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Rapid Communication

A Strategy to Identify Stable Membrane-Permeant Peptide Inhibitors of Myosin Light Chain Kinase

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Purpose. A peptide inhibitor of myosin light chain kinase (MLCK), termed membrane permeant inhibitor of myosin light chain kinase (PIK), has previously been demonstrated to correct paracellular barrier defects associated with *in vitro* cell models of infectious and inflammatory intestinal disease. The current study describes a strategy to identify stable analogues of PIK required for future *in vivo* studies that has resulted in the identification of two promising candidates.

Methods. Because PIK functions at an intracellular site of epithelial cells and is envisaged to be administered orally, hydrolysis patterns were determined for PIK in both extracts of homogenized Caco-2 (a human intestinal epithelial cell line) and in luminal secretions isolated from rat intestine. Based on these hydrolysis patterns, four peptides Ac-RKKYKYRRK-NH_2 (acetylated PIK), rkkykyrrk-NH_2 (D PIK), krrykykkr-NH_2 (Dreverse PIK), and RKKykyRRK- NH_2 (Dpalindrome PIK) were synthesised. Studies were carried out to determine the stability, activity, and selectivity of these PIK analogues.

Results. D PIK and Dreverse PIK had much longer half-lives of 3.6 and 13.4 h, respectively, compared to PIK, acetylated (Ac)-PIK, or Dpalindrome PIK. All PIK analogues inhibited MLCK potently, although D PIK was a slightly better inhibitor than the other analogues. Similarly, all PIK analogues enhanced paracellular barrier function in Caco-2 monolayers studied *in vitro*. No appreciable inhibition of cAMP-dependent protein kinase (PKA) or calcium/calmodulin-dependent protein kinase II (CaMPKII) was detected with any of the analogues.

Conclusions. PIK is quickly degraded within two enzyme-containing preparations that represent different aspects of the intestinal environment. The PIK analogues D PIK and Dreverse PIK demonstrated extended half-lives in these enzyme preparations while retaining the biological activity and specificity of the parent PIK peptide.

KEY WORDS: graft-versus-host disease; inflammatory bowel disease; intestinal paracellular permeability; myosin light chain kinase.

INTRODUCTION

Many diseases of the gastrointestinal (GI) tract are associated with disorders of epithelial cell paracellular permeability. Indeed, defects in this crucial function of the intestinal epithelia are particularly well established in conditions such as inflammatory bowel disease (IBD) and graft-versus-host disease (GVHD) (1). IBD is a chronic inflammatory condition of the GI tract of unknown origin that can be focused particularly in the small intestine in the case of Crohn's disease (CD) or in the large bowel in the case of ulcerative colitis (UC) (2). Current therapies for IBD include aminosalicylates and corticosteroids. Although immunosuppressive drugs, such as azathioprine, 6-mercaptopurine, methotrexate, and infliximab also benefit these patients, these drugs are not universally effective and can be coupled to a wide range of adverse effects. For example, infliximab, which has revolutionized CD therapy, is associated with reactivation of mycobacterial infections. Thus, non-immunosuppressive therapeutic options for the treatment of IBD and other diseases of the GI tract are needed (3).

Many diseases of the GI tract manifest with increased epithelial paracellular permeability that can be present in asymptomatic relatives of CD patients and may precede both disease presentation and relapse (4). These data suggest that returning epithelial permeability to normal levels might be effective in preventing disease development or, at least, relapse. We and others have studied the regulation of epithelial paracellular permeability in detail and have shown that contraction of the perijunctional ring of actin and myosin II is a crucial trigger for this regulation (5). We have shown that, in response to both physiologic and pathophysiologic stimuli, phosphorylation of myosin II regulatory light chain by myosin light chain kinase (MLCK), a Ca^{2+}/calmodulin (CaM)-dependent protein kinase, results in increased paracellular permeability (6,7). Although inhibition of MLCK can be accomplished using drugs such as ML-7 (8), these are limited in utility; such drugs target the ATP binding site of MLCK and

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ABBREVIATIONS: CD, Crohn disease; GVHD, graft-versus-host disease; IBD, inflammatory bowel disease; MLCK, myosin light chain kinase; PIK, membrane permeant inhibitor of myosin light chain kinase; TER, transepithelial electrical resistance; UC, ulcerative colitis.

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therefore inhibit other kinases, for example, protein kinase A and protein kinase C, through a similar mechanism, at doses only moderately higher than those needed for effective MLCK inhibition. Thus, the therapeutic utility of these compounds is limited (9).

Binding of Ca^{2+}/CaM to the regulatory domain of MLCK causes the MLCK regulatory domain to dissociate from the catalytic domain. This allows the catalytic domain to interact with and phosphorylate myosin II regulatory light chain (10). Consistent with this model of MLCK activation, fragments of the inhibitory domain effectively inhibit catalytic domain activity (11). Analysis of a peptide library identified a short peptide (RKKYKYRRK) that effectively inhibits MLCK. This peptide does not bind to calmodulin and is a specific inhibitor of MLCK in that it does not significantly inhibit calcium/calmodulin-dependent protein kinase II (CaMPKII) or cAMP-dependent protein kinase (PKA) activity (9). We studied this peptide further and found that, likely due to its sequence homology to well-established protein transduction domains, the peptide is readily able to enter the cytoplasm of human intestinal epithelial cells and inhibit intracellular MLCK. Thus, we named the peptide PIK, for membrane permeant inhibitor of myosin light chain kinase. We used an *in vitro* system to show that PIK reduces paracellular permeability after physiologic stimuli (e.g., Na^+-glucose co-transport) and prevented or corrected paracellular permeability defects induced by enteropathogenic *Escherichia coli* or by proinflammatory cytokines IFN-γ and TNF-α, respectively (7).

Based on these data, we concluded that PIK might be useful *in vivo*. Unfortunately, the composition of this peptide makes it a potential substrate for trypsin- and chymotrypsin-like proteases and thus a poor candidate for *in vivo* assessment. In the current report, we sought to verify this potential sensitivity to proteolysis and identify more-stable analogues of PIK that retained efficient and specific inhibition of MLCK. To do so, we first characterized PIK hydrolysis patterns in isolated rat intestine luminal secretions and homogenates of Caco-2 cells. This information was then used to design a series of peptide analogues that were characterized for stability, activity, and selectivity as potential candidates for future *in vivo* studies to examine the effect of MLCK inhibition in disease models of IBD. Our strategy to identify promising analogues used a mixture of 1) blocking terminal amino acids to limit carboxy- and amino-peptidases, 2) using D- rather than L-amino acids to reduce sensitivity to endoprotease activities, and 3) PIK sequence alterations to possibly improve substrate specificity. Two promising candidates for future *in vivo* studies were identified. Overall, these studies describe a method for the rational design of peptide analogues with improved stability while retaining required aspects of activity and selectivity.

MATERIALS AND METHODS

Materials

N^{α}-9-fluorenylmethoxycarbonyl (Fmoc)-amino acids, (2-1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexaflurophosphate (HBTU), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexaflurophosphate (PyBOP), hydroxybenzotriazole (HOBt), and rink amide MBHA resin [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin], non-radioactive protein kinase assay kits, protein kinase inhibitor 6-22 amide, and Ca^{2+}/calmodulin kinase II inhibitor 281-309 were from CN Biosciences Ltd. (Nottingham, UK). Monoclonal anti-phosphoserine clone PSR-45 was from Sigma-Aldrich Company Ltd. (Dorset, UK). Bio-Rad protein assay kit was obtained from Bio-Rad laboratories GmbH (Munich, Germany). All other chemicals and solvents were of analytical purity or high-performance liquid chromatography (HLPC) grade from Sigma-Aldrich Company Ltd. or Fisher Scientific (Leicestershire, UK).

Peptide Synthesis

Peptides were synthesised by solid-phase peptide synthesis techniques using Fmoc (9-fluorenylmethoxycarbonyl) chemistry using an automated Symphony Quartet Peptide Synthesizer (Zinsser analytic, Maidenhead, UK). Arginine guanyl group were protected by 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; lysine and tyrosine side chains were protected by *tert*-butoxycarbonyl and *tert*-butyl, respectively. Rink amide MBHA resin 100 mg (0.78 mmol/g) was swollen with dichloromethane for 30 min. De-protection of Fmoc-amino acids was accomplished by treatment with 20% (v/v) piperdine/dimethylformamide (DMF) for 20 min. Initial coupling reactions were performed by adding resin/amino acid/HOBt/PyBOP/*N,N,* diisopropylethylamine (DIEA) in equivalents of 1/5/5/4.9/10 and mixing for 2 h. Each subsequent coupling reaction was performed by *N*-α-Fmoc groups cleavage with 20% (v/v) piperdine/DMF for 12 min followed by mixing of 0.05 M Fmoc-amino acids dissolved in DMF with 0.1 M of HBTU and 0.4 M of 4-methylmorpholine for 30 min followed by resin washing in DMF. N-terminal acetylation was performed on some peptides prior to cleavage from the resin by treatment with 50% acetic anhydride, 25% pyridine, and 25% DMF.

Crude peptides were cleaved from the resin for 3 h in 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane, and 2.5% H_2O, roto-evaporated to remove solvents, precipitated with cold ether, dissolved with 2% acetonitrile, 2% acetic acid, and then lyophilized. Purification of desired peptides from crude material was achieved by semipreparative HPLC using a Vydac 218TP (Grace Vydac, CA, USA) C_{18} reversed-phase silica gel column (10 × 250 mm, 300 Å pore size, 5-μm particle size). Crude mixtures were separated using a 2% B to 50% B in 20 min gradient (flow rate = 2 ml/min) where eluent A was 0.3% TFA in water and eluent B was 0.3% TFA in acetonitrile. Separation of crude peptide mixtures was monitored at 280 nm. Collected peptide fractions were pooled, concentrated, and verified by liquid chromatography separation and mass spectrometry analysis (LC-MS). HPLC separation of peptides was performed on a 218TP C_{18} reversed-phase silica column (4.6 × 250 mm, 300 Å pore size, 5-μm particle size) using a 2% B to 50% B in 20 min gradient (flow rate = 0.5 ml/min) where eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in acetonitrile). Peptides were monitored at 280 nm and by positive electrospray ionization performed using a Thermo Finnigan LCQ DECA mass spectrometer (MS) and analyzed using Thermo Finnigan Xcalibar software from (Thermo Separation Products, Riveria Beach, FL, USA)

PIK Degradation

Luminal intestinal secretions were obtained by flushing isolated rat intestines (duodenum to ileum) with 10 ml of 20

mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7.4. Discharged contents were centrifuged to remove solids and the supernatant filtered through a 0.20-μm filter prior to determining total protein content. Caco-2 cells (passage numbers 30 to 40), obtained from ATCC, were grown for 2 to 3 weeks prior to disruption. This was achieved by rinsing the cells with PBS, lifted by brief trypsin treatment into a small volume of Dulbecco's modified Eagles's medium (DMEM), and washed twice with phosphate buffered saline (PBS). The final cell pellet was resuspended in a small volume of lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 20% glycerol at pH 7.4) and sonicated on ice. Protein concentrations of isolated intestinal fluid and lysed Caco-2 cell extracts were determined using the Bio-Rad Protein Assay. PIK peptides (1 mg/ml) in PBS were mixed with 0.1 mg protein of intestinal secretions or Caco-2 cell lysate on ice and incubated at 4°C and 37°C, respectively. At selected times, 100-μl aliquots were withdrawn and mixed with an equal volume of 0.5% TFA (in 50/50 water/acetonitrile) to terminate enzymatic reactions. Samples were centrifuged and supernatants analyzed by LC-MS analysis to determine the cleavage pattern of PIK.

In Vitro Kinase Assays

Confluent Caco-2 monolayers expressing the 215-kDa MLC kinase (6) were used as the source of MLC kinase, as described previously (7,12). After dilution in kinase reaction buffer (20 mmol/L morpholinepropanesulfonic acid, pH 7.4; 2 mmol/L $MgCl_2$; 0.35 mmol/L $CaCl_2$; and 0.2 μmol/L calmodulin), PIK peptides were added to the mixtures, and the reaction was initiated by the addition of γ^{32}P-ATP (ICN, Costa Mesa, CA, USA) and 5 μmol/L recombinant MLC and transferring the reaction tubes from ice to 30°C. Preliminary experiments demonstrated that the conditions used were within the linear range of the assay. MLC phosphorylation was determined by autoradiography of reaction mixtures separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

PKA activity was determined using a nonradioactive protein kinase assay kit by adding 20 U of PKA to 0.5, 1, 2.5, and 5 mM of PIK peptides and following the manufacturer's instructions. The protein kinase inhibitor 6-22 amide was used as a positive control. CaMPKII activity was determined using a peptide pseudo-substrate (Biotin-PLSRTLSVSS-NH_2) prepared by Fmoc solid-phase peptide synthesis as described previously (13). Biotinylated pseudo-substrate (0.5 μg/ml in PBS) was fixed to 96-well polystyrene microtiter plate wells previously coated with 100 μl of streptavidin (3 μg/ml in PBS) by overnight incubation at 4°C. Wells were then washed 3 times with 100 μl TBS containing 0.05% Tween-20 to remove unbound pseudo-substrate peptide. CaMPKII (20 U) was mixed with 0, 0.5, 1, 2.5, or 5 mM of a PIK peptide in 108 μl of CaMPKII reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM Na_2 EDTA, 100 μM ATP, 1.2 μM calmodulin, and 2 mM $CaCl_2$). After a 5-min preincubation at 30°C, 12 μl of kinase-PIK peptide sample was added to pseudo-substrate-coated wells along with 100 μl of CaMPKII reaction buffer. After 20 min of incubation at 30°C, 100 μl of 20% H_3PO_4 was added, and wells were washed 5 times with PBS. Phosphorylated pseudo-substrate was determined using a biotinylated monoclonal anti-phosphoserine antibody

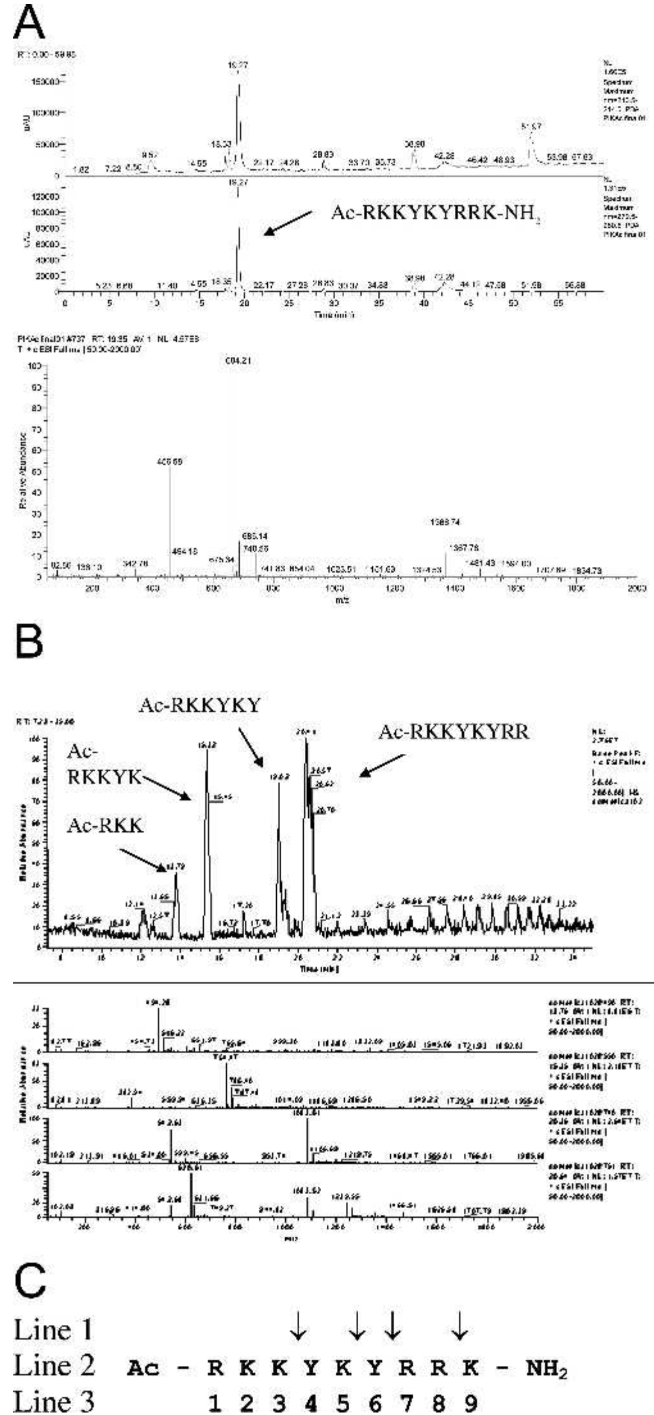


Fig. 1. (A) HPLC analysis of Ac-PIK sample detected at 214 and 280 nm along with LC-MS analysis of Ac-PIK showing breakdown pattern in positive ion mode. (B) Hydrolysis pattern determined by HPLC analysis of 1 mg/ml Ac-PIK following a 15-min incubation at 37°C with 2.5 μg/ml trypsin. Positive-ion mode LC-MS analysis was used to determine parent mass values for selected HPLC peaks. (C) Peptide site map of Ac-PIK showing initial sites of cleavage identified by LC-MS. Line 1 represents the prominent cleavage sites, line 2 amino acid composition of the peptide, and line 3 the peptide sequence number.

(100 μl of clone PSR-45, diluted 1/50,000 in PBS) followed by application of peroxidase-conjugated streptavidin and measurement of *o*-phenylenediamine (0.5 mg/ml) conversion (read at 492 nm).

In Vitro Cell-Based Studies

Caco-2 cells expressing SGLT1 were maintained and grown as polarized monolayers on collagen-coated 0.4-μm pore size polycarbonate membrane Transwell supports (Corning-Costar, Cambridge, MA, USA) as described previously (6). Electrophysiologic measurements were made using agar bridges with Ag-AgCl calomel electrodes and a voltage clamp (University of Iowa Bioengineering, Iowa City, IA, USA), as described previously (6,14). Fixed 50 μA currents were passed across Caco-2 monolayers allowing transepithelial resistance (TER) to be calculated using Ohm's law. Fluid resistance was subtracted from all values before subsequent analysis. All experiments used monolayers 18 days after reaching confluence and were performed at least 3 times with triplicate or greater samples in each experiment.

RESULTS AND DISCUSSION

Degradation of PIK

The amino acid sequence of PIK suggests vulnerability as a potential substrate for trypsin- and chymotrypsin-like hydrolysis. Due to the desire to administer PIK orally to target intestinal epithelial cells, we examined the potential for PIK to be degraded in intestinal luminal secretions and in the presence of enzyme activities associated with intestinal epithelial cells. Our method to monitor peptide breakdown using LC-MS was initially validated by incubating PIK with purified trypsin. The form of PIK used in these studies was acetylated at its N-terminus and amidated at its C-terminus (Ac-PIK; Ac-RKKYKYRRK-NH_2) to reduce susceptibility to amino- and carboxy-peptidases. LC-MS analysis of Ac-PIK demonstrated one prominent component with the expected molecular mass (1366 Da). A positive ion map of breakdown products for the parent peak was consistent with the composition of Ac-PIK (Fig. 1A). Incubation of 1 mg/ml Ac-PIK with 2.5 μg/ml trypsin for 15 min at 37°C resulted in the formation of several new peaks (Fig. 1B) that could be identified as Ac-RKKYK (763 Da), Ac-RKKYKYR (1083 Da), and Ac-RKKYKYRR (1239 Da). This breakdown pattern was consistent with anticipated tryptic proteolysis of Ac-PIK, as trypsin cleaves at the C-terminal side of positively charged amino acids, such as arginine (R) and lysine (K) residues. Selection of 2.5 μg/ml trypsin was for assay convenience and was not intended to infer anything about the intestinal concentration of this protease. These data confirm trypsin susceptibility of Ac-PIK. The data also validate the LC-MS method to analyze breakdown patterns of PIK. Because the Ac-PIK degradation products detected are typical of tryptic proteolysis, these data also confirm that the method we have used detects proteolytic cleavage products.

We next examined the stability of PIK (RKKYKYRRK-NH_2) in luminal fluids isolated from rat intestine. LC-MS analysis showed PIK to have the expected mass (1324 Da), and a positive ion breakdown map was consistent with its composition (Fig. 2). PIK incubated in rat intestinal fluid protein resulted in several cleavage products (Fig. 3A). Peptide bonds at the C-terminal sides of K and R residues were initially cleaved to produce products of KYKYRRK-NH_2 (1041

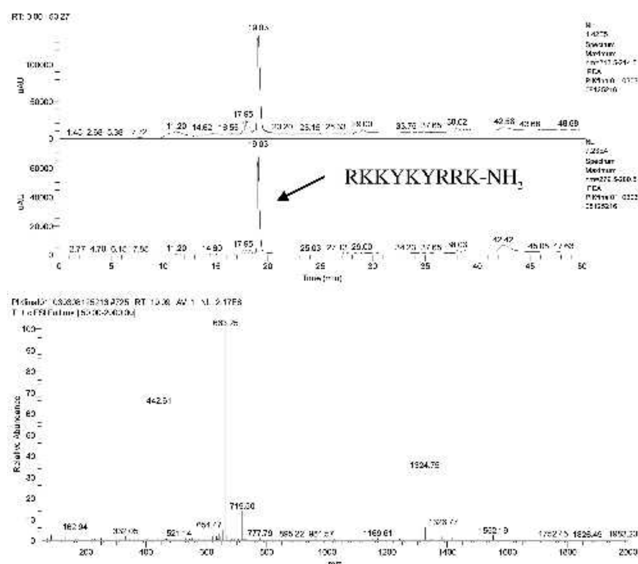
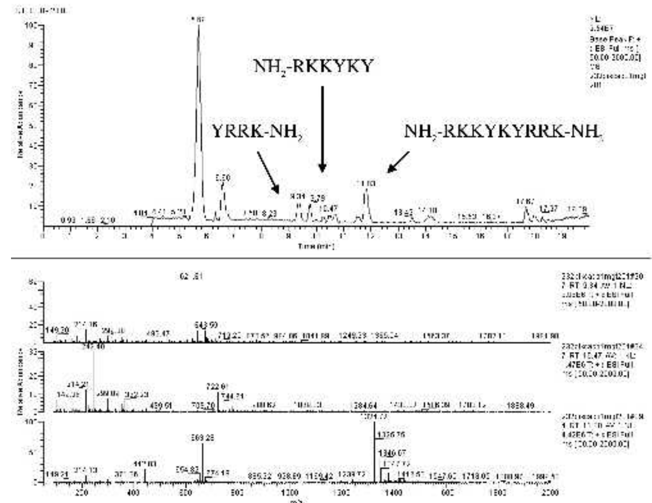


Fig. 2. HPLC analysis of PIK sample detected at 214 and 280 nm along with LC-MS analysis of PIK showing breakdown pattern in positive ion mode.

A



B

Fig. 3. (A) HPLC analysis of 1 mg/ml PIK hydrolysis pattern in rat intestinal fluid monitored at 214 nm. Reaction mixtures initially contained 1 mg/ml PIK and 0.1 mg/ml rat intestinal fluid protein and were incubated at 4°C for 15 min. Positive-ion mode LC-MS analysis was used to determine parent mass values for selected HPLC peaks. (B) Peptide site map of PIK showing initial sites of cleavage identified by LC-MS. Line 1 represents the prominent cleavage sites, line 2 amino acid composition of the peptide, and line 3 the peptide sequence number.

Da), NH_2-RKKYKYRR (1197 Da), RK-NH_2 (302 Da), and NH_2-RKK (431 Da). This information was used to generate a cleavage map for the most sensitive sites of PIK when incubated in rat intestinal fluid (Fig. 3B); these cleavage sites were characteristic of trypsin-like endopeptidase(s). Extended incubations in rat intestinal fluid led to the total hydrolysis of PIK with no detectable peptides remaining (result not shown). Based on the amino acid sequence of PIK, it is likely that non-trypsin-like proteases and peptidases participated in subsequent hydrolysis steps. PIK cleavage in rat intestinal fluid, however, is dominated initially by trypsin-like endopeptidase(s) that would act to cleave PIK at positively charged residues of the peptide.

We next characterized the stability of PIK in Caco-2 intestinal epithelial cell extracts, which contain a mixture of brush border and cytosolic proteases. LC-MS analysis of PIK incubated in a Caco-2 extracts (Fig. 4A) identified cleavage products of NH_2-RKKYK (722 Da) and YRRK-NH_2 (621 Da). Analysis of these peptides and the pattern of proteolysis needed to generate these peptides suggests the presence of chymotrypsin-like endopeptidase(s) that cleave PIK at the C-terminal sides of K^5 and Y^6 (Fig. 4B). The central palindrome $Y^4K^5Y^6$ of PIK is essential for its effective inhibition of MLCK (9). These protease/peptidase activities cleave this central palindrome sequence of PIK, which would act to neutralize its potential to inhibit MLCK.

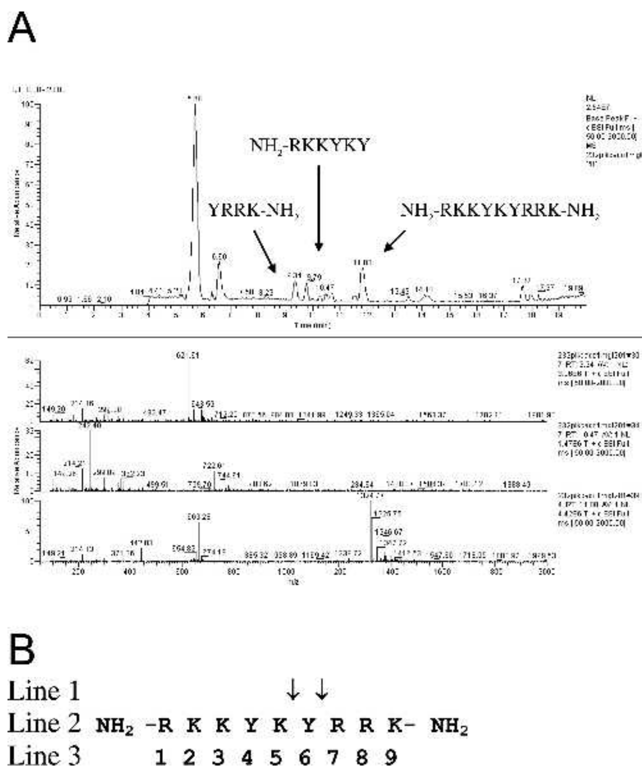


Fig. 4. (A) HPLC analysis of 1 mg/ml PIK hydrolysis pattern in Caco-2 lysate monitored at 214 nm. Reaction mixtures initially contained 1 mg/ml PIK and 0.1 mg/ml Caco-2 cell lysate protein and were incubated at 37°C for 15 min. Positive-ion mode LC-MS analysis was used to determine parent mass values for selected HPLC peaks. (B) Peptide site map of PIK showing initial sites of cleavage identified by LC-MS. Line 1 represents the prominent cleavage sites, line 2 amino acid composition of the peptide, and line 3 the peptide sequence number.

Table I. Half-Lives of PIK and PIK Analogues Incubated in Rat Intestinal Luminal Secretions[a]

Peptide	Half-life
PIK	0.2 min ± 0.00
Acetylated (Ac) PIK	0.2 min ± 0.01
Dpalindrome PIK	0.2 min ± 0.21
D PIK	3.6 h ± 2.60
Dreverse PIK	13.4 h ± 8.40

[a] Reaction conditions: The reaction mixtures contained 0.1 mg/ml of peptide and were incubated at 37°C with 0.2 mg/ml intestinal fluid protein; 100-μl aliquots were taken at various time points and the reaction terminated with an equal volume of 0.5% TFA (in 50/50 water/acetonitrile). Peptide decay was monitored by LC-MS with the percentage of peptide remaining at each time point being used to calculate half-life values by log-linear analysis. Half-lives calculated from triplicate samples (± standard deviation).

Stability of PIK Analogues

Based on the patterns of PIK protease susceptibility, we synthesized a series of analogues in an effort to obtain molecules with improved stability to protease and peptidase activities found in intestinal luminal secretions and associated with intestinal epithelial cells. In some instances, D-amino acids were used to replace L-amino acids to reduce PIK recognition by mammalian enzymes. Based on the degradation pattern shown above, we substituted the central palindrome $Y^4K^5Y^6$ of PIK to create Dpalindrome PIK (RKKykyRRK-

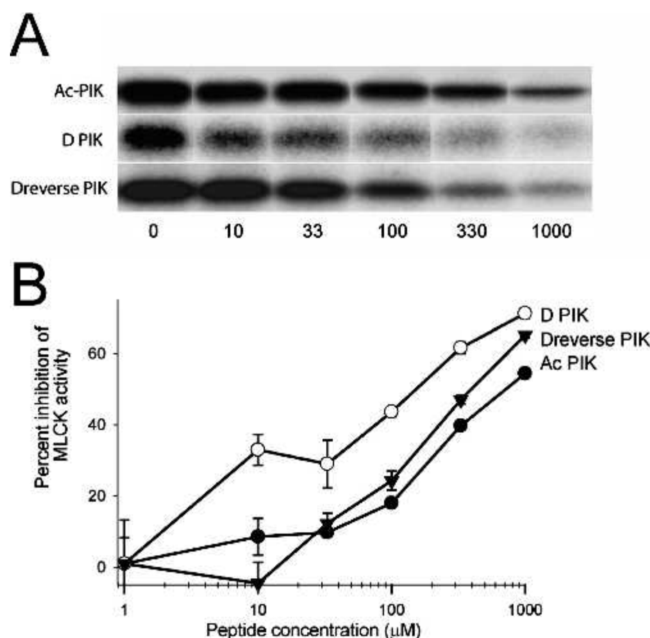


Fig. 5. Action of PIK analogues on MLCK function. (A) Representative example of experiment comparing MLC kinase activity for Ac-PIK to that observed using D PIK and Dreverse PIK. Increasing concentrations of peptides (0–1000 μM) were introduced into an *in vitro* kinase reaction with recombinant human enterocyte MLC and γ-^{32}P-ATP. Reaction mixtures were separated by SDS-PAGE and MLC phosphorylation assessed by autoradiography. (B) Percent inhibition of MLC kinase activity, demonstrated as a reduction of MLC incorporation of γ-^{32}P, from triplicate studies was averaged and normalized to γ-^{32}P incorporation in the absence of peptide.

NH_2; where capital letters indicate L-amino acids and lower case letter indicate D-amino acids). Because intestinal luminal secretions degraded PIK more effectively than the Caco-2 cell extracts, we analyzed degradation rates of PIK and PIK analogues by LC-MS during incubation with intestinal luminal secretions. The half-life of PIK, 0.2 min, was very short, consistent with rapid, multiple degradation events (Table I). Protection of the N-terminus by acetylation (Ac-PIK) did not increase its half-life, indicating that efforts to block aminopeptidase activities do not result in a stable PIK analogue. The half-life of Dpalindrome PIK was also similar to PIK. These data indicate that the positively charged residues flanking the central palindrome sequence of PIK are extremely sensitive to proteolysis.

We next synthesized two PIK analogues in which all of the L-amino acids were replaced by D-amino acids: D PIK (rkkykyrrk-NH2), which has the same amino acid sequence as PIK, while Dreverse PIK (krrykykkr-NH2) has the PIK sequence of amino acids in reversed order. This strategy to identify a stable PIK analogue was guided by observations that peptide synthesis using D-amino acids can result in loss of function that can be regained by sequence reversal (15). D PIK and Dreverse PIK showed 1000- and 4000-fold increases in half-life, to 3.6 h and 13.4 h, respectively, relative to the other PIK analogues when incubated in rat intestinal fluids (Table I).

Assessment of MLCK Inhibition

We have previously shown that PIK inhibits Caco-2 MLCK activity in a dose-dependent fashion (7). To determine if D PIK and Dreverse PIK shared this MLCK-inhibitory activity, we performed kinase assays using Caco-2 MLCK and recombinant intestinal epithelial myosin light chain. The activity of Dreverse PIK was indistinguishable from Ac-PIK, whereas D PIK appeared to have slightly enhanced MLCK inhibitory activity. Representative differences in the extent of myosin light chain phosphorylation are shown in Fig. 5A, and quantitative data from multiple experiments are shown in Fig. 5B. These results suggest that in the case of PIK, the use of D-amino acids does not require sequence reversal.

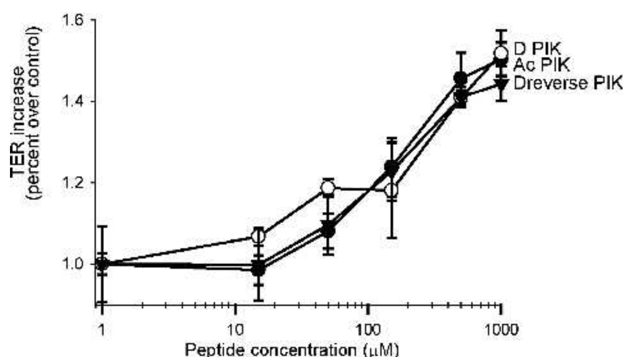


Fig. 6. Capacity of PIK analogues to affect transepithelial electrical resistant (TER) of Caco-2 monolayers *in vitro.* Dose-dependence of TER responses to D PIK (○) and PIK Dreverse (▼) parallels that of acetylated PIK (●) when added to the apical chamber of Caco-2 monolayers at increasing concentrations. TER values, determined 60 min after peptide addition, were normalized to those obtained after addition of vehicle alone. Mean ± SE.

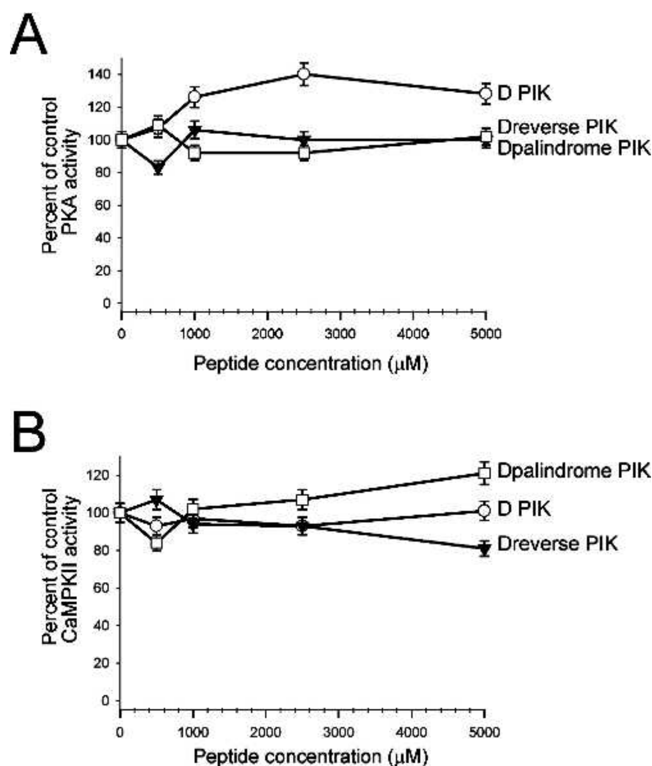


Fig. 7. Specificity of kinase inhibition by PIK analogues. Dose-dependence assessment of D PIK (○), Dreverse PIK (▼), or Dpalindrome PIK (□) to inhibit (A) c-AMP protein kinase A (PKA) or (B) calcium/calmodulin-dependent protein kinase II (CaMPKII). Percent of control activity of 20 units PKA was measured using a nonradioactive protein kinase assay kit (Calbiochem, Nottingham, UK). Percentage control activity of 20 units CaMPKII was determined by measurement of phosphorylated CaMPKII substrate. Mean ± SE, n = 3.

Effects on Paracellular Permeability

We showed previously that PIK is able to reduce paracellular permeability in a dose-dependent manner using an *in vitro* Caco-2 monolayer system where enhanced activity of Na^+-glucose co-transport results in a decrease in transepithelial electrical resistance (TER) (7). Having identified two PIK analogues with enhanced stability that each retained the ability to inhibit MLCK *in vitro*, we sought to assess the ability of these PIK analogues to increase TER of similar Caco-2 monolayers. In this assay, Ac-PIK, D PIK, and Dreverse PIK each produced similar increases in TER at concentrations up to 1 mM (Fig. 6). These results demonstrate that PIK analogues produced using D-amino acids (D PIK and Dreverse PIK) can produce a physiologic outcome similar to PIK molecules prepared from entirely L-amino acids (Ac-PIK). Because this effect on paracellular permeability requires PIK to be membrane permeant (7), these data also show that D PIK and Dreverse PIK are membrane permeant in a manner similar to PIK.

Specificity of PIK Analogues

Along with MLCK, PKA and CaMPKII are two other serine/threonine kinases that interact with calmodulin-mediated pathways. The PIK peptide sequence has previously been shown to discriminate between these kinases and spe-

cifically inhibit MLCK (9). If the more stable PIK analogues identified in our studies are to be useful *in vivo*, it is important that they also selectively inhibit only MLCK. Neither D PIK nor Dreverse PIK nor Dpalindrome PIK demonstrated significant inhibitory effects toward PKA (Fig. 7A) or CaMPKII (Fig. 7B) at concentrations up to 5 mM. Thus, both D PIK and Dreverse PIK, which were shown to have increased stability in rat intestinal fluid and Caco-2 cell lysate, were able to specifically inhibit MLCK, without affecting PKA or CaMPKII activities.

CONCLUSIONS

PIK represents a unique peptide-based drug candidate for the correction of paracellular permeability defects associated with intestinal disease. PIK is composed of a central palindrome YKY sequence that is required for its ability to inhibit MLCK. This sequence is flanked by positively charged amino acids that possibly act to properly position the central YKY sequence of PIK at a crucial site on MLCK (9). Presumably, these residues also make PIK membrane permeant, thereby allowing it to access intracellular MLCK (11). In its original form, however, PIK is highly susceptible to protease/peptidase hydrolysis. The methods presented outline a strategy for the identification of stable peptide analogues of PIK for assessment *in vivo*. Our data demonstrate two potential candidates that show comparable *in vitro* efficacy and specificity to PIK but have much greater stability in the presence of biological fluids shown to rapidly degrade PIK. Both of the peptide analogues identified represent promising candidates for *in vivo* studies were prepared completely from D-amino acids. These findings are consistent with previous studies demonstrating the stability of peptides derived from D-amino acids (15). These data are the first to demonstrate an effective approach for generating PIK peptide analogues composed of D-amino acids that retain the efficacy, specificity, and membrane permeability of the parent PIK peptide. Identification of these stable PIK peptide analogues now provide the opportunity to examine the role of myosin light chain kinase modulators in various *in vivo* models of epithelial inflammation and inflammation-associated diseases. Based on the *in vitro* data presented herein, a series of studies have been initiated using Dreverse PIK that suggest this analogue is sufficiently stable after intraluminal instillation in an *in vivo* rat model of graft-versus-host disease to effect a correction of intestinal barrier defect (Clayburgh *et al.,* manuscript submitted).

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