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Investigation of binding proteins for anti-platelet agent K-134 by Drug-Western method

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Abstract

K-134 ((–)-6-[3-[3-cyclopropyl-3-[(1*R*, 2*R*)-2-hydroxycyclohexyl]ureido]-propoxy]-2(1*H*)-quinolinone) is a novel anti-platelet agent with anti-hyperplastic activities. We found previously that K-134 is a potent phosphodiesterase-3 (PDE3) inhibitor. In the present study, we found other K-134-binding proteins by Drug-Western method. We isolated two clones that can bind directly to K-134, cofilin-2, and CD36 *in vitro*. Comparison of their amino acid sequences showed similarity over a short stretch [KxxxxVxIxWxxE] in part in the collagen-binding region of CD36. K-134 inhibited binding between CD36 and collagen type-I; however, other PDE3 inhibitors, cilostazol, amrinone, and an inactive derivative of K-134, 4S-OH-K-134, showed little or no effect on binding. It was strongly suggested that the direct binding between K-134 and CD36 is a characteristic effect of K-134, and the homologous stretch may be necessary for binding to K-134. These results also suggested that these interactions are involved in the mechanisms of the anti-platelet and anti-hyperplastic effects of K-134.

Keywords: K-134; Cofilin-2; CD36; GPIV; Anti-platelet agent; Anti-hyperplastic effects; Phosphodiesterase-3; Cilostazol

K-134 ((–)-6-[3-[3-cyclopropyl-3-[(1*R*, 2*R*)-2-hydroxycyclohexyl] ureido]-propoxy]-2(1*H*)-quinolinone) is a novel anti-platelet agent, and a 2(1*H*)-quinolinone derivative, similar to cilostazol, which is widely used as an anti-platelet agent. *In vivo* studies have demonstrated the anti-thrombotic and anti-hyperplastic effects of K-134. Moreover, these effects of K-134 are more potent than those of cilostazol [1]. The results of our previous study indicated that K-134 inhibited phosphodiesterase-3 (PDE3) activity with an IC_{50} value of 0.1 μM *in vitro* [2], and PDE3 was considered one of the target molecules of K-134 involved in these pharmacological effects.

Hidaka and colleagues have developed Drug-Western method [3], which is similar to the west-western and south-western methods using λ phage cDNA expression libraries, modified to use tag-conjugated drug as a screening probe; this is one of the easiest methods to find the binding partners of drugs. This method can be used to determine the binding affinity between expressed proteins and the drug based on the signal strength. In addition, this method can facilitate immediate cloning of the genes encoding drug-binding proteins. Therefore, we attempted to find novel target proteins of K-134 using Drug-Western method, and investigated the direct interactions between K-134 and these proteins *in vitro*.

Materials and methods

Preparation of drug probe. For conjugation between K-134 and bovine serum albumin (BSA) or biotin (Sigma, St. Louis, MO, USA), a functional amino group was chemically introduced into K-134 (KD-134 in Fig. 1). This derivative was conjugated with BSA (K-134-BSA in Fig. 1) using the

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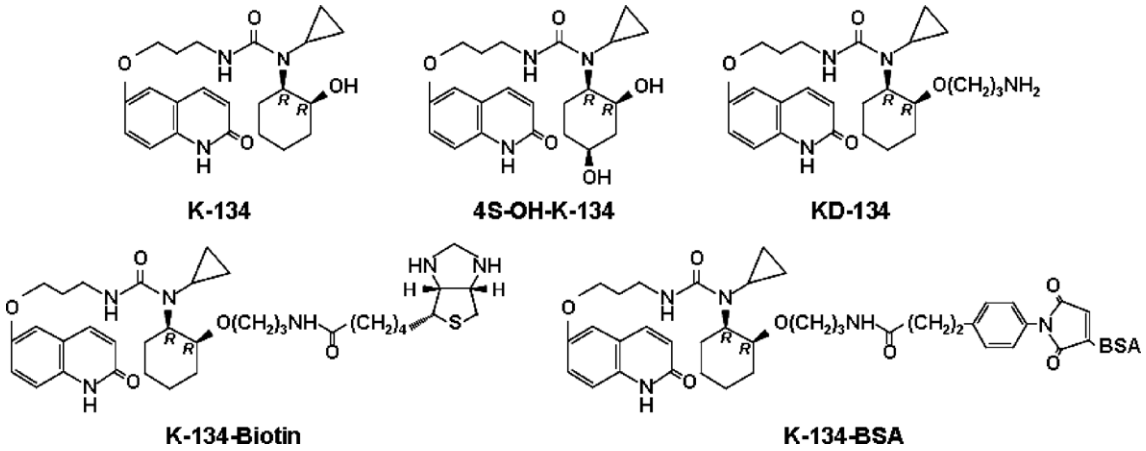

Fig. 1. Chemical structures of K-134 derivatives.

chemical cross-linker, sulpho-succinimidyl 4-(*p*-maleidophenyl)butyrate (Pierce Biotechnology, Inc., Rockford, IL, USA), in accordance with the supplier's instructions.

K-134-biotin was prepared by treatment of KD-134 with biotin in dimethylformamide containing tetraethylenediamine. The mixture was stirred at room temperature for 3 h and purified by thin layer chromatography (silica gel) to give K-134-biotin as a powder (Fig. 1).

Drug-Western method. Drug-Western method was described previously [3]. Briefly, a human placenta λTriple Ex cDNA library (Clontech, Palo Alto, CA, USA) was screened using K-134-BSA as a probe. The proteins expressed from the cDNA library were immobilised on nitrocellulose membranes (Hybond-C; Amersham Life Science, Buckinghamshire, UK), and the membranes were incubated with TBST (0.05% Triton X-100) containing K-134-BSA at room temperature for 4 h. After washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-BSA antibody (Rockland Inc., Gilbertsville, PA, USA) at 4 °C overnight. After washing with TBST, plaques containing proteins that bound to the drug were detected by enhanced chemiluminescence (SuperSignal WestFemo Maximum Sensitivity Substrate; Pierce Biotechnology, Inc.) using an imaging analyser (LAS-3000; Fujifilm, Tokyo, Japan). Library-derived phagemids were recovered by transfecting each of the phages into *Escherichia Coli* BM25.8 according to the manufacturer's instructions. The sequence of the cDNA insert was determined by the dideoxynucleotide chain termination method.

Interaction of K-134-biotin probe and recombinant human cofilin-2. Recombinant human cofilin-2 (8 μg/mL; Upstate, Lake Placid, NY, USA) was dissolved in carbonate buffer (0.1 M, pH 9.6). The solution was transferred to a 96-well plate (150 μL/well) and incubated for coating at 4 °C overnight. Each well was washed with PBS and incubated with 1% skimmed milk in PBS for blocking at 37 °C for 1 h. After washing with PBS, each well was incubated with 1 μM K-134-biotin probe in PBST (0.1% Tween 20) or a mixed solution of K-134-biotin (1 μM) and unlabelled KD-134 at 37 °C for 4 h. The wells were washed, HRP-conjugated avidin was added (avidin-HRP; Pierce Biotechnology, Inc.) and incubation was continued for a further 3 h at 37 °C. After washing the wells, 28.4 μM *o*-phenylenediamine containing 0.025% H_2O_2 (100 μL) was added and incubated at room temperature for 45 min. To stop the reaction, 1 M H_2SO_4 (25 μL) was added and the absorbance at 450 nm was measured using a microplate reader (MultiScan MS-UV; Lab Systems, Helsinki, Finland).

Interaction of K-134-biotin probe and recombinant human CD36. Recombinant human CD36/Fc chimera (3.3 μg/mL; R&D Systems, Inc., Minneapolis, MN, USA) was dissolved in carbonate buffer containing 10 mM dithiothreitol and incubated at 37 °C for 1 h. The solution was transferred to a 96-well plate (150 μL/well) and incubated for coating at 4 °C overnight. When K-134-biotin probe was incubated with various concentrations of KD-134, 3 μmol/L of K-134-biotin probe was used. The subsequent procedures were the same as described in the previous section.

Effects of K-134 on binding between CD36 and collagen type-I. The 96-well plate was incubated with collagen type-I (4 μg/mL; Calbiochem, Darmstadt, Germany) in carbonate buffer (0.1 M, pH 9.6) for coating at 4 °C overnight. After washing with PBS, a mixed solution of CD36/Fc chimera and test drug (K-134, 4S-OH-K-134, cilostazol or amrinone) was added to the wells and incubated at 37 °C for 4 h. Each well was washed with PBST and incubated with HRP-conjugated anti-Fc antibody (ICN Pharmaceuticals, Inc., Aurora, OH, USA) for 3 h. After washing with PBST, *o*-phenylenediamine solution (28.2 μM) containing 0.025% of H_2O_2 (100 μL) was added and incubated at room temperature for 35 min. The reaction was stopped with 1 M H_2SO_4 (25 μL) and absorbance at 450 nm was measured using a microplate reader.

Statistical analysis. SAS was used for statistical analyses. The statistical significance of differences between multiple groups was evaluated using Dunnett's test. Differences at $P < 0.05$ were regarded as significant.

Results

K-134 binds with cofilin-2 and CD36 obtained by Drug-Western method

The Drug-Western method was used to find novel-binding proteins of K-134, capable of explaining the mechanisms of its anti-platelet and anti-hyperplastic effects. We prepared K-134-BSA as a probe (Fig. 1). To conjugate K-134 with BSA, a functional amino group was chemically introduced into K-134 (KD-134, Fig. 1). KD-134 inhibited both ADP- and collagen-induced rat platelet aggregation, and it was suggested that the position of this linker was not involved in the function of K-134 (data not shown).

The positive clones that had significant signals of K-134-binding were isolated from approximately 1×10^6 plaques. We identified two clones encoding human cofilin-2 and CD36, respectively. We performed further experiments to characterise the direct binding of K-134 with cofilin-2 or CD36 *in vitro*.

Direct interaction of K-134 with cofilin-2 or CD36

Enzyme immunoassay (EIA) was performed to confirm the direct interaction between K-134 and cofilin-2 or CD36