Peptide Therapeutics

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Orally Active Peptidic Bradykinin B_1 Receptor Antagonists Engineered from a Cyclotide Scaffold for Inflammatory Pain Treatment**

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Chronic pain is a universal health issue associated with numerous medical conditions, for example after severe burns or following major surgery.[1] Compelling evidence suggests that bradykinin (BK) antagonists could be useful in treating chronic pain and inflammatory pain.[2] Bradykinin and its homolog kallidin (lysyl-BK or KD), which are collectively known as kinins, participate in many pathophysiological insults. They are short-lived peptide mediators and the most potent endogenous pain inducers.[3] Kinins are released during tissue injury or noxious stimulation and modulate pain through the activation of both the B_1 and the B_2 receptor, which are two G-protein-coupled receptors; the carboxypeptidase metabolites of kinins, des-Arg^9-BK and des-Arg^{10}-KD, activate the B_1 receptor.[2b,4] The B_1 receptor stimulates the chronic phase of the inflammatory pain response, while the B_2 receptor stimulates the acute phase owing to their differences in ligand dissociation, receptor desensitization, down-regulation as well as internalization.[2c,5] Emerging evidence also suggests that the B_1 receptor mediates various chronic pain responses through the activation of phospholipase C, thereby leading to the production of diacylglycerol and inositol triphosphate, which further activate protein kinase C and Ca^{2+} mobilization.[3]

Numerous BK-antagonist peptides have been discovered from natural sources and structure–activity studies.[6] Kinestatin isolated from frog skin and helokinestatin from lizard venom are examples of natural BK-antagonist peptides.[7] Structure–activity studies have shown that removing the C-terminal Arg residue and concurrently replacing the penultimate residue Phe to Leu of bradykinin to des-Arg^9-[Leu^8]-bradykinin or kallidin to des-Arg^{10}-[Leu^9]-kallidin (DALK) changes a bradykinin B_2 receptor agonist to a B_1 receptor antagonist (Figure 1).[8] To increase potency and in vivo stability, several laboratories also developed BK antagonists modified with unnatural amino acids.[6] An example is Icatibant (HOE140), an injectable B_2 receptor antagonist, which has recently gained approval by the European Commission for treating hereditary angioedema.[9] Thus far, no peptidic B_1 receptor antagonist has been approved for clinical use, and developing a BK-antagonist peptide as a long-lived and orally active therapeutic remains a major challenge.

The peptide-grafting strategy by inserting a bioactive BK-antagonist peptide into a proteinaceous natural-product scaffold is an attractive approach to develop an orally active

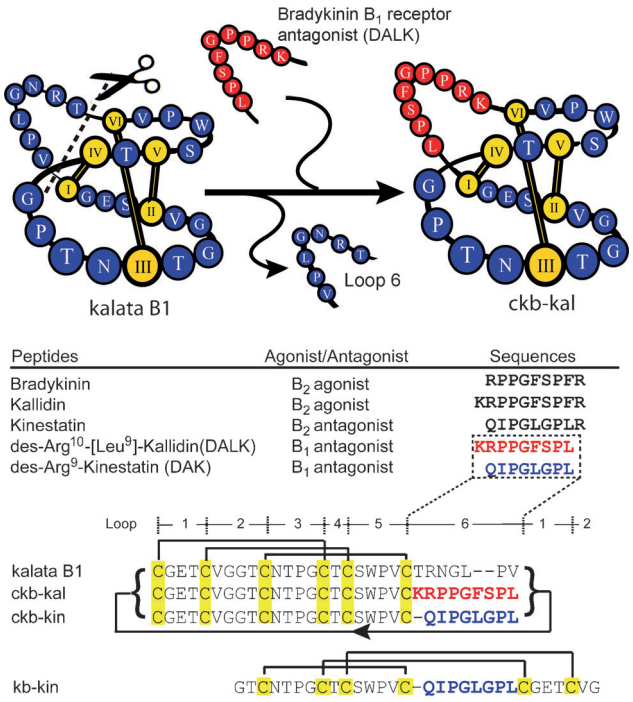

Figure 1. Scheme of engineered BK antagonists and their amino acid sequences. The peptidic B_1 receptor antagonists, DALK (kal) and DAK (kin), were used to replace the entire loop 6 of kalata B1 (ckb) in the design of two cyclic analogues ckb-kal and ckb-kin, respectively. A linear analogue of ckb-kin, kb-kin, was also synthesized as a comparison.

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bradykinin B_1 receptor antagonist for inflammatory pain treatment. As a group, cysteine-rich peptides (CRPs) are natural scaffolds and well-represented in venom toxins, enzyme inhibitors, and innate immune-defense molecules. They have compact structures, high sequence variability, and evolvability that confer them not only tolerance to peptide grafting but also stability to heat or enzyme treatment.[10] Vita et al. are among the pioneers to engineer a scorpion toxin CRP to become a metal-binding protein.[11] Also by using a scorpion toxin scaffold, Li et al. engineered an intracellular-active p53 inhibitor by exploiting the hot spots in the p53 and MDM2 protein–protein interface together with a cell-permeable cationic fragment.[12] Very recently, Craik and his coworkers successfully grafted a hexapeptide of an endothelial-growth-factor antagonist into a cyclotide scaffold.[13]

Cyclotides are plant CRPs that contain a cystine knot embedded in an end-to-end cyclic backbone.[14] The prototype, kalata B1, was discovered in 1973 by Lorents Gran because of its oxytocin-like activity of inducing uterotonic contraction, remarkably under oral administration.[15] Kalata B1 resists heating to 100 °C or acid denaturation and shows promise as a natural scaffold. Herein, we report the engineering of two novel orally active BK-peptide analgesics by using two sequence-dissimilar bradykinin B_1 receptor antagonists, des-Arg^{10}-[Leu^9]-kallidin (DALK) and des-Arg^9-kinestatin (DAK), grafted onto the kalata B1 scaffold (Figure 1).

Cyclotides contain six cysteine residues and six intercysteine loops, but only four of them, loops 2, 3, 5, and 6, are suitable for grafting a bioactive peptide. NMR spectroscopy studies have revealed that loop 6, the longest loop of kalata B1, is an extended loop that protrudes to the surface and is likely the most suitable loop for grafting a BK-antagonist peptide as a receptor-binding ligand.[14,16] Thus, we replaced the entire loop 6 of kalata B1 (ckb) with either DALK (kal) or DAK (kin) in designing two cyclic BK antagonists, ckb-kal and ckb-kin, respectively (Figure 1). An engineered linear analogue of ckb-kin, kb-kin, was also synthesized to determine the importance of the kalata backbone in the design of an orally active biologic.

The cyclic BK antagonists, ckb-kal and ckb-kin, were prepared by a stepwise solid-phase peptide synthesis using the conventional *tert*-butyloxycarbonyl (Boc) chemistry to afford their unprotected thioester precursors after HF cleavage and the removal of all protecting groups.[17] The thioester precursors were purified by reversed-phase (RP)-HPLC and subjected to the thia-zip cyclization[18] at pH 8 to form the cyclic amide backbone.[19] The linear peptide kb-kin was synthesized using the standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The oxidative folding of all three engineered BK antagonists was performed in tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) buffer containing 50% 2-PrOH (*v*/*v*) at pH 8.5 in the presence of a mixture of reduced (GSH) and oxidized gluthathione (GSSG), peptide/GSH/GSSG (1:100:10, mol/mol).[20] The folding progress monitored by RP-HPLC showed that the desired products with a cystine knot were obtained in 35–50% yield based on HPLC profile after 72 h of oxidative folding of crude product at room temperature. The overall yield of all three engineered BK antagonists was in the range of 13 to 20%. The cystine-knot connectivity was confirmed by a partial reduction and S-alkylation strategy followed by MS/MS sequencing (see the Supporting Information).[21]

All engineered BK antagonists were assayed for their serum stability and in vitro cytotoxicity. The half-life of bradykinin in human serum was reported to be (27 ± 10) s and that of des-Arg^9-bradykinin is relatively longer at 643 s.[22] All three engineered BK antagonists (cyclic and linear) showed remarkable stability in human serum for longer than six hours with more than 90% remained intact, whereas the control peptide, DALK, showed a half-life shorter than five minutes (Figure 2). The fact that the serum stability of the engineered BK antagonists is more than 350-fold higher than

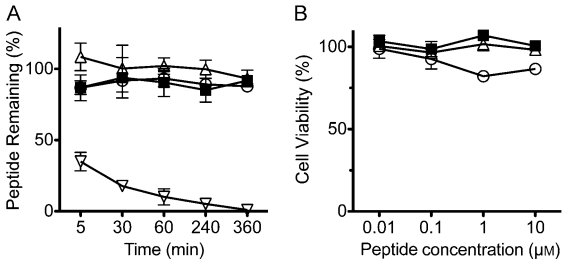

Figure 2. The stability assay (A) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay (B) of the engineered BK antagonists. A) All BK antagonists, ckb-kal (△), ckb-kin (■), and kb-kin (○), were stable in human serum over six hours, whereas the half-life of the control peptide DALK (▽) was shorter than five minutes. B) Both engineered cyclic BK peptides, ckb-kal (△) and ckb-kin (■), were nontoxic to NIH 3T3 fibroblast in 48 h as determined by the MTT assay, whereas the linear analogue kb-kin (○) showed 10% cell death at concentrations above 1 μM.

that of their corresponding BK-antagonist peptides strongly suggested that the cystine-knot arrangement of the kalata B1 scaffold without a cyclic backbone could provide stability to a grafted peptide to survive in circulation without being readily degraded by enzymes. Furthermore, cytotoxicity assays demonstrated that both ckb-kal and ckb-kin were generally nontoxic to NIH 3T3 fibroblast cells, although we found that approximately about 10% of the cells died after they were treated with more than 1 μM of kb-kin for 48 h (Figure 2). The cytotoxicity of kalata B1 is generally higher than that of the engineered peptides. The differences in cytotoxicity can be accounted for by the replacement of loop 6, which is the membrane-interacting region, and thus its replacement causes the loss of cytotoxicity of the engineered peptides.[23]

Activation of the bradykinin B_1 and B_2 receptors lead to the activation of phospholipase C and the release of inositol phosphates, thus triggering an increase in the level of intracellular Ca^{2+} ions. Under stimulation, the B_2 receptor gives a strong and transient response, while the B_1 receptor excitation is relatively lower but sustained.[2c,24] All three engineered BK antagonists, ckb-kal, ckb-kin, and kb-kin, significantly inhibited an increase of the intracellular Ca^{2+} level in Fura-2/AM-loaded HeLa cells after activation by a B_1 agonist (des-Arg^9-BK) but not by a B_2 agonist (BK; Fura-2/

AM is a calcium-chelating ratiometric fluorescent dye). In contrast, kalata B1 showed no inhibition effect (Figure 3). These results provide strong support that the engineered BK peptides are specific toward the B_1 receptor but not the B_2 receptor, since they did not inhibit the elevation of intracellular calcium level in the presence of BK, which is a B_2 agonist.

A competition receptor-binding assay was performed to confirm our findings for the receptor specificity of the engineered BK antagonists. The bradykinin B_1 receptor was cloned into the pCDNA3.1(−) expression vector and transfected into HEK 293 cells by using lipofectamine. The engineered BK antagonists, ckb-kal, ckb-kin, and kb-kin, were able to displace the fluorescently labeled agonist (fluorescein-des-Arg^9-BK) in a competitive manner (Figure 4).

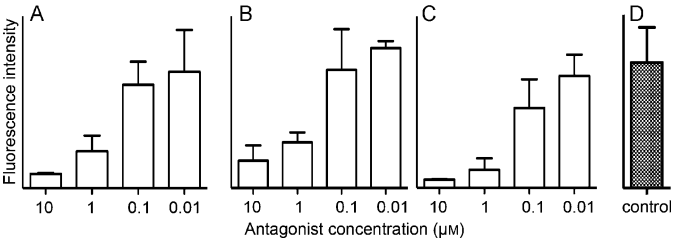

Figure 4. Competition receptor-binding assay on HEK 293 cells transfected with the bradykinin B1 receptor (BK1R). The replacement of a fluorescently labeled agonist by engineered BK antagonists causes a decrease in fluorescence intensity: A) ckb-kin, B) kb-kin, and C) ckb-kal at concentrations ranging from 0.01 to 10 μM. D) The intensity of the control (buffer) without an antagonist. The results were compared with the control to which no engineered peptides were added.

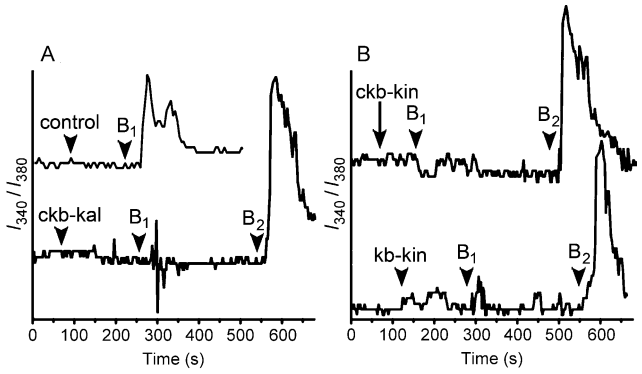

Figure 3. Inhibition effect of engineered BK antagonists on the intracellular Ca^{2+} level in Fura-2/AM-loaded HeLa cells, which is shown as the ratio of the fluorescence when the dye is excited at 340 and 380 nm (I_{340}/I_{380}). The traces are shifted vertically for clarity. The engineered BK peptides ckb-kal, ckb-kin, or kb-kin (10 nM) were added accordingly in separated culture dishes, followed by the addition of des-Arg^9-BK (B_1 agonist) and bradykinin (B_2 agonist) as arrows showed in the results. A) Injection of control (kalata B1) followed by injection of des-Arg^9-BK (B_1 agonist), which caused a sustained elevation of the intracellular Ca^{2+} level. The injection of ckb-kal (A, lower trace), ckb-kin (B, upper trace), and kb-kin (B, lower trace) and subsequent injection of des-Arg^9-bradykinin resulted in a minimal excitation of the Ca^{2+} level, whereas the injection of BK (B_2 agonist) caused a transit pattern of intracellular Ca^{2+} elevation.

The analgesic effect of ckb-kal, ckb-kin, and kb-kin were examined using the abdominal constriction assay in mice, which provides an in vivo model of visceral pain. The writhing action induced by acetic acid produces abdominal wall muscle constriction and elongation, thereby leading to hind limb extensions.[25] The number of extensions was recorded every minute for 15 min. Collier et al. reported that approximately 16% of mice are nonresponsive towards 0.5% acetic acid.[26] Thus, an exclusion criterion was set where mice in all groups that had fewer than five limb extensions during the 15 min observation were excluded from the analysis. Another characteristic of this assay is that it is not specific for either bradykinin B_1 or B_2 receptor. Porreca and co-workers reported that intraperitoneal (i.p.) injection of acetic acid into B_1 receptor-knockout mice showed approximately 50% reduction in the number of writhes.[27] The remaining 50% may be triggered by bradykinin B_2 receptors or other sensory mechanisms.

All three engineered BK antagonists, ckb-kal, ckb-kin, and kb-kin, were able to inhibit the writhing numbers significantly through i.p. injection, which was confirmed by two-way analysis of variance (ANOVA). Furthermore, we found that ckb-kal is the most potent antagonist in the writhing assay under both i.p. injection (1 mg kg^{-1}) and oral (p.o.) administration (10 mg kg^{-1}), with the maximum inhibition reaching 49 and 42%, respectively (Figure 5). However, ckb-kin is the weaker antagonist of the two engineered cyclic BK antagonists. The maximum inhibition values for i.p. and p.o. administration were 38 and 28%, respectively, while the linear analogue of cb-kin, kb-kin, showed 32 and 14% for i.p. and p.o. administration, respectively. The DALK control peptide showed a similar activity in the abdominal constriction assay as the rest of the cyclic engineered BK antagonists under i.p. administration with 38% inhibition, but no inhibition under p.o. administration (Figure 5). Furthermore, kalata B1, which was used as a negative control, showed no writhing inhibition activity at the maximum concentration of 1 mg kg^{-1} i.p. and 10 mg kg^{-1} p.o.

Among the four peptides tested, the cyclic peptide ckb-kal has the highest oral availability. Two-way ANOVA revealed that there was no significant difference between i.p. and oral administration for ckb-kal ($P = 0.94$). Similarly, the cyclic peptide ckb-kin also showed no significant difference between i.p. and p.o. administration ($P = 0.13$). In contrast, there was a significant difference between i.p. and p.o. administration observed in the linear analogue kb-kin ($P < 0.05$). Two-way ANOVA showed that the difference was highly significant ($P < 0.01$) between the different administration routes for the control peptide (DALK, i.p. versus p.o.).

Herein, the DALK peptide contains two positively charged amino acids and three Pro residues in the sequence, whereas the DAK peptide contains two Pro and no charged residues. Both the charged amino acids and Pro residues favor membrane interaction and cell permeation.[29] The difference of these two membrane-interacting residues present in the engineered cyclic peptides ckb-kal and ckb-kin could provide a plausible explanation for their bioavailability and hence the higher potency of ckb-kal over ckb-kin under oral admin-

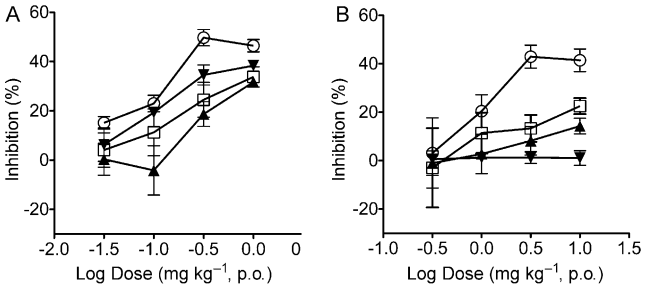

Figure 5. In vivo analgesic effect of engineered BK antagonists. Abdominal constriction assay on ckb-kal (○), ckb-kin (□), kb-kin (▲), and DALK (▼, kal) control at different concentrations under A) intraperitoneal injection (i.p.) and B) oral (p.o.) administration. Under i.p. injection, ckb-kal showed the highest inhibition effect, followed by DALK peptide, and kb-kin showed the lowest inhibition. Under oral administration, ckb-kal also showed the highest inhibition effect while the kb-kin and DALK showed little and no inhibition effect, respectively. All procedures were conducted under license from the Hong Kong Department of Health and following approval from The Chinese University of Hong Kong Animal Experimentation Ethics Committee (09/061/MIS).

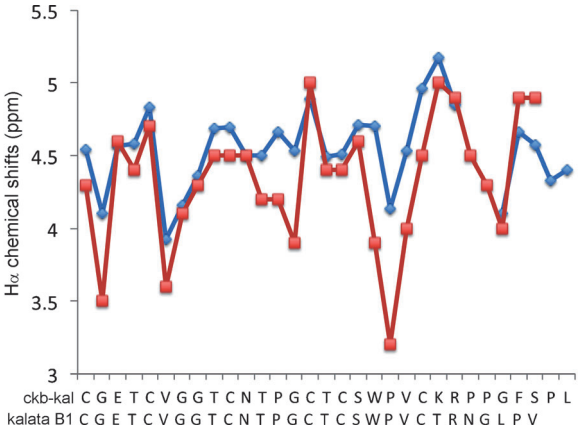

Figure 6. Chemical shift analysis of ckb-kal (blue) and kalata B1 (red). The result showed that the overall backbone structure of the engineered peptide, ckb-kal, is highly similar to that of kalata B1.

istration. The cyclic backbone of kalata B1 may be another contributing factor for oral bioavailability, because the linear analogue, kb-kin, exhibited much lower oral bioavailability than the cyclic analogues. A recent report by Clark et al. showed that by cyclizing a 16-residue conotoxin with a hydrophobic linker consisting of six aliphatic amino acids, the cyclic peptide became orally active, whereas the linear native conotoxin remained orally unavailable.[30] Taken together, our results suggest a compact structure and an elimination of N and C termini may improve oral bioavailability. Thus, our work provides further support for the potential of the cyclic cystine-knot peptide scaffold for designing orally active therapeutics.

It is noteworthy that the kalata B1 scaffold tolerates the grafting of two dissimilar and fairly large peptide fragments, the eight-residue DAK and the nine-residue DALK, onto the natural scaffold with minimal loss of the overall structural features while retaining the desired results of bioactivity and stability. Comparison of the chemical shifts of the hydrogen atoms of the peptide backbones of ckb-kal and kalata B1 showed that, except for the DALK region, the ckb-kal is highly similar to the kalata B1 (Figure 6).[31]

In summary, we have successfully demonstrated that the cyclotide kalata B1 could provide a superior natural scaffold for engineering orally active peptides as potentially useful therapeutics. However, further investigations are needed to determine the immunogenicity of these compounds under oral administration. The grafting of BK-antagonist peptides DALK or DAK into the kalata B1 scaffold were performed, and the stability was enhanced substantially. Measurements of the intracellular Ca^{2+} level revealed that two of our designed cyclic antagonists are specific blockers for the bradykinin B_1 receptor, but not for B_2. In vivo abdominal constriction assay showed significant inhibition of pain response in our animal model under i.p. administration in a dose-dependent manner. However, under oral administration, only the cyclic analogues were able to inhibit the writhing action, whereas the linear analogue as well as the DALK peptide itself showed poor or no inhibition, respectively. It appears that the cystine-knot constraint of the linear kb-kin also produces effect after p.o. administration, but the combination of cyclization and the cystine-knot constraint produces a stronger effect.

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