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Cell-Penetrating Bisubstrate-Based Protein Kinase C Inhibitors

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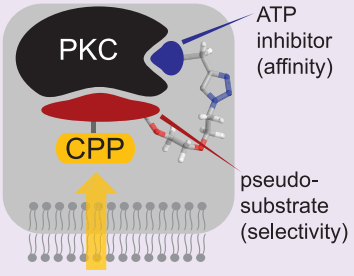
Supporting Information

ABSTRACT: Although protein kinase inhibitors present excellent pharmaceutical opportunities, lack of selectivity and associated therapeutic side effects are common. Bisubstrate-based inhibitors targeting both the high-selectivity peptide substrate binding groove and the high-affinity ATP pocket address this. However, they are typically large and polar, hampering cellular uptake. This paper describes a modular development approach for bisubstrate-based kinase inhibitors furnished with cell-penetrating moieties and demonstrates their cellular uptake and intracellular activity against protein kinase C (PKC). This enzyme family is a longstanding pharmaceutical target involved in cancer, immunological disorders, and neurodegenerative diseases. However, selectivity is particularly difficult to achieve because of homology among family members and with several related kinases, making PKC an excellent proving ground for bisubstrate-based inhibitors. Besides the pharmacological potential of the novel cell-penetrating constructs, the modular strategy described here may be used for discovering selective, cell-penetrating kinase inhibitors against any kinase and may increase adoption and therapeutic application of this promising inhibitor class.



Protein kinases are important pharmaceutical targets through their role in vital cellular processes, e.g., apoptosis and differentiation, and several protein kinase inhibitors have reached the market for conditions such as cancer.[1−4] However, many current compounds exclusively target the high-affinity but highly conserved ATP pocket, leading to poor selectivity.[5] Alternatively, inhibitors targeting the peptidic substrate binding groove have been envisaged. These pseudosubstrates mimic the substrate sequence but lack the phosphorylation site.[6] Although pseudosubstrates display selectivity, they often lack potency. Bisubstrate-based kinase inhibitors that simultaneously target the ATP binding site for affinity and the peptide binding groove for selectivity can meet this challenge.[7−12] Typical bisubstrate-based inhibitors consist of a peptidic part connected to an ATP-competitive aromatic moiety and display markedly improved selectivity and affinity compared to their constituents. Although such constructs are successful in cell-free assays, they tend to be large and polar, hindering cellular delivery.

One delivery strategy for large, polar compounds involves attaching cell-penetrating peptides (CPPs).[13−15] These are small peptides (typically <30 amino acids and positively charged) able to cross the cell membrane, e.g., the HIV-derived Tat peptide[16] and antennapedia homeodomain derived penetratin.[17] A recent example involved a CPP-conjugated acyltransferase inhibitor, intracellularly delivered and active inside mice.[18] A CPP-conjugated pseudosubstrate inhibitor of PKC has been successfully applied to influence neuron growth.[19] However, as stated above attaching an ATP-competitive moiety yielding a bisubstrate-based inhibitor may greatly enhance potency. Alternatively, since many serine/threonine kinase substrates contain multiple arginine residues and oligoarginines are CPPs, a bisubstrate-based inhibitor was described comprising an ATP-analogue connected to oligoarginine, which was active intracellularly.[12] Unfortunately, the generic oligoarginine sequence is unlikely to be suitable for obtaining selective inhibitors of any given kinase. This may be especially problematic for tyrosine kinases, which often lack positive charges in their substrates. This provides a strong argument supporting the development strategy described here.

Here we present CPP-conjugates of bisubstrate-based PKC inhibitor **1** (Figure 1, panel a), the development and evaluation of which we described recently.[20−22] The PKC family comprises 12 extremely homologous isozymes and is a sought-after therapeutic target involved in, e.g., cancer, immunological disorders, and neurodegenerative diseases, although isozyme selectivity is particularly difficult to achieve.[23−28] In a cell-free assay **1** displayed good affinity and

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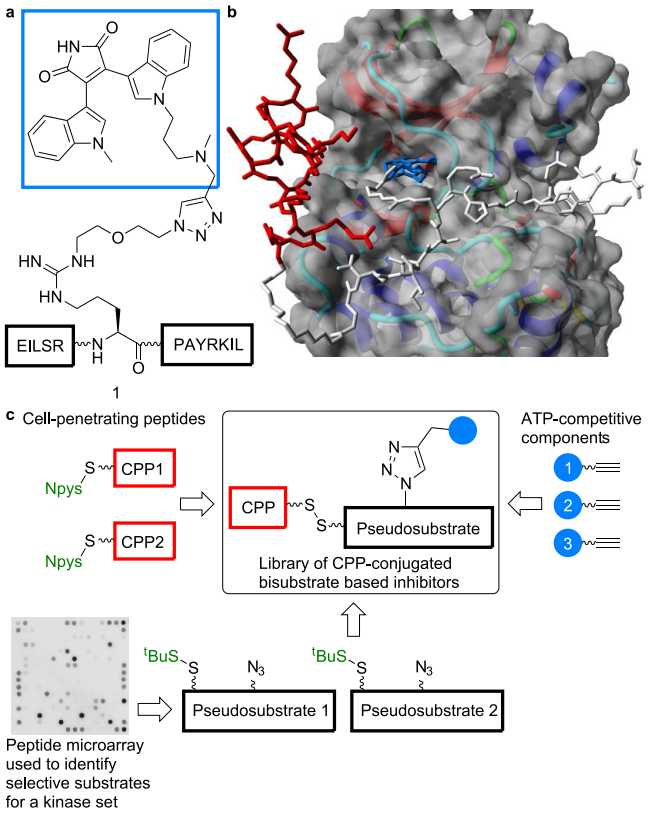

Figure 1. (a) Bisubstrate-based inhibitor **1**. (b) Model of conjugate **7a** with PKCθ (CPP in red, ATP-competitive part in blue, and pseudosubstrate in white). (c) Modular discovery strategy of CPP-conjugated bisubstrate-based inhibitors.

Table 1. Phosphorylation Inhibition Activity (Microarray)[a]

		IC$_{50}$ (μM)[c]			% inhibition ([I] = 0.4 μM)			
enzyme	substrate[b]	**1**	pseudosubstrate	**5**	**7a**	**7b**	**13a**	**13b**
PKCα	CREB1	–	–	1.8 ± 0.6	–	–	–	–
	KPCB	–	–	2.9 ± 0.9	–	–	–	–
	MARCKS	–	–	1.9 ± 0.5	–	–	–	–
PKCζ	CREB1	0.33 ± 0.03	–	–	53 ± 5	55 ± 5	48 ± 3	51 ± 3
	KPCB	0.23 ± 0.02	–	–	46 ± 2	43 ± 2	50 ± 5	51 ± 5
	MARCKS	0.59 ± 0.03	–	–	57 ± 5	48 ± 11	64 ± 13	59 ± 8
PKCθ	CREB1	0.81 ± 0.02	–	2.9 ± 0.3	45 ± 2	49 ± 4	46 ± 5	45 ± 4
	KPCB	0.45 ± 0.01	–	2.3 ± 0.3	63 ± 4	69 ± 3	69 ± 1	59 ± 3
	MARCKS	0.17 ± 0.03	–	2.9 ± 0.4	65 ± 2	63 ± 4	64 ± 3	55 ± 6

[a]Protocol and data set for new compounds in Supporting Information. [b]CREB1 (cAMP response element binding protein 126–138), KPCB (PKC splice isoform βII 19–31), MARCS (myristoylated alanine-rich C-kinase substrate 152–164), and RS6 (40S ribosomal protein S6 228–240). [c]Previously reported data.[20,21]

selectivity both among PKC isozymes (Table 1) and with respect to related protein kinase A. It gave an affinity improvement of at least a 100-fold compared to its pseudosubstrate component and 10-fold compared to the ATP-competitive part.[21] A recent review[28] cited lack of PKC isozyme selectivity and problematic delivery as reasons why, even after over 20 years of drug discovery attention, the full therapeutic potential of PKC has not yet been realized. In this light, the approach described here is particularly valuable.

Our development strategy for cell-penetrating bisubstrate-based inhibitors is shown in Figure 1, panel c. A suitable pseudosubstrate component is selected by treating peptide microarrays with any set of kinases to identify a substrate peptide with the desired selectivity profile.[21] This is furnished with an azido group for connecting an ATP-competitive part and an orthogonal attachment point for a CPP-component. In this example a disulfide linkage was used; however, other conjugation strategies may be employed, e.g., native chemical ligation.[29] An advantage of the disulfide linker is potential reductive cleavage inside the cell liberating the unconjugated bisubstrate-based inhibitor.[19] Using the chemoselective azide–alkyne click reaction,[30,31] the modified pseudosubstrates are connected to ATP-competitive components, which may be conveniently based on existing inhibitors furnished with an alkyne moiety.[22] Finally, CPPs with an activated cysteine moiety are linked to the ATP-pseudosubstrate constructs. Figure 1, panel b shows a model of inhibitor **1** conjugated to the Tat-peptide bound to PKCθ based on several crystal structures of PKC–inhibitor complexes. The view is along the peptide binding groove containing the pseudosubstrate part

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(white) with the ATP-competitive component (blue) in a deep binding pocket in the center. The CPP-part (red) is situated outside the kinase on the left.

Thus, a diverse three-dimensional library of cell-penetrating bisubstrate-based inhibitors can be rapidly generated and evaluated. Here, intracellular delivery of the constructs was investigated in HeLa cells with confocal microscopy and FACS experiments, followed by PKC activity assays based on the well-known PKC substrate Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS).[32−36] Since the approach described here is applicable to any given kinase, it may lead to a significant leap in adoption of the promising bisubstrate-based kinase inhibitors, e.g., in a therapeutic setting.

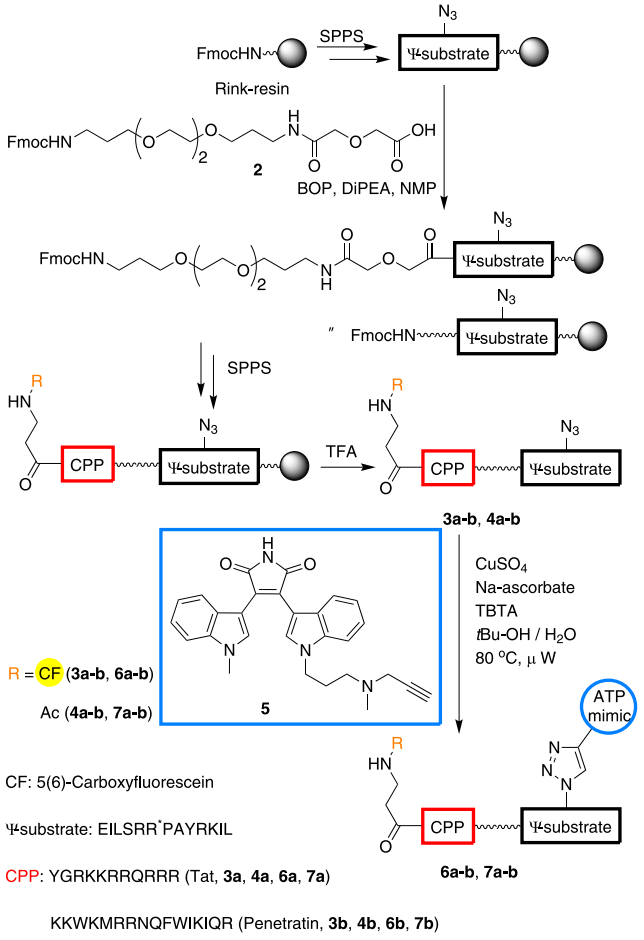
RESULTS AND DISCUSSION

Synthesis. Bisubstrate-based PKC inhibitor **1**[20−22] formed the basis of all CPP-conjugates. Many CPPs and conjugation methods have been applied with varying and sometimes unpredictable rates of success. Therefore, we decided to use different CPPs, the Tat-peptide[16] and penetratin,[17] as well as different attachment strategies: linear synthesis onto the *N*-terminus of the pseudosubstrate and attachment through a disulfide. An advantage of the first method is its straightforwardness; however, the disulfide method is modular and allows different CPPs to be evaluated through a divergent synthesis. Furthermore, the disulfide linkage may be reductively cleaved inside the cell, e.g., by glutathione, yielding the unmodified bisubstrate-based inhibitor.[19]

Scheme 1 shows the synthesis of linear Tat-conjugates **6a** and **7a** and penetratin-conjugates **6b** and **7b**. First, the pseudosubstrate component comprising a modified arginine residue furnished with an azide moiety (as in Figure 1, panel a) was prepared by solid phase peptide synthesis (SPPS).[20,37] At the *N*-terminus spacer **2**[38] was coupled, after which the CPP component was assembled by SPPS. Finally, either an acetyl moiety or the widely used carboxyfluorescein label was coupled. Unlabeled constructs **7a,b** were used in intracellular activity measurements to exclude potential influence of the hydrophobic fluorescent moiety. An additional potential disadvantage of fluorescein is its tendency to bleach. However, this would only lead to underestimates of delivery efficiency and does not impact intracellular activity measurements. Introduction of the carboxyfluorescein moiety proved capricious; however, good yields were obtained with its hydroxysuccinimidyl ester. After acidic cleavage from the resin, CPP-pseudosubstrate complexes **3a,b** and **4a,b** were obtained. These were linked to ATP-competitive part **5** by microwave-assisted copper-catalyzed azido cycloaddition (CuAAC),[39,40] yielding CPP-bisubstrate-conjugates **6a,b** and **7a,b**. All compounds could be fully purified by preparative HPLC, except fluorescently labeled penetratin construct **6b**, which could not be separated from excess **5** even after repeated attempts using different conditions, although the peaks appear well separated on analytical HPLC. For the cellular uptake experiments this was considered unproblematic since **5** does not carry the fluorescein label, is therefore not visible, and will at most lead to an underestimate of the penetrating ability of **6b**. All compounds for subsequent PKC activity assays were purified to homogeneity.

Disulfide-linked conjugates **13a,b** were prepared as shown in Scheme 2. First, CPPs furnished with a cysteine residue **9a,b** were synthesized by SPPS followed by cleavage in the presence of **8**.[19] Fluorescent pseudosubstrate **10** was prepared by SPPS, incorporating a *t*Bu-disulfide protected cysteine residue. This

Scheme 1. Synthesis of Linear CPP-Conjugated Bisubstrate-Based Inhibitors 6a,b and 7a,b



protecting group survives cleavage, resulting in a protected cysteine residue in **10**, which allows CuAAC chemistry and can be subsequently deprotected using tributylphosphine.[41−43] Thus, pseudosubstrate **10** was conjugated to **5**, deprotected to yield **12**, and conjugated to CPP components **9a,b** by stirring in an ammonium acetate buffer, affording CPP-bisubstrate-conjugates **13a,b**.

The inhibitory potency and selectivity of the CPP-conjugates against the target PKC isozymes was gauged with the same microarray assay used to discover **1** (Table 1).[30−32] An inhibitor concentration similar to the IC_{50} of **1** was applied for all inhibitors, and the expected inhibition profile was obtained, demonstrating that the CPPs used behave as neutral carriers.

Cellular Uptake. To gauge the uptake properties of the new inhibitors, HeLa cells were incubated with fluorescein-labeled constructs **6a,b** and **13a,b** at 10 μM. After careful washing, nuclear counterstain Dapi was applied for inspection with a confocal microscope (Figure 2, panel a). Presence inside the cell was clear for all conjugates, and no co-localization with the nucleus was observed. There was an apparent difference between linear conjugates **6a,b** where fluorescence was evenly spread throughout the cytosol and disulfide conjugates **13a,b** where fluorescence was concentrated in discrete small areas. This may indicate endosomal uptake, which has been suggested for CPPs.[44,45] No significant differences in appearance were observed between the two CPPs.

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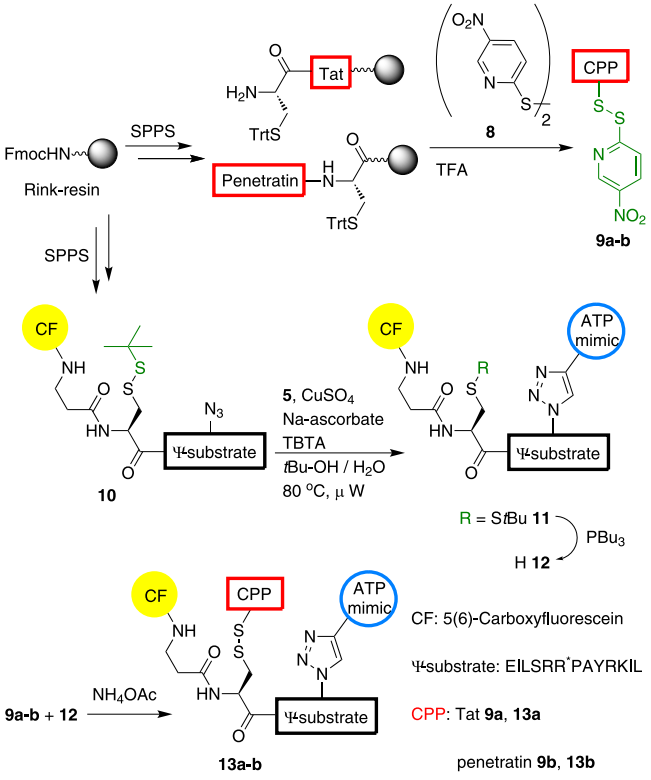
Scheme 2. Preparation of Disulfide CPP-Conjugated Bisubstrate-Based Inhibitors 13a,b



These microscopy results were in agreement with FACS experiments on linear constructs **6a,b** (Figure 2, panel b; full data in Supporting Information). For both CPPs fluorescein was associated with 60% of the cells (85% of viable cells). Viability in the presence of our compounds was assessed by staining with propidium iodide. No significant increase in dead cell counts compared with an untreated control was observed, indicating tolerance of the CPP-conjugates at 10 μM.

Intracellular activity. The intracellular activity of CPP-bisubstrate-conjugates **7a,b** and **13a,b** was investigated using an ELISA assay and Western blots. Inhibitory activity was gauged by decreased phosphorylation of well-known PKC substrate MARCKS.[32−36] Briefly, HeLa cells were treated with the compound of interest and subsequently stimulated with phorbol-12-myristate-13-acetate (PMA). After careful washing, the cells were lysed and analyzed. In the ELISA setup used, an anti-MARCKS antibody first captured phosphorylated and nonphosphorylated MARCKS. Then, the amount of captured PKC phosphorylated MARCKS was quantified with a specific antibody against MARCKS phosphorylated at positions S152 and S156.[46]

Although MARCKS is commonly used to gauge PKC activity, it is also phosphorylated by other kinases.[47,48] We have determined substrate specificity profiles for 30 kinases with the substrate microarray used for the discovery of **1**, which indeed showed that, e.g., kinases PKB, IKKα, and PKR phosphorylated MARCKS to an extent. Since the core inhibitor's specificity was established previously,[20−22] our aim was demonstrating intracellular activity, and this aselectivity was considered unproblematic. Furthermore, a negative control (no PMA stimulation and therefore minimal PKC acitivity) showed negligible MARCKS phosphorylation. In any case, no fully PKC-specific substrate is known.

For the Western blots, the lysates were run on SDS-PAGE, blotted, and stained using the phospho-MARCKS antibody. Conditions were optimized (PMA concentration, antibody incubation time, and sample load) to obtain clear bands. β-Actin was quantified as a loading control.

The ELISA results are summarized in Figure 3, panel a. MARCKS phosphorylation is shown relative to an untreated, stimulated sample with full PKC activity (positive control, 100%) and an untreated, nonstimulated sample with minimal PKC activity (negative control, 0%). Linear Tat-conjugate **7a**, linear penetratin-conjugate **7b**, and disulfide Tat-conjugate **13a** inhibited PKC intracellularly with similar IC_{50}'s (Figure 3, panel b; 10 ± 4 μM, 19 ± 7 μM, and 17 ± 6 μM respectively).

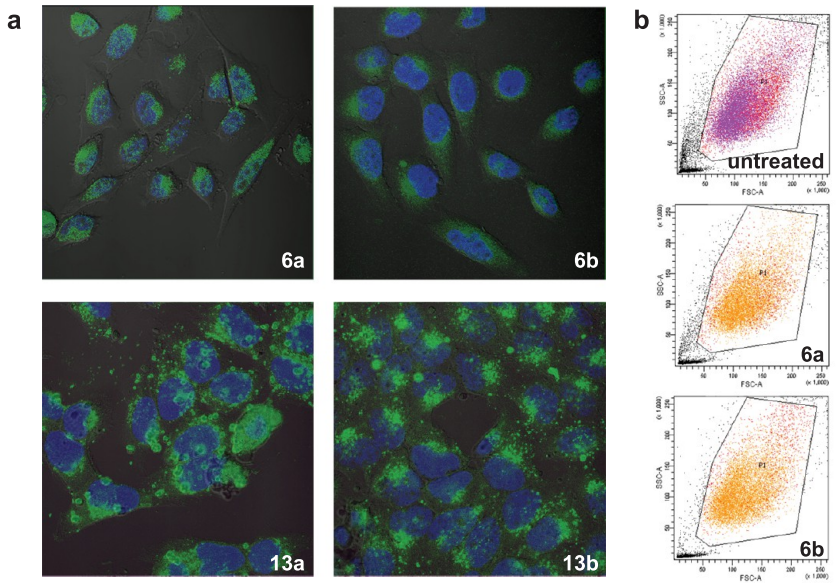


Figure 2. Uptake by HeLa cells. (a) Overlaid confocal microscopy images of cells treated with 10 μM fluorescently labeled inhibitors **6a,b** and **13a,b** (green). Dapi counterstaining was used to identify the nucleus (blue). (b) FACS results for cells treated with fluorescently labeled inhibitors **6a,b** showing fluorescein stained cells in yellow.

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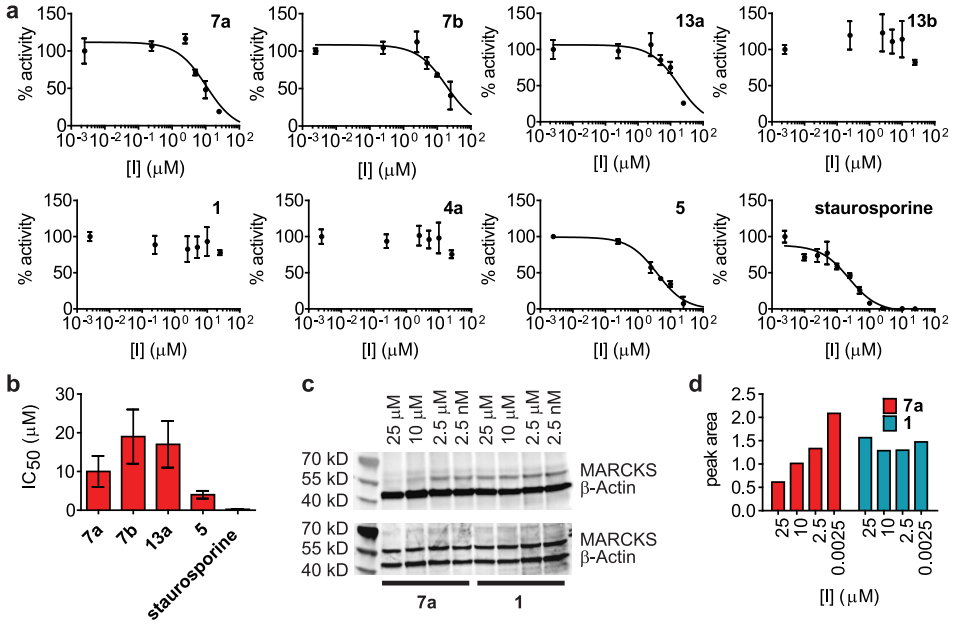

Figure 3. Intracellular activity assay measuring MARCKS phosphorylation. (a) ELISA assay of phospho-MARCKS after exposure to inhibitors **7a**,**b**, **13a**,**b**, non-CPP-conjugated bisubstrate-based inhibitor **1**, Tat-conjugated pseudosubstrate **4a**, ATP-competitive component **5**, and known PKC inhibitor staurosporine. All data points were measured in duplicate. (b) IC_{50} values of all active compounds obtained with the ELISA assay. (c) Western blots for Tat-conjugated inhibitor **7a** (left) and corresponding non-CPP-conjugated inhibitor **1** (right). The top blot is stained with phospho-MARCKS antibody, and the bottom blot with full-MARCKS antibody. (d) Analysis of band intensities of the Western blot relative to β-actin.

Surprisingly, disulfide penetratin-conjugate **13b** only gave weak inhibition even at 25 μM, although both corresponding linear construct **7b** and corresponding Tat compound **13a** inhibited PKC.

In order to evaluate the biostability of the inhibitors, **4a**, **7a**, and **13b** were treated with human pooled serum, and aliquots taken at different time points were analyzed with HPLC (see Supporting Information). Judged from the resulting degradation time courses, the serum half-life of Tat-conjugates **4a** and **7a** was approximately 8 h. However, penetratin-conjugate **13b** had a half-life of less than 1 h, which may explain its poor inhibitory activity.

All data points were corrected for total protein content determined with the BCA assay. Amounts measured were consistent (typically <10% deviation) except for **7b** at 25 μM concentration. An Alamar Blue assay was performed with all compounds at all concentrations (see Supporting Information). Only treatment with 25 μM **7b** gave significantly decreased metabolic activity (45%). The other constructs were tolerated at all concentrations tested.

As negative controls, HeLa cells were treated with **1** which comprises the pseudosubstrate and the ATP-competitive component but no CPP, as well as Tat-conjugate **4a** which lacks an ATP-competitive part. Since this pseudosubstrate inhibits PKC orders of magnitude more weakly,[21] **4a** should not give inhibition. Indeed, both **1** and **4a** were unable to significantly inhibit PKC at concentrations up to 25 μM. **4a**'s lack of potency provides a strong argument supporting bisubstrate-based inhibitors. Since treatment with **1** gave no inhibition, the activity observed is intracellular and not due to residual inhibitor present after lysis. In addition, although **1** contains both lipophilic and positively charged amino acids and therefore resembles CPPs, these structural features were insufficient for intracellular delivery. Conversely, the TAT peptide resembles PKC substrates and has indeed been found to be an inhibitor.[49,50] However, since no inhibition was observed for **4a**, which comprises the TAT peptide, TAT's potential activity does not play a role in the conjugated form applied here, and the inhibition by **7a**,**b** and **13a** must be attributed to the bisubstrate component. Similar inhibitory activity has not been described for penetratin, and **13b**'s lack of potency indicates this transporter is inert here as well. In any case, the potential for effects caused by the carrier increases the attractiveness of the modular approach presented here since multiple CPPs may be conveniently evaluated. In contrast, both ATP-competitive component **5** and well-known PKC inhibitor staurosporine[51] inhibited PKC intracellularly (IC_{50} 3.8 ± 0.7 μM and 0.23 ± 0.06 μM, respectively) as expected.

The potency of the CPP-bisubstrate-conjugates was approximately 20-fold lower than that of **1** measured in a cell-free assay with recombinant PKC (IC_{50} 0.5 μM against a MARCKS derived peptide substrate),[20] indicating that intracellular delivery is still the limiting process, and increasing the incubation time may be beneficial. In contrast, the potency of ATP-competitive component **5** was similar to that measured in the cell-free assay (IC_{50} 2 μM against MARCKS),[20] indicating that this lipophilic compound readily crosses the cell membrane.

The Western blot experiments gave similar results to the ELISA assay (an example is shown in Figure 3, panel c, remaining blots in Supporting Information). Apart from the β-actin loading control, one band was visible, corresponding to phosphorylated MARCKS. MARCKS runs anomalously on SDS-PAGE depending on conditions.[32−36] To confirm its identity, a blot was stained with an antibody against full-length MARCKS, giving a band at the same position. For the phospho-MARCKS antibody, band intensity diminished with increasing concentration of **7a**,**b** and **13a** corresponding to a

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lower amount of phosphorylation and therefore increased PKC inhibition. **13b** had no influence on band intensity, corroborating the ELISA data. When band intensities were determined relative to β-actin (Figure 3, panel d), inhibition curves were obtained similar to the ELISA data (see Supporting Information). As expected, nonpenetrating compound **1** had no influence on MARCKS phosphorylation. With the antibody against full-length MARCKS, the MARCKS band relative to β-actin is much more intense, independently of inhibitor concentration, indicating that only a fraction of available MARCKS is phosphorylated.

Although the selectivity of underlying inhibitor **1** was established earlier,[20−22] the new CPP-conjugates might somehow aselectively inhibit many kinases when inside HeLa cells. Therefore, HeLa lysates treated with **7b** and **13a** (25 μM) were evaluated with the microarray assay used for discovery of **1** (see Supporting Information). A large number of substrates were phosphorylated, indicating that there is still copious kinase activity and the conjugates therefore display selectivity. In fact, the profile obtained was similar to that of an untreated HeLa sample as expected for a selective compound. Interestingly, one of the differences observed is diminished phosphorylation of some PKC substrates found in our initial PKC profiling experiment, e.g., MARCKS.[21]

In conclusion, CPPs were successfully applied for the intracellular delivery of selective bisubstrate-based PKC inhibitors. Confocal microscopy and FACS showed that CPP-bisubstrates were taken up and tolerated by HeLa cells. An ELISA assay and Western blots based on phospho-MARCKS demonstrated the intracellular activity of the CPP-bisubstrates. The resulting convenient, modular development strategy may be used for discovering selective, cell-penetrating kinase inhibitors against any kinase. This is a prerequisite for potential therapeutic application and therefore represents a significant step forward for adoption of these promising inhibitors.

METHODS

General. All reactions were carried out at ambient temperature, and all reagents were used as obtained unless stated otherwise. Dichloromethane (DCM), *N*-methyl-2-pyrrolidone (NMP), and *N*,*N*-diisopropyl-*N*-ethylamine (DiPEA) were stored on molecular sieves (4 Å). Electron spray MS was performed on a Shimadzu LCMS-QP8000 instrument in positive ionization mode, and MALDI-TOF MS on a Shimadzu Kratos AXIMA-CFR instrument with $hACTH_{18-39}$ ($M + H^+$ 2465.198) as external reference and α-cyano-4-hydroxycinnamic acid, pure or mixed with 10% 2-(4-hydroxyphenylazo)benzoic acid for disulfide compounds,[52] as matrix. Preparative HPLC was performed on a Gilson preparative HPLC system with a C8 column (Alltech Altima XL C8, 100 Å, 10 μm, 250 mm × 22 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/MeCN 95/5 v/v) to 100% buffer B (0.1% TFA in H_2O/MeCN 5/95 v/v) and analytical HPLC on a Shimadzu HPLC workstation with a C8 column (Alltech Altima XL C8, 90 Å, 5 μm, 250 mm × 4.6 mm) and the same gradient. Microwave-assisted reactions were conducted in closed vessels inside a Biotage Initiator reactor. Mass spectra and HPLC traces for all compounds are included in the Supporting Information.

General Procedure A. Agitation was by nitrogen bubbling. *Fmoc-deprotection*: Tentagel S RAM resin was treated with 20% piperidine in NMP (5.0 mL, 3 × 8 min), followed by washing with NMP (5.0 mL, 3 × 2 min) and DCM (5.0 mL, 3 × 2 min). *Coupling*: The resin was treated with Fmoc-Xxx-OH (4 equiv), benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) (4 equiv), and DiPEA (8 equiv) in NMP (5.0 mL) for 1 h, followed by washing with NMP (5.0 mL, 3 × 2 min) and DCM (5.0 mL, 3 × 2 min). The modified arginine residue[20,37] was coupled using the amino acid (2 equiv), BOP (2 equiv) and DiPEA (4 equiv) in NMP (5 mL) overnight. *Cleavage and deprotection*: The resin was stirred with a mixture of TFA (9.5 mL), triisopropylsilane (TIS) (0.25 mL), and H_2O (0.25 mL) for 3 h at RT and removed by filtration. The crude peptide was precipitated in cold methyl-*tert*-butylether (MTBE)/hexanes (40 mL, 1/1 v/v). After centrifugation (3000 rpm, 5 min) and decantation, the pellet was washed with ether (3 × 40 mL), dissolved in *tert*-BuOH/water (1/1, v/v), lyophilized, and purified by preparative HPLC.

General Procedure B. The peptide was treated with **5** (1.1 equiv), $CuSO_4.5H_2O$ (1.5 equiv), sodium ascorbate (2.0 equiv), and TBTA (0.05 equiv) in *tert*-BuOH/H_2O (3.0 mL, 1/1 v/v) under microwave irradiation at 80 °C for 45 min, lyophilized, and purified by preparative HPLC.

CF-βAla-Tat-pseudosubstrate 3a and CF-βAla-Pen-pseudosubstrate 3b. The peptides were synthesized according to general procedure A (0.125 mmol scale). The fluorescent label was introduced using 5(6)-carboxyfluorescein *N*-hydroxysuccinimidyl ester (118 mg, 0.25 mmol) and DiPEA (174 μL, 0.50 mmol) in NMP (5.0 mL, overnight). **3a** (38.3 mg, 7.6%, 95% per step) and **3b** (29.0 mg, 4.9%, 95% per step) were obtained as yellow powders. **3a**: *m*/*z* (MALDI) 4017.5 ($M + H^+$, $C_{179}H_{292}N_{62}O_{44}$ requires 4017.3). **3b**: *m*/*z* (MALDI) 4702.8 ($M + H^+$, $C_{219}H_{342}N_{64}O_{50}S$ requires 4703.6).

Ac-βAla-Tat-pseudosubstrate 4a and Ac-βAla-Pen-pseudosubstrate 4b. The peptides were synthesized according to general procedure A (0.125 mmol scale). The *N*-terminus was acetylated with Ac_2O (0.5 M), DiPEA (0.125 M), and hydroxybenzotriazole monohydrate (0.015 M) in NMP (5.0 mL, 2 × 10 min). **4a** (98.6 mg, 21 mg%, 97% per step) and **4b** (87.4, 16%, 97% per step) were obtained as white powders. **4a**: *m*/*z* (ESI) 925.6 ($M + 4H^{4+}$, $C_{160}H_{284}N_{62}O_{39}$ requires 925.6), 740.8 ($M + 5H^{5+}$, requires 740.7), 617.6 ($M + 6H^{6+}$, requires 617.4), 529.0 ($M + 7H^{7+}$, requires 529.3). **4b**: *m*/*z* (ESI) 1097.3 ($M + 4H^{4+}$, $C_{200}H_{334}N_{64}O_{45}S$ requires 1097.1), 878.5 ($M + 5H^{5+}$, requires 877.9), 732.5 ($M + 6H^{6+}$, requires 731.8).

CF-βAla-Tat-bisubstrate 6a and CF-βAla-Pen-bisubstrate 6b. These compounds were prepared from **3a** (10 mg, 2.49 μmol) and **3b** (4.1 mg, 0.87 μmol) according to general procedure B. **6a** (0.45 mg, 4%) and **6b** (0.67 mg, 15%) were obtained as orange powders. **6a**: *m*/*z* (ESI) 1117.8 ($M + 4H^{4+}$, $C_{207}H_{318}N_{66}O_{46}$ requires 1117.1), 894.4 ($M + 5H^{5+}$, requires 893.9), 745.6 ($M + 6H^{6+}$, requires 745.1), 639.5 ($M + 7H^{7+}$, requires 638.8). **6b**: *m*/*z* (ESI) 1289.4 ($M + 4H^{4+}$, $C_{247}H_{368}N_{68}O_{52}S$ requires 1288.7), 1031.8 ($M + 5H^{5+}$, requires 1031.2), 860.0 ($M + 6H^{6+}$ requires 859.5).

Ac-βAla-Tat-bisubstrate 7a and Ac-βAla-Pen-bisubstrate 7b. These compounds were prepared from **4a** (11.1 mg, 3.0 μmol) and **4b** (13.2 mg, 3.0 μmol) according to general procedure B. **7a** (5.2 mg, 42%) and **7b** (1.1 mg, 7.6%) were obtained as orange powders. **7a**: *m*/*z* (MALDI) 4149.1 ($M + H^+$, $C_{188}H_{310}N_{66}O_{41}$ requires 4149.4). **7b**: *m*/*z* (MALDI) 4835.9 ($M + H^+$, $C_{228}H_{360}N_{68}O_{47}S$ requires 4835.8).

Cys(Npys)-Tat 9a and Pen-Cys(Npys) 9b. The peptides were synthesized according to general procedure A (0.25 mmol scale). 2,2′-Dithiobis(5-nitropyridine) **8** (388 mg, 1.25 mmol) in TFA/TIS/H_2O (5 mL, 92.5/5/2.5 v/v/v) was used for cleavage and deprotection. **9a** (90.7 mg, 20%, 94% per step) and **9b** (212.8 mg, 34%, 97% per step) were obtained as yellow powders. **9a**: *m*/*z* (ESI) 606.3 ($M + 3H^{3+}$, $C_{72}H_{126}N_{36}O_{16}S_2$ requires 606.0). **9b**: *m*/*z* (ESI) 835.0 ($M + 3H^{3+}$, $C_{112}H_{176}N_{38}O_{22}S_3$ requires 834.8), 626.4 ($M + 4H^{4+}$, requires 626.3).

CF-βAla-Cys(*St*Bu)-pseudosubstrate 10. The peptide was synthesized according to general procedure A (0.25 mmol scale). The fluorescent label was coupled overnight using 5(6)-carboxyfluorescein *N*-hydroxysuccinimidyl ester (236 mg, 0.50 mmol) and DiPEA (348 μL, 1.0 mmol) in NMP (5.0 mL). **10** (234.8 mg, 40%, 97% per step) was obtained as a yellow powder. *m*/*z* (ESI) 1174.6 ($M + 2H^{2+}$, $C_{108}H_{163}N_{29}O_{26}S_2$ requires 1174.1), 783.3 ($M + 3H^{3+}$, requires 783.1), 587.6 ($M + 4H^{4+}$, requires 587.6).

CF-βAla-Cys(*St*Bu)-bisubstrate 11. This-conjugate was prepared from **10** (45 mg, 19.2 μmol) according to general procedure B. **11** (17.8 mg, 33%) was obtained as an orange powder. *m*/*z* (ESI) 1399.0 ($M + 2H^{2+}$, $C_{136}H_{189}N_{33}O_{28}S_2$ requires 1399.2), 933.4 ($M + 3H^{3+}$, requires 933.1), 700.6 ($M + 4H^{4+}$, requires 700.1).

CF-βAla-Cys-bisubstrate 12. **11** (20 mg, 7.14 μmol) was dissolved in TFE/H_2O (2.0 mL, 95/5 v/v), tributylphosphine (9.0 μL, 35.8 μmol) was added under an Ar-atmosphere, and the reaction mixture was stirred for 3 h. The peptide was precipitated in MTBE/EtOAc (3/1 v/v) and centrifuged. The pellet was washed with MTBE/EtOAc (3/1 v/v) and dissolved in buffer A. Purification by preparative HPLC yielded **12** (10 mg, 52%) as an orange powder. *m/z* (ESI) 1355.8 (M + $2H^{2+}$, $C_{132}H_{181}N_{33}O_{28}S$ requires 1355.2), 903.8 (M + $3H^{3+}$, requires 903.8), 677.7 (M + $4H^{4+}$, requires 678.1).

Tat-disulfide-bisubstrate 13a and Pen-disulfide-bisubstrate 13b. A mixture of CH_3CN and a 1 M ammonium acetate buffer (pH 5) (1/1 v/v) was degassed with N_2 for 30 min. Either **9a** (4.0 mg, 2.21 μmol) or **9b** (5.5 mg, 2.21 μmol) was added together with **12** (6.0 mg, 2.21 μmol) under an Ar-atmosphere, and the reaction mixture was stirred under N_2 atmosphere (2 h). Purification by preparative HPLC afforded **13a** (7.4 mg, 77%) and **13b** (6.6 mg, 59%) as orange powders. **13a**: *m/z* (MALDI) 4369.9 (M + H^+, $C_{199}H_{303}N_{67}O_{42}S_2$ requires 4370.3). **13b**: *m/z* (MALDI) 5054.5 (M + H^+, $C_{239}H_{353}N_{69}O_{48}S_3$ requires 5054.7).

Cell Culture. HeLa cells (American Type Culture Collection cat. no. CCL-2) were grown in monolayer in RPMI 1640 medium (PAA) containing 10% heat-inactivated fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (PAA) in a CO_2 incubator (Binder). The medium was replaced twice a week, and cells were passaged every 4 days in a 1:10 ratio.

Confocal Laser Scanning Microscopy. A total of 20,000 cells were seeded per well of a 16-well Lab-tek chamber slide (Nalge Nunc) in culture medium. One day after seeding, cells were washed 2× with culture medium, incubated with 10 μM inhibitor for 1 h in a CO_2 incubator, washed with PBS (PAA), and fixed with 3.7% paraformaldehyde (Sigma-Aldrich) for 30 min at 37 °C. The cells were washed and counterstained with Dapi (Sigma-Aldrich) for 5 min. After washing, confocal slides were mounted on glass cover slides using FluorSave (Calbiochem) and inspected with a Zeiss LSM 710 microscope.

Fluorescence Activated Cell Sorting (FACS). HeLa suspension (500 μL, 10^6 cells mL^{-1}) was added to a vial. Cells were washed 2× with cold PBS and incubated with 10 μM of inhibitor at 37 °C for 1 h, followed by washing 3× with PBS and treatment with propidium iodide. A minimum of 10,000 events per sample were recorded on a FACSCanto II (BD Biosciences) machine. Propidium positive cells were excluded.

HeLa Cell Incubation and Lysis. One day after seeding 100,000 cells per well in 24-wells tissue culture plates (Corning), regular medium was removed and replaced by serum-free medium for 1 h. Medium was replaced by serial dilutions of test compounds in serum-free medium followed by incubation for 1 h in a CO_2 incubator. Cells were washed 2× with serum free medium, stimulated with 0.1 μM PMA (Sigma-Aldrich) for 10 min at RT, and washed 2× with PBS on ice. Finally, cells were lysed in RIPA buffer (Teknova) containing protease and phosphatase inhibitor mix (Thermo Sci.) for 10 min on ice. The lysates were collected and total protein content was determined using the Pierce BCA protein assay kit (Thermo Sci.).

ELISA. The PathScan Phospho-MARCKS (Ser152/156) Sandwich ELISA Kit (Cell Signaling Technology) was used. Briefly, wells containing immobilized MARCKS-antibody were incubated overnight with the lysate of interest at 4 °C. After washing, an antiphospho-MARCKS-biotin-conjugate was added (1 h, 37 °C), followed by streptavidin-horseradish peroxidase (30 min, 37 °C). Finally, peroxidase substrate 3,3′,5,5-tetramethylbenzidine (TMB) was added (10 min, 37 °C) followed by an acidic stop solution. Absorption at 450 nm was determined as a measure of phospho-MARCKS production using a BioTek μQuant plate reader.

Western Blot. The lysate of interest (9 μg total protein per lane) was run on an 8−16% gradient polyacrylamide gel (Thermo Science) under denaturing conditions and transferred onto a nitrocellulose membrane using an iBlot system (Invitrogen). The membrane was blocked overnight at 4 °C with 5% BSA in PBS (50 mL) and washed 3 × 10 min with 0.1% Tween-20 in PBS (10 mL). After incubation overnight at 4 °C with a 1:1000 solution of the primary antibody [phospho-MARCKS antibody (Millipore), β-actin antibody (Thermo Scientific), or full-MARCKS antibody (Millipore)] in PBS (7 mL), the membrane was washed 3 × 10 min with 0.1% Tween-20 in PBS (10 mL). A 1:1000 solution of HRP-conjugated goat anti-rabbit (Thermo Sci.) in PBS (5 mL) was added (1 h, RT) followed by washing 1 × 10 min with 0.1% Tween-20 in PBS (10 mL) and 2 × 10 min with PBS (10 mL). After staining with the Femto SuperSignal substrate kit (Thermo Sci.), blots were visualized using a Bio-Rad ChemiDoc imager.

■ ASSOCIATED CONTENT

Supporting Information

MS spectra and HPLC chromatograms for all compounds, viability data, selectivity control experiments, and full data sets for FACS and Western Blot experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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