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Antiplatelet effect of a newly developed AMP-activated protein kinase activator YLF-466D

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ABSTRACT

AMP-activated protein kinase (AMPK) acts as a major regulator of cellular energy homeostasis. In platelets, AMPK activation stimulates endothelial nitric oxide synthase (eNOS) and its downstream signaling, and thereby inhibits platelet aggregation. In this study, a newly developed AMPK activator 3-[[(3*E*)-3-[(4-chlorophenyl)phenylmethylene]-2,3-dihydro-2-oxo-1*H*-indol-1-yl]methyl]-benzoic acid (YLF-466D) was tested for its antiplatelet activity. Treatment of isolated platelets with YLF-466D resulted in AMPK activation in a concentration-dependent manner in a range of 50–150 μM. Under the same experimental condition, YLF-466D effectively inhibited aggregation induced by platelet agonists including thrombin, ADP and collagen. Such AMPK activation and aggregation inhibition were abolished by pretreatment with the AMPK inhibitors compound C (CC) and ara-A, indicating that antiaggregatory effect of YLF-466D is mediated by AMPK. YLF-466D induced an activation-dependent eNOS phosphorylation at Ser1177, an elevation of cyclic nucleotides cGMP and cAMP, and subsequent phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser239 and Ser157. All these events were prevented by CC and ara-A. In addition to isolated platelets, YLF-466D attenuated whole blood aggregation induced by collagen. Taken together, YLF-466D is capable of inhibiting platelet aggregation by activating AMPK and its downstream eNOS-cGMP-PKG signaling axis. This study reconfirms the antiplatelet activity of AMPK activators and suggests the potential application of YLF-466D to antiplatelet therapy, although the in vivo and clinical validation remains to be assessed.

1. Introduction

AMP-activated protein kinase (AMPK) is a serine/threonine kinase acting as a master regulator of cellular energy homeostasis. AMPK is activated in response to stresses that deplete cellular ATP supplies like low glucose, hypoxia, ischemia, and heat shock. Activated AMPK positively regulates signaling pathways that replenish cellular ATP supplies, for instance, fatty acid oxidation and autophagy, and negatively regulates ATP-consuming biosynthetic processes such as gluconeogenesis, lipid and protein synthesis (Hardie, 2007). AMPK is involved in diverse signaling pathways mediating a wide range of biological activities including cell growth, apoptosis and aging, as well as metabolisms of glucose, fatty acid and protein. AMPK is currently regarded as a potential therapeutic target for type 2 diabetes, obesity, and various cancers. Accordingly, a number of AMPK activators have been developed, although most of them have yet to reach the clinic (Steinberg and Kemp, 2009).

Among currently available small molecule AMPK activators, a 2-imino-4-thiazolidinone derivative PT1 and a thienopyridone A-769662 have attracted much attention in the aspects of novelty, specificity and action mechanism (Cool et al., 2006; Pang et al., 2008; Steinberg and Kemp, 2009). However, there is still a lot of room for improvement with regard to efficacy and bioavailability. Recently, a novel alkene oxindole derivative YLF-466D, 3-[[(3*E*)-3-[(4-chlorophenyl)phenylmethylene]-2,3-dihydro-2-oxo-1*H*-indol-1-yl]methyl]-benzoic acid, (formerly known as compound 24) was developed by structural optimization of PT1 (Yu et al., 2013) (Fig. 1). Compared with PT-1 and A-769662, YLF-466D possesses higher potency toward AMPK, improved bioactivity and favorable bioavailability, and thus it needs further study for clinical application.

Platelets are blood cells mediating physiological hemostasis. However, dysregulation of platelet activity, potentially caused by xenobiotics such as drugs and toxicants or by disease conditions, is involved in atherosclerosis and inflammation by leading to thrombosis, and ultimately contributes to the development of acute coronary syndrome, stroke and the ischemic complications of peripheral vascular disease. Therefore, platelets serve as a primary therapeutic target for atherothrombotic disorders, and antiplatelet therapies play a key role in treating various cardiovascular

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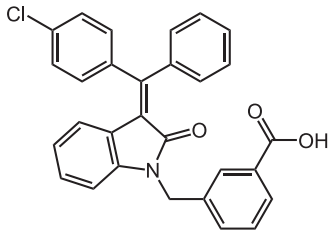

Fig. 1. Chemical structure of YLF-466D.

diseases (Michelson, 2010). Current antiplatelet drugs including a cyclooxygenase inhibitor aspirin, ADP receptor P2Y12 antagonists, phosphodiesterase (PDE) inhibitors, and integrin αIIbβ3 antagonists are widely and successfully used in the clinic. However, as with most drugs, they still have significant drawbacks such as low efficacy, bleeding problems and interindividual variability in their responses. These clinical limitations require further improvements in antiplatelet drugs, and novel targets are being suggested, which includes glycoprotein (GP) VI, GP1b, a serotonin receptor $5HT_{2A}$ and P-selectin (Michelson, 2010).

Fleming et al. (2003) and us have suggested that AMPK is a potential target and its activator may be a possible candidate for antiplatelet drug (Fleming et al., 2003; Liu et al., 2013). Indeed, activating AMPK by a pharmacological activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) attenuated platelet aggregation by stimulating the eNOS-NO/sGC-cGMP/PKG signaling pathway. These studies provided the rationale for the application of AMPK activators to antiplatelet drugs. In the present study, YLF-466D was examined in an isolated platelet system to demonstrate its antiplatelet activity and to evaluate a developmental potential as an antiplatelet drug.

2. Materials and methods

2.1. Reagents

YLF-466D was purchased from MedChem Express (Princeton, MA, USA). Collagen and ADP were obtained from Chrono-log Corp. (Havertown, PA, USA). Protease inhibitor cocktail and phosphate inhibitor cocktail tablets were from Roche Diagnostics (Indianapolis, IN, USA). cGMP and cAMP assay kits and bicinchoninic acid (BCA) protein assay kits were from R&D Systems (Minneapolis, MN, USA) and Pierce Biotechnology (Rockford, IL, USA), respectively. Anti-AMPKα, anti-phospho-AMPKα (Thr172), anti-phospho-eNOS (Ser1177), anti-vasodilator-stimulated phosphoprotein (VASP), anti-phospho-VASP (Ser239), anti-phospho-VASP (Ser157) and horseradish (HRP)-conjugated goat anti-rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA, USA). The following chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA): thrombin, adenine 9-β-D-arabinofuranoside (ara-A), diethylammonium (Z)-1-(*N*,*N*-diethylamino) diazen-1-ium-1, 2-diolate (DEA NONOate), adenosine. Other chemicals and sources were as follows: anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); 6-[4-(2-piperidin-1-ylethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-α]-pyrimidine (compound C) (Merck KGaA, Darmstadt, Germany); anti-eNOS antibody and Immobilon Western detection reagent (Millipore, Billerica, MA, USA); HRP-conjugated donkey anti-goat IgG (Bethyl Laboratories, Montgomery, TX, USA). All other chemicals used were of the highest purity available and purchased from standard suppliers.

2.2. Animals

All animal experiments were conducted in accordance with protocols approved by the Ethics Committee of Animal Service Center at Dongguk University. Male Sprague–Dawley rats (5–6 weeks of age) were purchased from Daehan Biolink (Eumseong, Korea), and acclimated for 1-week before experiments. The laboratory animal facility was maintained at a constant temperature and humidity with a 12 h light/dark cycle. Food and water were provided ad libitum.

2.3. Preparation of washed platelets

Washed platelets (WP) were prepared as described previously (Liu et al., 2013). Briefly, blood was collected from the abdominal aorta of rats anesthetized with ether. Acid–citrate–dextrose (ACD; 85 mM trisodium citrate, 66.6 mM citric acid and 111 mM glucose) was used as an anticoagulant (ACD:blood = 1:6). After centrifugation at 250 × g for 15 min, platelet-rich plasma (PRP) was obtained from supernatant. PRP was centrifuged further at 500 × g for 10 min, and the platelet pellet was washed once with washing buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM $MgCl_2$, 0.8 mM NaH_2PO_4, 10 mM HEPES and 5 mM EDTA, pH 6.5). WP was prepared by suspending the platelet pellet in suspension buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM $MgCl_2$, 0.8 mM NaH_2PO_4, 10 mM HEPES, 5.6 mM dextrose and 1 mM $CaCl_2$, pH 7.4). Platelet number was counted using a Neubauer hemocytometer (Paul Marienfeld, Lauda-Königshofen, Germany) and the concentration was adjusted to 2×10^8 platelets/ml.

2.4. Platelet aggregation study

Platelet aggregation experiments were performed in a four-channel aggregometer (Chrono-log Corp.). WP were treated with the testing materials for the indicated times, and platelet aggregation was induced by either 0.12–0.14 U/ml thrombin, 2.5 μg/ml collagen, or 16 μM ADP which is the minimal concentration inducing submaximal aggregation.

2.5. Assessment of AMPK, eNOS and VASP activation in platelets

Activation of AMPK, eNOS, and VASP was examined by conventional Western blot analysis with activation-dependent, phospho-specific antibodies and suitable HRP-conjugated secondary antibodies. To avoid protein loss during membrane stripping, phospho-protein and total protein were detected separately with the same samples in different gels. After treating the WP with the testing materials, platelet pellet was obtained by centrifugation of WP at 12,000 × g for 2 min. Platelet pellet was lysed by lysis buffer (50 μM HEPES, 50 μM NaCl, 50 μM sucrose, 1% Triton X-100, protease inhibitor cocktail and phosphatase inhibitor cocktail). Protein content was quantified with a BCA protein assay kit, and cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to a polyvinylidene difluoride membrane, immunoreactive proteins were detected with primary antibodies, HRP conjugated secondary antibodies, and Immobilon Western detection reagents as described previously (Liu et al., 2013). Chemiluminescence images were obtained and analyzed with a ChemiDoc XRS + system and Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Analysis of cGMP and cAMP in platelets

WP treated with testing materials were disrupted by sonication on ice. Lysed platelets were treated with 10% ice-cold trichloroacetic acid and kept on ice for 30 min. After centrifugation at 12,000 × g for 10 min, the supernatant was extracted three times with water-saturated ether. The water layer containing cyclic nucleotides was lyophilized in a centrifugal vacuum concentrator (Hanil Science Industrial, Incheon, Korea). Dried samples were