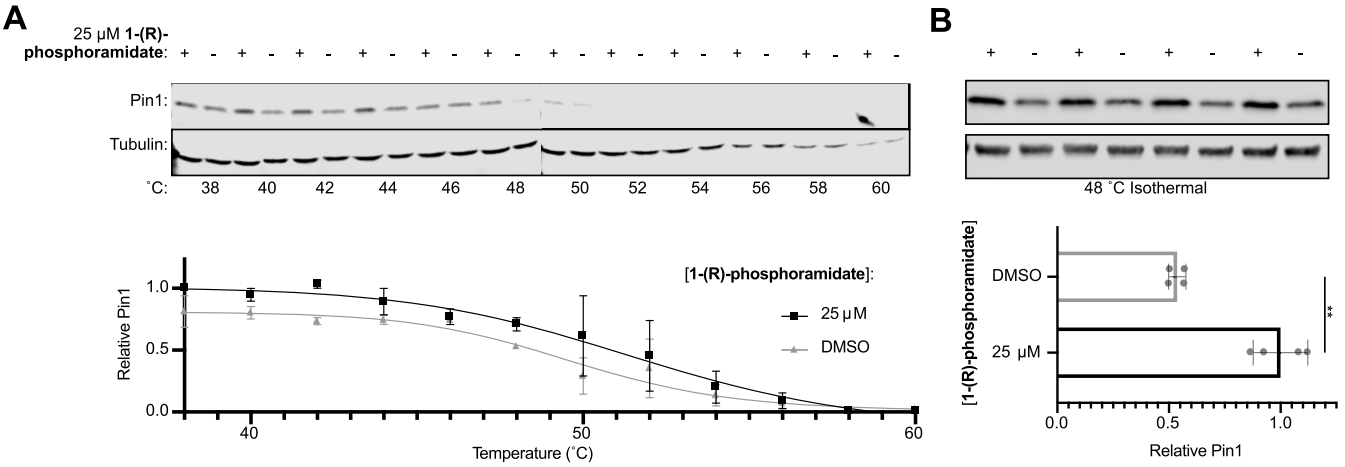

Figure 4. Treatment with 1-(R)-phosphoramidate leads to Pin1 target engagement, by CETSA. (A) K562 cells were treated with 1-(*R*)-phosphoramidate, heated at temperatures between 38 to 60 °C, and the soluble fraction assayed for Pin1 abundance by Western blot. Treatment with 1-(*R*)-phosphoramidate led to stabilization of Pin1, compared to the mock treated (quantified below). Results are the average of three independent experiments, and error bars represent SEM. (B) Similar results were observed in MDA-MB-231 cells, treated at a fixed temperature (48 °C) in biological quadruplicates (quantified below). **p value <0.01.

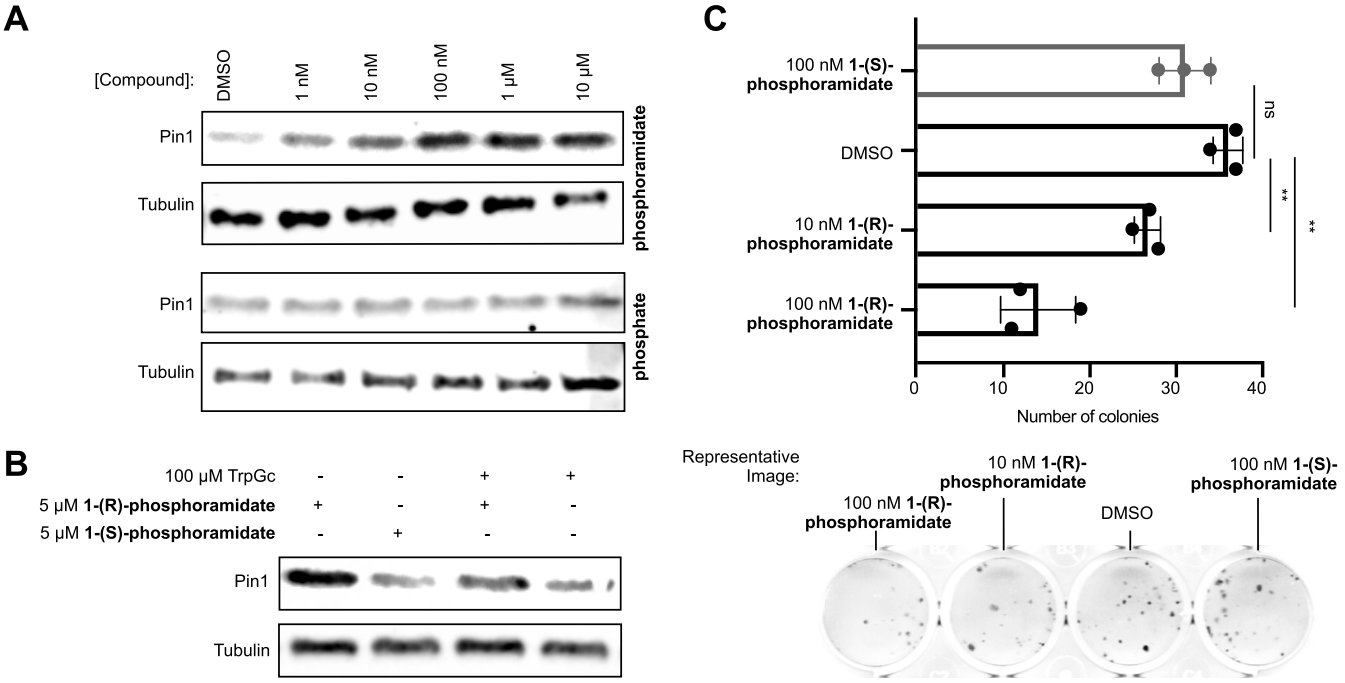

Figure 5. 1-(R)-Phosphoramidate is released in cells, leading to Pin1 binding and inhibition. (A) Treatment of MDA-MB-231 cells with 1-(*R*)-phosphoramidate for 72 h results in a dose-responsive increase in Pin1 abundance. (B) Treatment with a Hint1 inhibitor, TrpGc, suppresses this 1-(*R*)-phosphoramidate activity. (C) 1-(*R*)-phosphoramidate, but not the control, inhibited colonogenic activity in treated PC3 cells. Results are the average of experiments performed in triplicate, and the error bars represent SD. A representative dose series is shown below. **p value <0.01.

by catalytic peptidyl−prolyl isomerization. One of the best characterized biological roles for Pin1 is in tumor colony formation; as Pin1 knockdown is reported to inhibit colonogenic potential.[36] Using PC3 neuroendocrine prostate cancer cells, we tested whether treatment with 1-(*R*)- or 1-(*S*)-phosphoramidate might reduce colony formation. We found that 1-(*R*)-phosphoramidate, but not 1-(*S*)-phosphoramidate, inhibited colony formation (Figure 5C), suggesting that isomerase activity is indeed important for this role. Importantly, treatment with 1-(*R*)-phosphoramidate did not significantly reduce proliferation of more confluent PC3 cells after 72 h, based on either ATP-Glo or MTT assays (Supporting Information Figure 4). Thus, Pin1 plays a role in colony formation at low density, but it does not seem to have a major function in survival signaling at high cell density. Indeed, knockdown of Pin1 or inhibition by other approaches shows a similar result.[27,36,37]

Together, these results suggest that 1-(*R*)-phosphoramidate is liberated by Hint1 in cells, releasing 1-(*R*)-phosphate, which then binds Pin1's catalytic site and inhibits some of its functions. Thus, we propose that 1-(*R*)-phosphoramidate will be a useful chemical probe, enabling future studies into Pin1's enigmatic roles in cancer. Such future studies would also benefit from understanding which phosphoramidate diastereomer is a better

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ACS Med. Chem. Lett. 2020, 11, 1704−1710

substrate for Hint1, as well as quantitative measurements of its cytosolic conversion kinetics.

More generally, this work provides initial evidence that the "ProTide" approach is more broadly applicable than previously appreciated. We speculate that additional evaluation of Hint1's substrate envelope might open this approach to additional scaffolds. For example, molecular recognition in kinase/phosphatase signaling, 14−3−3 scaffolding and nucleoside metabolism involves selective binding of phosphates.[38] As envisioned, this pro-drug approach rebalances the interplay between permeability and potency, allowing chemical probes to include the natural phosphate that is so important in molecular recognition.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00170.

Experimental details, supporting figures (S1−S5), and analytical characterization. (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Hint1, histidine triad nucleotide binding protein 1; Pin1, petidiyl−prolyl isomerase NIMA interacting 1; CES1, carboxylesterase 1; FP, fluorescence polarization; ITC, isothermal titration calorimetry; UPLC, ultrahigh performance liquid chromatography; MS, mass spectrometry; CETSA, cellular thermal shift assay; PAMPA, parallel artificial membrane permeability assay

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