Drug Delivery

Design, Construction, and Intracellular Activation of an Intramolecularly Self-Silenced Signal Transduction Inhibitor**

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The AB protein toxins are among the most potent and lethal cell death inducing agents known. This protein family includes such notorious cytotoxins as ricin, and the cholera, diphtheria, tetanus, and botulinum toxins.[1] These, and related family members, share a variety of structural and mechanistic motifs. The A polypeptide acts on a specific intracellular target in a fashion that compromises cellular viability. By contrast, the B moiety serves as a transporter that delivers the A component to the intracellular environment. In addition, the B polypeptide is typically conjoined to the A moiety through a disulfide bridge, which suppresses the biological activity of the latter. However, upon intracellular reduction of the disulfide, the A protein is released into its fully activated state.

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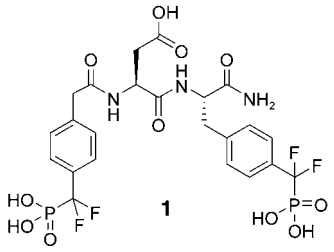
It occurred to us that the AB protein toxin mechanism by which a highly toxic, but membrane-impermeable, protein is delivered to a cell in an inert form might prove applicable to other inhibitory agents as well. We describe herein a strategy that employs a cell-penetrating peptide (CPP)[2] to 1) suppress the activity of an appended inhibitor, 2) deliver the membrane-impermeable inhibitor to the intracellular environment, and, once inside the cell, 3) release the inhibitor in its active form.

We previously described the protein tyrosine phosphatase (PTP) 1B inhibitor **1**.[3] This species is the most potent



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(inhibitory constant, $K_i = 2.4$ nM) and selective PTP1B inhibitor (1000–10000-fold versus a panel of phosphatases) reported to date. The negatively charged aryldifluorophosphonate functional groups participate in key active-site and near-active-site interactions that are responsible for the high PTP1B potency displayed by **1**.[4] Not surprisingly, the negatively charged inhibitor is not cell-permeable.[5] We anticipated that a cell-permeable derivative of **1** could be prepared by coupling it to the CPP ((D)-Arg)$_8$ moiety to generate **2** (Scheme 1). Polycationic CPPs are widely used to transport cargo through cell membranes.[2] However, we also expected that electrostatic interactions between the positively charged CPP and the negatively charged cargo might have the desirable effect of suppressing inhibitory efficacy. Consequently, effective inhibition of PTP1B, a cytoplasmically oriented endoplasmic reticulum (ER)-embedded protein,[6] should only transpire upon release of the cargo from the CPP and only if release occurs within a PTP1B-available region of the cell. By contrast, a corresponding construct that lacks the disulfide bridge (**5**) should be unable to effectively inhibit PTP1B.

Compound **2** was directly prepared on the Rink resin by sequential coupling of eight (D)-Fmoc-Arg(Pbf) residues (Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), FmocNH$(CH_2)_2SS(CH_2)_2CO_2H$, compound **3**, Fmoc-Asp-(*t*Bu)OH, and compound **4** (Scheme 1).[7] Simultaneous deprotection and cleavage mediated by CF_3CO_2H furnished **2**. Compound **2** is an extraordinarily ineffective inhibitor of PTP1B, with a K_i value (8.2 μM) that is three orders of magnitude lower than that of **1**. This appears to be a consequence of intramolecular interactions between the PTP1B-directed portion of **2** and its disulfide-linked ((D)-Arg)$_8$ appendage. NMR experiments under aqueous conditions confirm that the key active-site-directed aryldifluorophosphonate moiety is engaged in through-space interactions with the ((D)-Arg)$_8$ cell-permeabilizing sequence (see Supporting Information). Furthermore, the intramolecular nature

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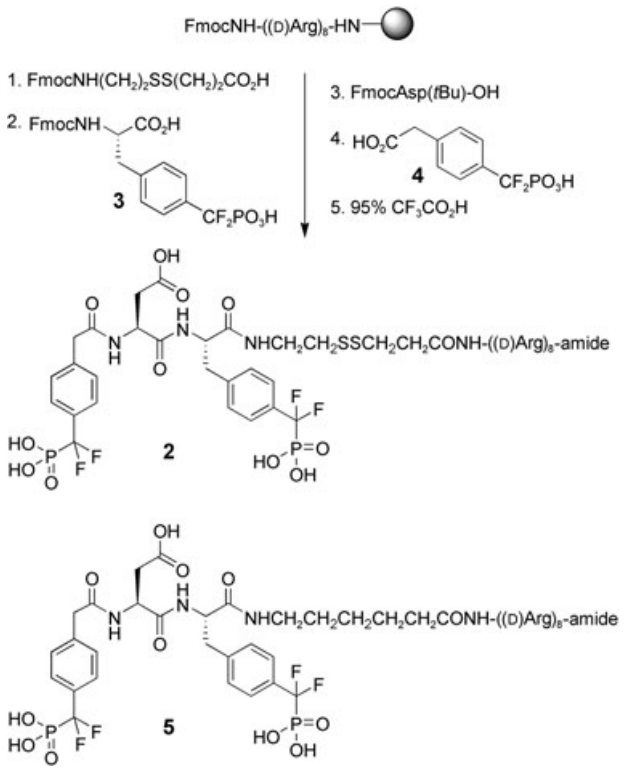

Scheme 1. Solid-phase synthesis of the CPP-S-S-(PTB1-B inhibitor) constructs **2** and **5**. Peptides were prepared on the Rink amide resin by using 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids and a standard coupling protocol involving 1-hydroxybenzotriazole/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/*N*-methylmorpholine for the activation of acids.

enhancements in IRβ and IRS-1 phosphotyrosine levels have been reported with a PTP1B inhibitor.[5]

We performed an analogous study using a Chinese hamster oocyte (CHO) cell line transfected with an expression plasmid encoding human IR (CHO/HIRc). We previously found that compound **1** has no effect on IRβ and IRS-1 phosphotyrosine levels in this cell line, since its five negative charges render it membrane-impermeable.[5] The CHO/HIRc cell line was exposed to compounds **2** and **5** over a selected range of concentrations for 1 h and subsequently treated either with or without insulin (10 nM) for 10 min. Cell lysates were then resolved by SDS-PAGE, electrotransferred to nitrocellulose membranes, and probed with anti-pTyr antibody (Figure 1). Compound **5** has no effect on IRβ or IRS-1

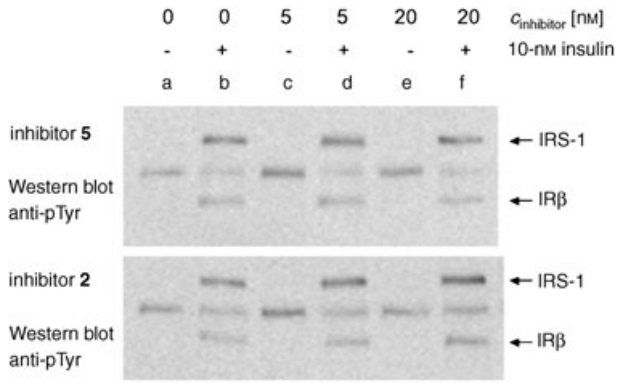

Figure 1. Effect of PTP1B inhibitors **5** (upper gel) and **2** (lower gel) on the tyrosine phosphorylation status of IRS-1 and IRβ. Lane a (no inhibitor, without insulin); lane b (no inhibitor, with insulin); lane c (5-nM **5** and **2**, without insulin); lane d (5-nM **5** and **2**, with insulin); lane e (20-nM **5** and **2**, without insulin); lane f (20-nM **5** and **2**, with insulin).

of this interaction is confirmed by the fact that the inhibitory efficacy of free **1** is not affected by added $((D)\text{-Arg})_8$ (at concentrations up to 20 μM). Finally, the masking effect of the positively charged permeabilizing sequence is due to electrostatic interactions, since a corresponding cell-permeable fatty-acid-derivatized PTP1B inhibitor is fully active.[5]

The disulfide bridge present in **2** is absent in the otherwise structurally equivalent derivative **5**. Compound **5** was synthesized in a fashion analogous to that outlined in Scheme 1 with the exception that $FmocNH(CH_2)_5CO_2H$ was employed in step 1. As expected, both compounds **2** and **5** ($K_i = 9.0$ μM) are equally poor PTP1B inhibitors under nonreducing conditions.

We determined the cellular effects of compounds **2** and **5** on insulin signaling to assess the efficacy of the delivery strategy mimicking AB protein toxins. Insulin-mediated activation of the insulin receptor (IR) produces a wide array of cellular responses, including enhanced phosphorylation of the insulin receptor β (IRβ) subunit and the insulin receptor substrate-1 (IRS-1). Previous studies have demonstrated that PTP1B serves as a negative regulator of the insulin-activated signaling pathways by catalyzing the dephosphorylation of IRβ and IRS-1.[5] For example, the antisense-mediated reduction of PTP1B in rat hepatoma cells generates a 2.3-fold and 1.5-fold increase in IRβ and IRS-1 phosphotyrosine levels, respectively.[8] In addition, significant phosphotyrosine levels, even at concentrations as high as 100 nM (data not shown). This is not surprising given that the latter concentration is 90 times lower than the in vitro K_i of **5**. By contrast, even at 5 nM, compound **2** enhances the phosphotyrosine content of IRβ and IRS-1 1.7- and 1.4-fold, respectively. An even more pronounced effect is observed at 20 nM (2.4- and 2.2-fold, respectively). These concentrations are nearly three orders of magnitude lower than the K_i value displayed by **2** under nonreducing conditions in vitro. These results are consistent with the notion that the disulfide bridge linking the PTP1B inhibitory component ($K_i = 2.4$ nM) with the CPP has been cleaved and that the liberated inhibitor has ready access to the cytoplasmically oriented protein target.

In summary, we have shown that a CPP module can be used to biochemically silence the biological activity of appended cargo.[9] This property, which is a characteristic feature of AB protein toxins, should restrict the desired biological effect to the targeted intracellular environment. We are currently investigating the scope of this approach as well as the utility of compound **2** for the treatment of diabetes and obesity.[10]

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