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Effect of chemical modification on the ability of pyrrolidinium fullerene to induce apoptosis of cells transformed by JAK2 V617F mutant

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ABSTRACT

JAK2 V617F mutant, a gene responsible for human myeloproliferative neoplasms (MPNs), causes not only cellular transformation but also resistance to various anti-cancer drugs. We previously reported that pyrrolidinium fullerene markedly induced the apoptosis of JAK2 V617F mutant-induced transformed cells through the reduction of apoptosis signal-regulating kinase 1 (ASK1), following inhibition of the c-Jun N-terminal kinase (JNK) pathway. In the current study, we found that the replacement of the 2-hydrogen atom (−H) or N-methyl group (−CH_3) by the butyl group (−C_4C_9) caused the more than 3-fold potent cytotoxic effects on cells transformed by the JAK2 V617F mutant. Strikingly, these chemical modification of pyrrolidinium fullerene resulted in more marked reduction of ASK1 protein and a more potent inhibitory effect on the JNK signaling cascade. On the other hand, when modified with a longer alkyl group, the derivatives lacked their cytotoxicity. These observations clearly indicate that the modification of pyrrolidinium fullerene with a suitable length of alkyl group such as butyl group enhances its apoptotic effect through inhibition of the ASK1-MKK4/7-JNK pathway.

1. Introduction

The myeloproliferative neoplasms (MPNs), which include polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF), are clonal hematopoietic stem cell diseases characterized by uncontrolled proliferation of terminally differentiated myeloid cells. A 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. In the majority of MPN patients, the JAK2 gene has a homozygous G → T transversion, which results in a valine-to-phenylalanine substitution at codon 617 of JAK2 (V617F) [1–3].

Previously, we found that the JAK2 V617F mutant induces the cytokine-independent survival of erythroid progenitor cells [4]. The JAK2 V617F mutant was reported to induce the activation of signaling pathways, signal transduction and activator of transcription 3 (STAT3) and STAT5 and kinases, Akt, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in a cytokine-independent manner. We demonstrated that STAT5, Akt and JNK are critical signal transducers for the proliferation and the transforming ability induced by JAK2 V617F mutant [5–7]. In addition, it was shown that cells transformed by the JAK2 V617F mutant exhibited resistance to various anti-cancer drugs, such as bleomycin (BLM), which generates DNA double-strand breaks, and mitomycin C (MMC) and cisplatin (CDDP), which are DNA cross-linking drugs [8–11], suggesting that the JAK2 V617F mutant causes not only the activation of survival signals against apoptosis induced by cytokine removal but also the resistance to various anti-cancer drugs. Therefore, the rapid development of effective therapeutic drugs for MPNs is required.

Fullerene (C_{60}) was discovered by Kroto et al. in 1985 as the third allotropic form of carbon after diamond and graphite [12]. Fullerene is a spherical molecule 0.7 μm in diameter and a new type of organic compound with a cage-like structure [13]. Chemical modification with several hydrophilic groups increases the solubility of fullerene, and the derivatives of water-soluble fullerene were reported to possess various biological and pharmacological properties [14–18]. As a typical derivative, pyrrolidinium fullerene exhibits anti-proliferative activity to various cancer cells [19]. Previously, we reported that pyrrolidinium fullerene markedly induced apoptotic cell death of cells transformed by the JAK2 V617F mutant through reduction of the protein expression of apoptosis

Abbreviations: ASK1, apoptosis signal-regulating kinase 1; c-IAP1, cellular inhibitor of apoptosis protein 1; DCFH, dichlorodihydrofluorescein; DMSO, dimethyl sulfoxide; Epo, erythropoietin; ERK, extracellular signal-regulated kinase; EpoR, erythropoietin receptor; ET, essential thrombocytosis; JAK2, Janus kinase 2; JNK, c-Jun N-terminal kinase; MKK4/7, mitogen-activated protein kinase kinase 4/7; MPNs, myeloproliferative neoplasms; PMF, primary myelofibrosis; PV, polycythemia vera; STAT, signal transducers and activators of transcription; ROS, reactive oxygen species.

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signal-regulating kinase 1 (ASK1) and inhibition of the JNK pathway [20]. ASK1, one of the mitogen-activated protein kinase kinase kinases (MAPKKKs), was previously demonstrated to stimulate the JNK signaling cascade mediating the activation of JNK upstream kinases, named MKK4/7 [21].

In the current study, we focused on the relationship between the chemical structure of the derivatives of pyrrolidinium fullerene and their apoptotic effects. Our study suggests the possibility that the chemical modification of fullerene would emphasize their utility as anti-cancer drugs.

2. Materials and methods

2.1. Chemicals and reagents

Pyrrolidinium fullerene and its derivatives were synthesized as previously described [15,17,19]. Anti β-actin antibody and anti-HA antibody (3F10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Roche (Indianapolis, IN), respectively. Anti-phospho JAK2 antibody (Y1007/Y1008), anti-phospho-STAT3 antibody (Y705), anti-STAT3 antibody, anti-phospho-STAT5 antibody (Y694), anti-STAT5 antibody, anti-phospho-ERK1/2 antibody (T202/Y204), anti-ERK1/2 antibody, anti-phospho-Akt (S473), anti-Akt antibody, anti-ASK1 antibody, anti-phospho-MKK4 antibody (S257/T261), anti-MKK4 antibody, anti-phospho-MKK7 antibody (S271/T275), anti-MKK7 antibody, anti-phospho-JNK antibody (T183/Y185), anti-JNK antibody and anti-cleaved caspase 3 antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated secondary antibodies were from Dako (Glostrup, Denmark). 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) and α-tocopherol were purchased from Sigma Inc. (St. Louis, MO).

2.2. Retroviral infection and cell cultures

Ba/F3 cells were infected with retroviruses coding a mutant of murine JAK2 c-HA (V617F) with murine EpoR as described previously [4]. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (BioWest, Nuaillé, France), 100 units/ml penicillin (Nacalai Tesque, Tokyo, Japan) and 100 μg/ml streptomycin (Nacalai Tesque).

2.3. Western blotting

Cells were harvested in ice-cold PBS and lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA pH 8.0, 0.5%

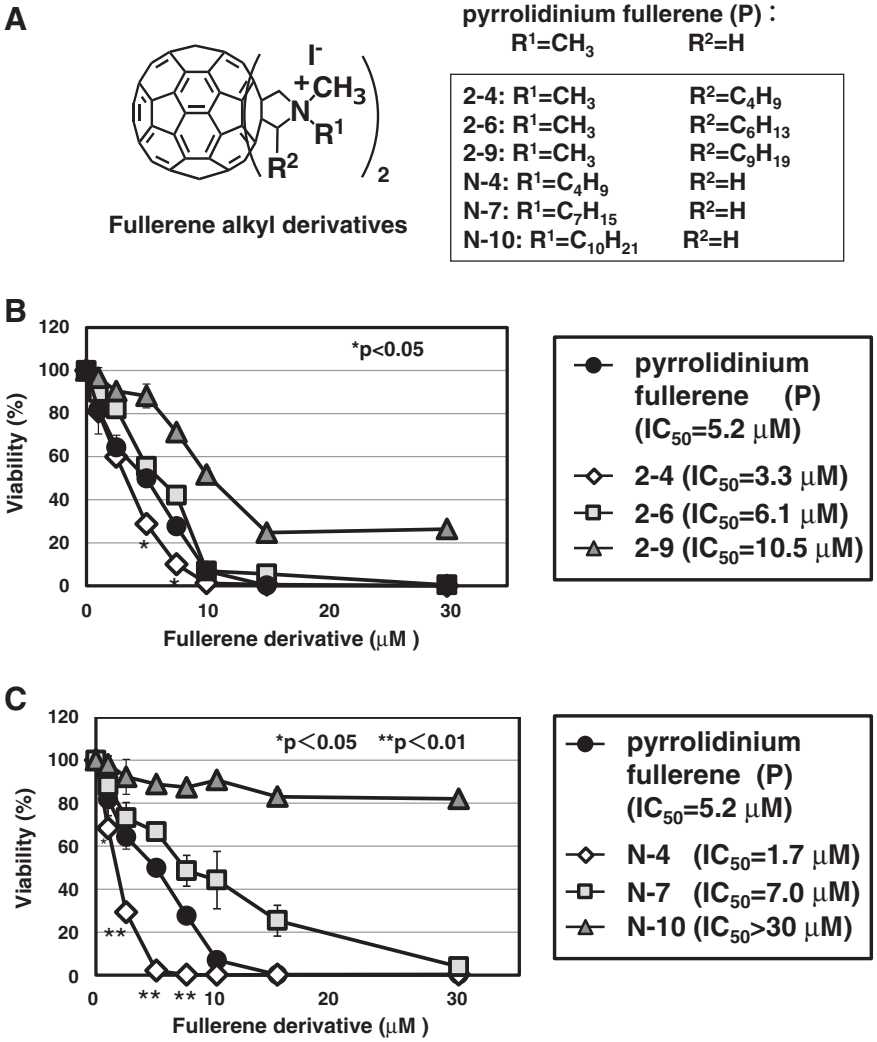

Fig. 1. Pyrrolidinium fullerene and its derivatives exhibit cytotoxicity to VF-Ba/F3 cells. (A) Structures of fullerene derivatives. Pyrrolidinium fullerene (P) is C_{60}-bis *N*, *N*-dimethylpyrrolidinium iodide. In pyrrolidinium fullerene (P), the 2-hydrogen atom (−H) or N-methyl group ($-CH_3$) was substituted with various alkyl groups and named 2-4, 2-6, 2-9, N-4, N-7, and N-10, respectively. (B, C) VF-Ba/F3 cells were treated with different concentrations (1, 2.5, 5, 7.5, 10, 15, 30 μM) of pyrrolidinium fullerene (P) and its derivatives for 24 h. Cell viability was determined by trypan blue staining. Results are the mean ± S.D. of three independent experiments. * and ** indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively (vs pyrrolidinium fullerene (P)).