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Design of a bioactive cell-penetrating peptide: when a transduction domain does more than transduce‡

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The discovery of cell-penetrating peptides (CPPs) has facilitated delivery of peptides into cells to affect cellular behavior. Previously, we were successful at developing a phosphopeptide mimetic of the small heat shock-like protein HSP20 . Building on this success we developed a cell-permeant peptide inhibitor of mitogen-activated protein kinase-activated protein kinase 2 (MK2). It is well documented that inhibition of MK2 may be beneficial for a myriad of human diseases including those involving inflammation and fibrosis. During the optimization of the activity and specificity of the MK2 inhibitor (MK2i) we closely examined the effect of cell-penetrating peptide identity. Surprisingly, the identity of the CPP dictated kinase specificity and functional activity to an extent that rivaled that of the therapeutic peptide. The results reported herein have wide implications for delivering therapeutics with CPPs and indicate that judicious choice of CPP is crucial to the ultimate therapeutic success. Published in 2009 by John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: mitogen-activated protein kinase-activated protein kinase 2; cell-penetrating peptide; inhibitor specificity; peptide therapeutics

Introduction

Over the past 20 plus years since the simultaneous discovery by Frankel and Pabo [1] and by Green and Loewenstein [2] that the TAT protein from the HIV virus could be taken up by cells, a tremendous body of work investigating the mechanism and limitations of the uptake has been generated [3–6]. In addition, several investigators have identified new sequences that act as CPPs [7–10] (commonly used CPPs also listed in Ref. 11). The mechanism of entry into the cell by these cell-permeating peptides is still an active topic of investigation [11–13]. Less widely investigated is the effect of the identity or primary structure of the CPP on the activity of the molecule to which it is attached.

MAPKAP-K2 (MK2), controls gene expression at both the transcriptional and post-transcriptional levels as well as cytoskeletal architecture [14]. Two MAP kinases, p38α, and p38β, activate MK2 [15]. Environmental stresses including heat shock, hypoosmolarity, and hypoxia and inflammatory cytokines, such as TGF-β1, IL-1, TNF-α, IL-6, and GM-CSF, activate the p38/MK2 pathway [16–23]. When activated, MK2 increases the translation and stability of cytokine mRNA and causes actin reorganization [16,24,25].

Inhibiting MK2 may be beneficial for a myriad of human diseases including endotoxic shock [26], pancreatitis [27], asthma [28], localized inflammatory disease [28], atherosclerotic cardiovascular disease [29], Alzheimer's disease [30,31], cancer [32], neural ischemia [31], rheumatoid arthritis [33], and inflammatory bowel disease [34]. Thus, an MK2 inhibitor may have an enormous impact on treating human disease. While several small molecule inhibitors of MK2 are under development, none has yet been approved by the United States FDA [35].

We previously reported on an MK2 inhibitor peptide, WLRRIKAWLRRIKALNRQLGVAA, [36] that was derived from the sequence published by Hayess and Benndorf [37]. However, the initial work with this peptide, while demonstrating functional activity, also demonstrated poor cell viability *in vitro*. This observation led us to explore the potential origin of toxicity.

Work by Lukas *et al*. suggests that highly basic peptides can serve as inhibitors of MLCK [38]. This information coupled

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Abbreviations used: *CPP, cell penetrating peptide; TGF-β1, transforming growth factor beta-1; IL-1, interleukin-1; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; GM-CSF, granulocyte macrophage-colony stimulating factor; FDA, Food and Drug Administration; FMOC, Fluorenylmethyloxycarbonyl; HBTU, O-Benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluorophosphate; NMM, N-Methylmorpholine; MALDI TOF, Matrix-assisted laser desorption/ionization time of flight; DTT, Dithiothreitol; Sox, 9-hydroxy-5-(N,N-dimethylsulfonamido)-2-methylquinoline; MK2, mitogen-activated protein kinase-activated protein kinase 2 (also commonly known as MAPKAP-K2); MK3, mitogen-activated protein kinase-activated protein kinase 3; CaMKI, Calcium/calmodulin-dependent protein kinase I; MK5, mitogen-activated protein kinase-activated protein kinase 5 (also commonly known as PRAK); SAPK2a, mitogen-activated protein kinase 14 subunit alpha (also commonly known as p38α or MAPK14α); p38β, mitogen-activated protein kinase 14 subunit beta (also commonly known as SAPK2b or MAPK14β); IRAK4, interleukin-1 receptor-associated kinase 4; MLCK, myosin light chain kinase (also commonly known as MYLK); PKBβ, thymoma viral proto-oncogene 2 (also commonly known as Akt2); PKCδ, Protein kinase C delta; ROCK-I, Rho-associated coiled-coil containing protein kinase 1.*

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with the apparent toxicity of the highly basic WLRRIKAWLRRIKALNRQLGVAA led us to hypothesize that basic CPPs may lead to nonspecific kinase inhibition. To further investigate the impact of CPP on activity, alternate CPPs were examined. Two of these CPPs were based on HIV's TAT protein – YGRKKRRQRRR and YARAAARQARA [39–42]. The original CPP, WLRRIKAWLRRIKA, was also tested along with its non-functional monomer, WLRRIKA. Two additional, novel CPPs were introduced during this work, KAFAKLAARLYR and FAKLAARLYR. These two CPPs are based on the antithrombin III heparin-binding domain [43,44]. We have shown that all of the CPPs tested in these studies enter cells (data not shown). Kinase inhibition was evaluated using radiometric assays to better identify how modifications to the CPP influenced MK2 inhibition and to better determine the specificity of complete (CPP and therapeutic domain coupled) peptides.

These studies suggest that care must be taken in choosing CPPs for the delivery of bioactive molecules into cells. The sequence of the CPP can influence the kinase inhibition activity and specificity. In addition, progress has been made toward designing a peptide-based inhibitor with improved potency and specificity for MK2. Future studies will address the biological activity of our novel MK2 inhibitor peptides in cell culture and *in vivo*.

Table 1. Peptides tested in fluorescent-based kinase activity assay

Peptide	Percentage of KALNRQLGVAA reaction velocity
Alanine substitution	
KALARQLGVAA	−61 ± 2
D-amino acid substitution	
KALdNRQLGVAA	−5 ± 10
Other modifications	
KKKALNRQLGVAA	−9 ± 8
WLRRIKAWLRRIKALNRQLGVAA	−132 ± 10
CPP Domain	
WLRRIKA (non-functional)	+306 ± 21
WLRRIKAWLRRIKA	−83 ± 4
YGRKKRRQRRR	+44 ± 17
YARAAARQARA	+149 ± 13

Concentration of 100 μM was used for all peptides. Percentages represent the percentage change in MK2 reaction velocity *versus* the unsubstituted peptide, KALNRQLGVAA, at a concentration of 100 μM. At this concentration, KALNRQLGVAA inhibited 73% of MK2 activity. Error is reported as the SD between three samples.

Experimental

Peptide Synthesis and Purification

Peptides were synthesized on Rink-amide or Knorr-amide resin (Synbiosci Corp., Livermore, CA) using standard FMOC chemistry [45] on a Symphony® Peptide Synthesizer (Protein Technologies Inc., Tucson, AZ). The coupling reagent for the amino acids (Synbiosci Corp.) was HBTU/NMM (Anaspec - Freemont, CA; Sigma - St. Louis, MO). Following synthesis, the peptide was cleaved from the resin with a trifluoroacetic acid-based cocktail, precipitated in ether, and recovered by centrifugation. The recovered peptide was dried *in vacuo*, resuspended in MilliQ purified water, and purified using an FPLC (ÄKTA Explorer, GE Healthcare, Piscataway, NJ) equipped with a 22/250 C18 prep-scale column (Grace Davidson, Columbia, MD). An acetonitrile gradient with a constant concentration of either 0.1% trifluoroacetic acid or 0.1% acetic acid was used to achieve purification. Desired molecular weight was confirmed by time-of-flight MALDI mass spectrometry using a 4800 *Plus* MALDI TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA).

Fluorescence-Based Kinase Activity Assay

The Omnia® Kinase Assay for MAPKAP-K2 kit (Invitrogen, Carlsbad, CA) was used to determine the reaction velocity for MK2 in the presence and absence of each of the peptides listed in Table 1. The kit contains a proprietary reaction buffer to which the following were added (final concentrations are given): 1 mM ATP, 0.2 mM DTT, 10 μM MAPKAP-K2 Sox-modified peptide substrate, 5 ng MK2, and the peptide inhibitor of interest (final volume of 50 μl). Human MK2 was purchased from Millipore. The reactions were performed at 30 °C in the wells of a low-protein-binding 96-well plate provided with the kit, and fluorescence readings (excitation = 360 nm, emission = 485 nm) were taken every 30 s for 20 min using a SpectraMax M5 Spectrophotometer (Molecular Devices, Sunnyvale, CA). Reaction velocity was determined for each reaction well from the slope of a plot of relative fluorescence units *versus* time. Each inhibitor peptide was tested at least at four concentrations, 12.5, 25, 50, and 100 μM in triplicate.

Radiometric IC_{50} and Kinase Activity Determination

A commercial radiometric assay service was used to test the specificity and potency of complete peptides (CPP and therapeutic domain coupled). In these assays, a positively charged substrate is phosphorylated with a radiolabeled phosphate group from an ATP if the kinase is not inhibited by an inhibitor peptide. The positively charged substrate is attracted to a negatively charged filter membrane, quantified with a scintillation counter, and compared to a 100% activity control. ATP concentrations within 15 μM of the apparent K_m for ATP were chosen because an ATP concentration near the K_m allowed for the kinases to have the same relative phosphorylation activity and because Hayess and Benndorf showed that the mechanism of the original inhibitor peptide was not to compete with the ATP binding site [37]. Individual conditions for each assay (reference inhibitors, buffer conditions, ATP concentration, substrate, etc.) and information about each kinase tested can be found on Millipore's website at http://www.millipore.com/drugdiscovery/dd3/kpservices. IC_{50} values for inhibitor peptides were determined using Millipore's IC_{50} *Profiler* Express service. The IC_{50} value was estimated from a 10-point curve of one-half log dilutions. For peptides that were tested for specificity, the concentration that inhibited approximately 95% of MK2 activity was chosen to profile against a battery of kinases related to MK2, cell viability, or human disease from Millipore's Kinase *Profiler* service. In both assays, compounds were supplied in DMSO. Every kinase activity measurement was conducted in duplicate.

Mesothelial Cell Culture

Immortalized human pleural mesothelial cells (ATCC CRL-9444) were grown in Medium199 with Earle's BSS and 0.75 mM L-glutamine (Mediatech Inc., Manassas, VA), 1.25 g/l sodium bicarbonate (Sigma), 3.3 nM epidermal growth factor (EGF) (MBL International, Woburn, MA), 40 nM hydrocortisone (Sigma), 870 nM insulin (MBL International), 20 mM HEPES (Sigma), trace elements mixture B (Mediatech Inc., Waltham, MA), 10% fetal

bovine serum (FBS) (Hyclone), and 1% penicillin/streptomycin (Mediatech Inc., Waltham, MA). Passage number four mesothelial cells were used in live–dead assays.

Live–Dead Assay

Live–dead assays based on Molecular Probe's LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells were used to assess the toxicity of various MK2 inhibitor peptides on human pleural mesothelial cells. Mesothelial cells were seeded in a Corning CellBind®, black well, clear-bottom, 96-well plate. Upon reaching 90–100% cell confluence, 200 µl of the appropriate media with treatment was added to each well. Cells were incubated at 37 °C and 5% CO_2 for 24 h. One-half hour prior to the 24-h time point, 70% methanol was added to untreated cells to kill and permeabilize the cells. These cells were used for determining the background of Calcium-AM (CA) and the maximum dead signal for ethidium homodimer-1 (EthD-1). Conversely, untreated live cells were used to determine the background of EthD-1 and the maximum live signal for Calcein-AM. At the 24-h time interval, the cells were washed two times with 200 µl of PBS. Then, 100 µl of the appropriate stain stock was added to each well. Controls received the optimal concentration of either EthD-1 (8 µM) or CA (4 µM). All treated wells received the same optimal concentrations of EthD-1 and CA in the same stock. Samples were incubated for the appropriate optimal time interval at 37 °C and 5% CO_2. The optimal stain concentrations and time intervals were determined via staining confluent cells with variable concentrations of stain and measuring fluorescence for each stain at variable time intervals. Thus, the optimal time for dye incubation was chosen as the time that allowed for EthD-1 saturation and was still in the linear range of CA. All fluorescence was measured with a Spectramax M5 Microplate Reader (Molecular Devices). CA required an excitation wavelength of 494 nm and an emission wavelength of 517 nm. EthD-1 required an excitation wavelength of 528 nm and an emission wavelength of 617 nm.

Results

Amino Acid Substitutions and Deletions

Using a fluorescence-based kinase assay, peptides with select amino acid deletions and one alanine and D-amino acid substitution were examined (Table 1). The modifications to the original sequence described by Hayess and Benndorf were made for ease and cost of peptide synthesis. Both the D-amino acid and alanine substitutions showed that the asparagine was not critical for MK2 inhibition. In fact, replacing the asparagine with an alanine enhanced MK2 inhibition. Only moderate decreases in inhibition were seen when two *N*-terminal lysines were removed. Thus, further peptide studies were completed on peptides synthesized with only one *N*-terminal lysine and with either the asparagine from the original peptide or an alanine substituted for the asparagine.

Inhibition of MK2 – Synergy between the CPP and Therapeutic Domains

The MK2 inhibitor peptide, KKKALNRQLGVAA, described by Hayess and Benndorf [37], was used as a control. The IC50 of the control peptide was compared to our novel cell permeant version containing the WLRRIKAWLRRI CPP. The cell permeant version dramatically enhanced MK2 inhibition when coupled with the therapeutic domain (Table 1). Since this CPP was not designed to inhibit MK2, we hypothesized that the CPP itself might serve as a general kinase inhibitor.

Table 2. Effect of CPP on IC_{50} of MK2 inhibitor peptides

Peptide	IC_{50} (µM)
WLRRIKAWLRRIKAL*N*RQLGVAA	0.74
FAKLAARLYRKAL*A*RQLGVAA	1.8
KAFAKLAARLYRKAL*A*RQLGVAA	4.4
YARAAARQARAKAL*A*RQLGVAA	22
YARAAARQARAKALNRQLGVAA	5.8

Concentration of peptide selected yielded between 2-8% MK2 activity.

Table 3. Effect of CPP on inhibitor peptide specificity for MK2

Peptide	Concentration tested (µM)	Percentage of 43 kinases tested with less than 20% activity
WLRRIKAWLRRIKAL*N*RQLGVAA	30	47
FAKLAARLYRKAL*A*RQLGVAA	100	37
KAFAKLAARLYRKAL*A*RQLGVAA	100	28

Concentration of peptide selected yielded between 2-8% MK2 activity.

CPPs Alone Can Inhibit MK2

To determine whether CPPs alone would inhibit MK2, we tested the ability of three CPPs to inhibit MK2 (Table 1 and Supporting Information Figure S1). Of the three functional CPPs tested, YARAAARQARA [41] showed the least MK2 inhibition, and unlike the other CPPs tested, the level of MK2 inhibition did not vary over the concentration range investigated. One of the most widely known and used CPPs, YGRKKRRQRRR, inhibited 61.2% of MK2 activity at a concentration of 100 µM. Even at a concentration of 25 µM, this CPP inhibited 47.7% of MK2 activity. While minimal MK2 inhibition occurred with YARAAARQARA, the CPP WLRRIKAWLRRIKA potently inhibited MK2. In fact, WLRRIKAWLRRIKA was a much more potent inhibitor of MK2 activity than any therapeutic domain sequence. Interestingly, WLRRIKA, a truncated version of the CPP WLRRIKAWLRRI, was a very poor inhibitor of MK2. Overall, the functional CPPs tested had varying ability to inhibit MK2.

CPP Choice Affects Potency

In five complete peptide inhibitors of MK2, CPP choice dramatically affected potency (Table 2). The IC_{50} of the original peptide developed by Hayess and Benndorf was 31 µM (IC_{50} curve not shown). Thus, all CPPs displayed synergistic efficacy with their respective therapeutic domains. However, the WLRRIKAWLRRI CPP had an IC_{50} value more than one order of magnitude lower than the peptide with the same therapeutic domain coupled to the YARAAARQARA CPP.

CPP Choice and Substitution of Asparagine for Alanine Affect Specificity

The three most potent inhibitors of MK2 (Table 2) were tested against 43 diverse human kinases related to MK2, cell viability, or human disease at a single concentration that yielded 2-8% of normal MK2 activity (Table 3 and Supporting Information

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Table 4. Effect of five complete inhibitor peptide variants on ten human kinases

Peptide sequence	WLRRIKAWLRRIK-AL**N**-RQLGVAA	YARAAARQARA-KAL**N**RQLGVAA	FAKLAARLYRKA-L**A**RQLGVAA	KAFAKLAARLYR-KAL**A**RQLGVAA	YARAAARQARA-KAL**A**RQLGVAA
Concentration of peptide inhibitor (μM)	30	300	100	100	300
Human kinase	Percentage kinase activity				
MK2	2 ± 1	10 ± 3	5 ± 2	8 ± 1	0 ± 0
MK3	16 ± 2	19 ± 3	10 ± 1	17 ± 1	5 ± 1
CaMKI	9 ± 1	8 ± 1	0 ± 2	0 ± 2	2 ± 0
MK5	67 ± 9	81 ± 3	131 ± 4	148 ± 4	86 ± 2
SAPK2a (p38α)	61 ± 7	100 ± 6	30 ± 6	59 ± 8	66 ± 3
IRAK4	12 ± 1	68 ± 3	13 ± 4	16 ± 2	23 ± 2
MLCK	4 ± 1	66 ± 9	1 ± 1	2 ± 0	9 ± 0
PKBβ	18 ± 2	96 ± 1	16 ± 5	28 ± 4	17 ± 2
PKCδ	11 ± 0	105 ± 2	40 ± 2	24 ± 3	101 ± 3
ROCK-I	0 ± 1	95 ± 7	25 ± 2	29 ± 0	27 ± 4

Concentrations selected were intended to yield between 0–10% MK2 activity. Error is reported as the SD between two samples.

Table S1). Even at over three times the concentration, peptides with the FAKLAARLYR and KAFAKLAARLYR CPPs were more specific than the peptide with the WLRRIKAWLRRI CPP.

To further explore this specificity phenomenon, five complete inhibitor peptides were tested against 10 human kinases (Table 4). Based on the results of the radiometric and fluorometric assays, the YARAAARQARA CPP was a poor inhibitor of MK2 and, thus, may generally be a poor kinase inhibitor. Also, while the substitution of an alanine for an asparagine had only a modest effect on MK2 inhibition, we wanted to evaluate the effect of this substitution on specificity. The kinases chosen for this testing were selected for the following reasons. Three of the selected kinases are structurally similar to MK2 and, therefore, may also be inhibited by the MK2 inhibitor peptides; thus, data with different CPPs would indicate whether the CPP affected relative activity and specificity within kinase families. MAPKAP-K3 (MK3) shares 75% of MK2's amino acid identity, and MK2 and MK3 phosphorylate many of the same substrates with similar kinetics [23]. MK2 and MK3 share 35–40% identity with CaMKI [46,47]. MK5 shares 40% amino acid identity with MK2 and MK3 [16]. SAPK2a (equivalent to p38α MAP kinase) was selected to determine if the peptide inhibited the upstream kinase that phosphorylates MK2 *in vivo* [48–51]. MLCK is a substrate of MK2 *in vitro* that was inhibited strongly in our previous kinase screening [52]. Thus, we were able to investigate the effects of the CPPs within a kinase signaling cascade. Finally, IRAK4, PKBβ, PKCδ, and ROCK-I were selected because they represent a diverse array of kinases and because the three peptides listed in Table 3 and tested previously dramatically inhibited their kinase activity.

The results of this study demonstrated dramatic differences among the specificity of the five tested kinase inhibitor variants (Table 4). The concentrations selected for the assay yielded between 0–10% MK2 activity according to IC_{50} data (Table 2). The peptides in the first two peptide columns in Table 4 show a direct comparison of the inhibitors with the WLRRIKAWLRRI and YARAAARQARA CPPs and the arginine containing inhibitor peptide domain. For 8 of the 10 kinases, MK3 and CaMKI as the exceptions, the YARAAARQARA CPP containing inhibitor peptide showed significantly reduced nonspecific inhibition. Even with the more specific YARAAARQARA CPP, inhibition of MK3 and CaMKI is not surprising since these kinases are part of the same kinase family and have significant sequence homology as described above. The next three peptides in Table 4 provide a direct comparison of YARAAARQARA, FAKLAARLYR, and KAFAKLAARLYR CPPs with the inhibitor domain containing the asparagine to alanine substitution. Even with the asparagine to alanine substitution, the YARAAARQARA containing inhibitor shows increased specificity as the other two peptides antagonize MK5 activity and suppress PKCδ activity while YARAAARQARA containing peptides do not. Finally, comparison of the two YARAAARQARA containing peptides shows that while the asparagine to alanine amino acid substitution does not affect MK2 inhibition, it dramatically affects specificity. While this substitution affects specificity, the YARAAARQARA containing peptide even with the alanine substitution is significantly more specific than the WLRRIKAWLRRI-peptide containing the original asparagines residue. All peptides inhibited MK2 and MK3; however, in each case, the inhibition of MK2 was greater than that of MK3. Also, inhibition of CaMKI was equal to or greater than MK2 inhibition. None of the inhibitors except perhaps FAKLAARLYRKALARQLGVAA was a good inhibitor of SAPK2a (p38α).

In this study, CPPs that provided more specificity were used at higher concentrations to achieve the same level of inhibition of MK2. Even at three to ten times the concentrations of the other peptides, the peptides with the YARAAARQARA CPP inhibited IRAK4, MLCK, and PKCδ to a lesser degree. Also, although WLRRIKAWLRRIKALNRQLGVAA was used at the lowest concentration, this peptide had the least specificity.

Peptide Specificity Correlates with Peptide Toxicity

Table 5 shows that less specific peptides were much more lethal. The peptide with the WLRRIKAWLRRI CPP killed nearly every cell within 24 h at a concentration of only 40 μM. However, the CPP that caused the complete peptide to be most specific, YARAAARQARA, could be used at concentrations in excess of the highest peptide concentration tested, 3mM, without resultant cell death.

Table 5. Effect of complete inhibitor peptide on mesothelial cell viability

Peptide	Maximum non-lethal concentration (μM)[a]
WLRRIKAWLRRIKALNRQLGVAA	<40[b]
KAFAKLAARLYRKALARQLGVAA	230
FAKLAARLYRKALARQLGVAA	300
YARAAARQARAKALARQLGVAA	>3000

[a] Highest concentration of peptide that resulted in live–dead equal to that of untreated cells.
[b] Only about 4% of cells live at this concentration.

Discussion

Clearly, these kinase assays have limitations that preclude a direct correlation of the results with *in vivo* activity. The kinase profiling was performed on individual kinases at one concentration while cells contain a plethora of kinases with which the inhibitor may interact. Furthermore, kinase expression varies widely within and between cells. The described studies did not examine whether inhibition of MK2 would be preferred over inhibition of other kinases, thus, potentially affecting specificity. While these studies provided a great deal of useful information about kinase and peptide inhibitor interactions, more studies in competitive *in vitro* environments and *in vivo* will be needed to establish the therapeutic value of these peptide-based kinase inhibitors.

While this study focused primarily on the effects of the CPP on the specificity of potency of kinase-inhibitor peptides, even single amino acid substitutions within the kinase inhibition domain of the peptide can significantly impact specificity. Both fluorescence-based and radiometric assays demonstrated that an asparagine to alanine substitution in the therapeutic domain had minimal impact on inhibitor potency for MK2 (the minor activity differences seen between fluormetric and radiometric assay results can be explained by slight differences in assay conditions and ATP concentrations). However, the radiometric assay showed that this substitution decreased the specificity of the peptide. The drastic change in specificity caused by this modification would be nearly impossible to predict *a priori*.

The more surprising results came from examining CPPs used to deliver the therapeutic domain. CPPs alone inhibited varying amounts of MK2 activity. All assays also showed that the conjugation of each tested CPP to the MK2 inhibition sequence enhanced the efficacy of the therapeutic domain. Investigators have reported this phenomenon with other CPPs [53]. At this point, the mechanism of this enhanced efficacy is unclear. The CPP may help block the ATP binding site, enhance the binding of the therapeutic domain to the substrate binding site, bind to regulatory allosteric sites, or simply change the conformation of the kinase to reduce kinase activity. The crystal structure of MK2 reveals that the C-terminal regulatory domain of MK2 has high helical character, occupies the substrate binding pocket, and may act as a pseudosubstrate [54]. While the CPPs in this paper share little sequence homology with MK2's C-terminal regulatory domain, they probably do form helix configurations and may enhance peptide binding to the substrate binding site of MK2 [41,54]. The CPPs themselves do not appear to act as substrate mimics for MK2 since they share very little homology to known substrates of MK2 [55].

Perhaps, more importantly, this is the first report to our knowledge showing that CPP selection can dramatically affect peptide specificity in addition to activity. Moreover, while these peptides inhibited additional kinases within the MK2 family, the peptides also inhibited kinases with diverse structures and evolutionary origin. While these assays showed that no peptide was completely specific for MK2, even small molecular inhibitors of kinase activity approved by the United States FDA lack specificity in similar kinase activity assays [56–59]. Complete specificity for a target kinase may not even be desirable since inhibiting a single kinase may not be sufficient to counter the actions of compensatory pathways.

The data presented cannot explain why different CPPs lead to varying specificity and IC50s, but both charge and hydrophobicity likely contribute to this phenomenon. While the mechanism of entry is believed to vary for different CPPs, most CPPs are positively charged and have electrostatic interactions with the phospholipids, gangliosides, glycosaminoglycans, and polysialic acid attached to the plasma membrane [60–63]. In addition to charge, hydrophobicity has been shown to be important for cell penetration [64]. Thus, the identity of hydrophobic amino acid within the primary sequence of the CPPs may also play a role in therapeutic potency and specificity. Nonetheless, we have shown not only that the sequence of the inhibitor domain affects kinase specificity, but also that the choice of CPP coveys dramatically different pharmacological activity to kinase inhibitor peptides. Future studies will examine the role of charged and hydrophobic residues within the primary structure of CPPs to further elucidate the phenomenon that leads to change in therapeutic potency and toxicity of the delivered therapeutic.

Of the peptides tested, less specific peptides were much more lethal to pleural mesothelial cells. The reason that more specific peptides could be used at higher concentrations is not clear. One would expect peptides with higher specificity to inhibit fewer kinases crucial to cell function inducing less toxicity. The fact that knocking out MK2 is not lethal in mice indicates that even extremely high concentrations of a specific inhibitor of MK2 should not be lethal and adds support to this theory [26,65]. In contrast, investigators generally agree that different peptide CPPs enter cells in variable ways [11,12,66]. Thus, the lethality of less specific peptides may not be only a function of lack of inhibitor specificity but also a function of the peptide's ability to enter the cell cytoplasm. Finally, both arginine and tyrosine contribute to nonspecific protein-protein interactions, and this phenomenon may also be playing a role in both specificity and toxicity of the peptides. Regardless, CPP choice was largely responsible for increased inhibitor peptide toxicity. Peptides with identical therapeutic domains but different CPPs had vastly different toxicity profiles.

We believe that these results have wider implications for delivering cargos with CPPs. The CPPs themselves may inhibit multiple kinases in the cell. One of the most widely known and used CPPs, YGRKKRRQRRR, had a substantial impact on MK2 activity. Thus, the results of cell and animal studies using CPPs have likely been somewhat confounded by the biological activity of the CPP in cells. Furthermore, these studies show that CPP selection can have a dramatic impact on therapeutic specificity and can actually be used to enhance the efficacy of therapeutic cargos. Additionally, less specific peptides that differed from more specific peptides only in CPP identity were lethal to cells at lower concentrations *versus* more specific peptides. As a result, future

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work will warrant careful consideration of the diverse properties of CPPs as well as judicious selection of a CPP for specific applications.

Declaration of Interest

A. Panitch, B. L. Seal, and C. M. Brophy have a financial conflict of interest. Moerae Matrix, Inc. has an option to license the technology described in the manuscript, and A. Panitch, B. L. Seal, and C. M. Brophy own greater than 5% interest in the company.

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Supporting information

Supporting information may be found in the online version of this article.

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