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Fullerene derivative prevents cellular transformation induced by JAK2 V617F mutant through inhibiting c-Jun N-terminal kinase pathway

Megumi Funakoshi-Tago [a,*,1], Tatsuaki Nagata [a,1], Kenji Tago [b], Masaki Tsukada [a], Kazuyuki Tanaka [a], Shigeo Nakamura [c], Tadahiko Mashino [d], Tadashi Kasahara [a]

[a] Department of Biochemistry, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan
[b] Division of Structural Biochemistry, Department of Biochemistry, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi-ken 329-0498, Japan
[c] Department of Chemistry, Nippon Medical School, 2-297-2 Kosugi-cho, Nakahara-ku, Kawasaki, Kanagawa 211-0063, Japan
[d] Department of Medicinal Chemistry and Bio-organic Chemistry, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan

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ABSTRACT

The constitutively activated mutation (V617F) of tyrosine kinase Janus kinase 2 (JAK2) is found in the majority of patients with myeloproliferative neoplasms (MPNs). The development of a novel chemical compound to suppress JAK2 V617F mutant-induced onset of MPNs and clarification of the signaling cascade downstream of JAK2 V617F mutant will provide clues to treat MPNs. Here we found that a water-soluble pyrrolidinium fullerene derivative, C_{60}-bis (N, N-dimethylpyrrolidinium iodide), markedly induced apoptosis of JAK2 V617F mutant-induced transformed cells through a novel mechanism, inhibiting c-Jun N-terminal kinase (JNK) activation pathway but not generation of reactive oxygen species (ROS). Pyrrolidinium fullerene derivative significantly reduced the protein expression level of apoptosis signal-regulating kinase 1 (ASK1), one of the mitogen-activated protein kinase kinase kinases (MAPKKK), resulting in the inhibition of upstream molecules of JNK, mitogen-activated protein kinase kinase 4 (MKK4) and mitogen-activated protein kinase kinase 7 (MKK7). Strikingly, the knockdown of ASK1 enhanced the sensitivity to pyrrolidinium fullerene derivative-induced apoptosis, and the treatment with a JNK inhibitor, SP600125, also induced apoptosis of the transformed cells by JAK2 V617F mutant. Furthermore, administration of both SP600125 and pyrrolidinium fullerene derivative markedly inhibited JAK2 V617F mutant-induced tumorigenesis in nude mice. Taking these findings together, JAK2 V617F mutant-induced JNK signaling pathway is an attractive target for MPN therapy, and pyrrolidinium fullerene derivative is now considered a candidate potent drug for MPNs.

1. Introduction

The classic Philadelphia chromosome-negative myeloproliferative neoplasms (Ph-MPNs), which include *polycythemia vera* (PV), *essential thrombocytosis* (ET) and *primary myelofibrosis* (PMF), are clonal disorders arising from hematopoietic stem cells or progenitor cells, and are characterized by uncontrolled proliferation of terminally differentiated myeloid cells. Clinical manifestations include variable degrees of erythrocytosis, thrombocytosis and leukocytosis, or cytopenias, extramedullary hematopoiesis (e.g. splenomegaly), increased risk for thrombosis and transformation to acute myeloid leukemia (AML) [1,2]. In 2005, a novel somatic mutation of the tyrosine kinase Janus kinase 2 (JAK2) gene was identified in more than 90% of PV patients and in approximately 50% of ET and PMF patients [3,4]. This dominant gain-of-function mutation is a guanine to thymidine substitution at nucleotide 1849 of the *JAK2* gene, which results in a valine-to-phenylalanine substitution at codon 617 of JAK2. Recently, it has been reported that this point mutation of JAK2 could induce an MPN-like phenotype utilizing conditional knock-in mice, demonstrating that V617F mutation of the JAK2 gene is a cause of MPNs [5].

In hematopoietic cells, JAK2 regulates various cytokine-induced signaling pathways, including erythropoietin (Epo) [6]. JAK2 possesses seven Janus homology (JH) domains, from JH1 to JH7. JH1 is the C-terminal kinase domain and JH2 is a catalytically inactive pseudokinase domain [7]. In the inactive form of JAK2, JH1 is reported to be associated with JH2, which is reported to suppress the kinase activity of JH1 [8]. Although no detailed structural study has been completed, the V617F mutation results in a lack of autoinhibition, possibly by destabilizing the JH1–JH2 interaction [9].

Abbreviations: Cdk4, cyclin-dependent kinase 4; DCFH, dichlorodihydrofluorescein; DMSO, dimethyl sulfoxide; Epo, erythropoetin; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; i.p., intraperitoneal; i.v., intravenous; Jak, Janus kinase; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MPNs, myeloproliferative neoplasms; OdC, ornithine decarboxylase; PBS, phosphate-buffered saline; RT-PCR, reverse transcription–polymerase chain reaction; ROS, reactive oxygen species; suppressor of cytokine signaling, SOCS; s.c., subcutaneous; STAT, signal transducers and activators of transcription; TAE, Tris-acetic acid-EDTA.

* Corresponding author. Tel./fax: +81 3 5400 2697.
E-mail address: tago-mg@pha.keio.ac.jp (M. Funakoshi-Tago).

[1] These authors contributed equally to the study.

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Previously, we found that the JAK2 V617F mutant induces the cytokine-independent survival of erythroid progenitor cells [10]. In addition, we clarified that JAK2 V617F mutant requires Epo receptor (EpoR) as a scaffolding protein to exhibit transforming activity [11]. JAK2-induced phosphorylation of tyrosine residues at 343 and 479 in EpoR is essential for the recruitment and activation of STAT5 and the Akt signaling pathway, respectively. We demonstrated that STAT5 and Akt are critical signal transducers for a proliferative advantage and the transforming ability of JAK2 V617F mutant [12,13]; however, the details of the mechanism by which JAK2 V617F mutant causes tumorigenesis have not yet been brought to light, and it is expected that elucidation of the signaling pathway induced by JAK2 V617F mutant would provide clues to understanding the pathogenic mechanism of MPNs and developing novel therapeutic procedures.

In 1985, fullerene (C60) was discovered as a new type of carbon allotrope that has been utilized for micro-scale devices in electronic and mechanical applications [14]. Chemical modification with several hydrophilic groups increases the solubility of fullerene, and water-soluble fullerene derivatives are reported to possess various biological and pharmacological properties, as described below. (1) Pyrrolidinium fullerene derivative induced apoptosis of human promyeloleukemia cells, HL60, through the generation of reactive oxygen species (ROS) [15]. (2) Malonic acid fullerene derivative has excellent antioxidant activity [16]. (3) Proline-modified fullerene derivative has inhibitory activity on human immunodeficiency virus (HIV)-reverse transcriptase [17] (Fig. 1).

In this study, we analyzed the effects of fullerene derivatives on JAK2 V617F mutant-mediated transformation. Pyrrolidinium-type full-erene derivative potently induced apoptotic cell death of JAK2 V617F mutant-transformed cells through inhibition of the JNK activation pathway and prevented JAK2 V617F mutant-induced tumorigenesis in vivo. These observations suggest that JNK could be a therapeutic target for MPNs and show the potential of the fullerene derivative to be a novel anti-MPN drug.

2. Materials and methods

2.1. Reagents

Three types of fullerene derivative were synthesized as previously described [15–17]. Recombinant human erythropoietin (Epo) (ESPO® 3000) was purchased from Kirin Brewery Co. (Tokyo, Japan). Antibodies against c-Myc, Odc and β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA antibody (3 F10) was purchased from Roche (Indianapolis, IN). Other primary antibodies and peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA).

2.2. Plasmids

Murine JAK2-HA was subcloned into retroviral plasmids, murine stem cell virus (MSCV)-GFP and MSCV-Hygro (Clontech, CA, USA). Murine EpoR c-Flag was subcloned into MSCV-Puro (Clontech, CA, USA) [12]. The sequences of oligonucleotides used for constructing shRNA retroviral vector were as follows: sh-ASK1: 5′-gatccccgtgcagagactgagagtaattcaagagattactctcagtctctgcacttttta-3′ and 5′-agcttaaaaagtgcagagactgagagtaatctcttgaattactctcagtctctgcacggg-3′. Underlined sequences correspond to the sequence of murine ASK1 (from 4728 to 4746 in the 3′-untranslated region).

2.3. Cell cultures

Ba/F3 cells were infected with empty virus (−), wild-type murine JAK2 c-HA or a mutant of murine JAK2 c-HA (V617F) with full-length murine EpoR c-FLAG as described previously [10,11]. These cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (BioWest, France), 100 units/ml penicillin and 100 μg/ml streptomycin (Nacalai Tesque), with or without 5 units/ml Epo.

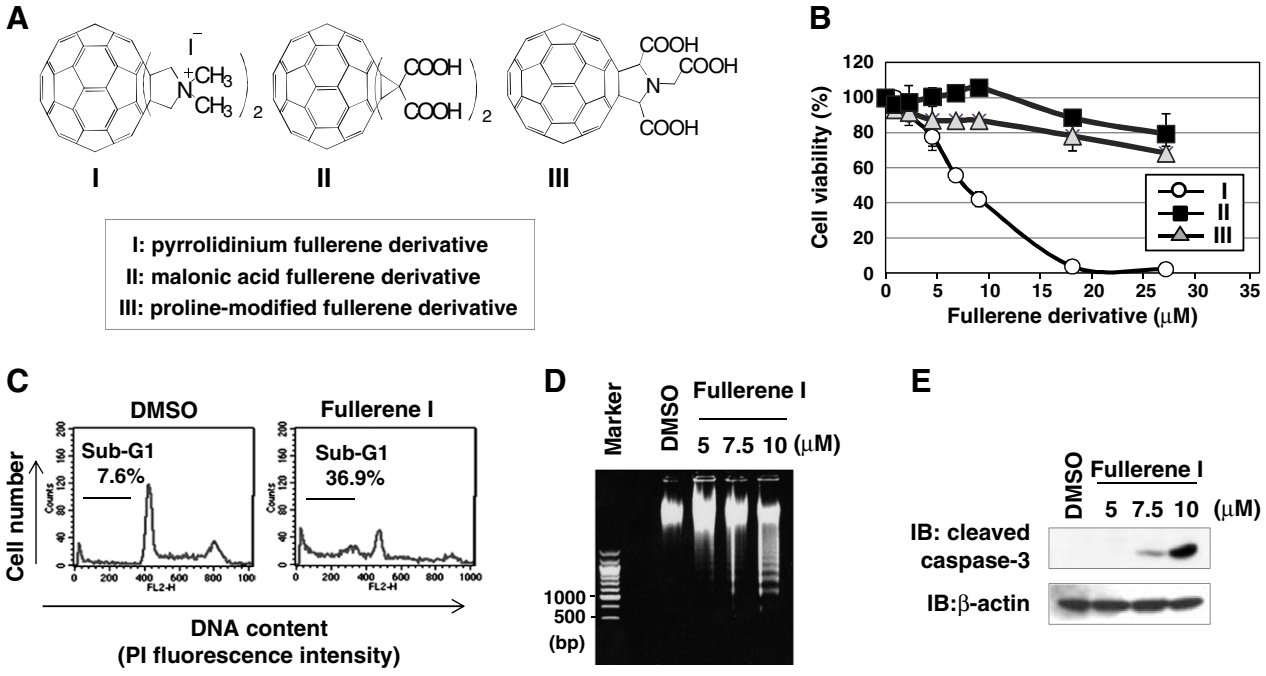

Fig. 1. Pyrrolidinium-type fullerene derivative significantly induces apoptosis in BaF3 cells transformed by JAK2 V617F mutant. (A) Structure of fullerene derivatives. I: pyrrolidinium fullerene derivative: C_{60}-bis (*N,N*-dimethylpyrrolidinium iodide), II: malonic acid fullerene derivative: C_{60}-dicyclopropane-1,1,1′,1′-tetracarboxylic acid, III: proline-modified fullerene derivative: C_{60}-N-carboxymethyl-2,5-dicarboxy-pyrrolidine. (B) Ba/F3-VF cells were treated with fullerene derivatives for 24 h. The cell viability was determined by trypan blue staining. Results are the mean ± S.D. of three independent experiments. (C) Ba/F3-VF cells were treated with DMSO (0.1%) or fullerene derivative I (7.5 μM) for 24 h. Cell cycle was analyzed by flow cytometry. (D, E) Ba/F3-VF cells were treated with DMSO (0.1%) or fullerene derivative I (5, 7.5, 10 μM) for 24 h. (D) DNA was isolated from cells and subjected to agarose gel electrophoresis. (E) Cell lysates were immunoblotted with anti-cleaved caspase-3 antibody or anti-β-actin antibody.