

## ORIGINAL ARTICLE

# Inhibition of adult T-cell leukemia cell proliferation by polymerized proanthocyanidin from blueberry leaves through JAK proteolysis

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## Abstract

We have previously reported that the proanthocyanidin (PAC) fraction of blueberry leaf extract (BB-PAC) inhibits the proliferation of HTLV-1-infected adult T-cell leukemia (ATL) by inducing apoptosis. In the present study, we further analyzed the structure of BB-PAC and elucidated the molecular mechanism underlying the inhibitory function of HTLV-1-infected and ATL cells. After hot water extraction with fractionation with methanol-acetone, BB-PAC was found to be concentrated in fractions 4 to 7 (Fr7). The strongest inhibition of ATL cell growth was observed with Fr7, which contained the highest BB-PAC polymerization degree of 14. The basic structure of BB-PAC is mainly B-type bonds, with A-type bonds (7.1%) and cinchonain I units as the terminal unit (6.1%). The molecular mechanism of cytotoxicity observed around Fr7 against ATL cells was the degradation of JAK1 to 3 and the dephosphorylation of STAT3/5, which occurs by proteasome-dependent proteolysis, confirming that PAC directly binds to heat shock protein 90 (HSP90). JAK degradation was caused by proteasome-dependent proteolysis, and we identified the direct binding of PAC to HSP90. In addition, the binding of cochaperone ATPase homolog 1 (AHA1) to HSP90, which is required for activation of the cofactor HSP90, was inhibited by BB-PAC treatment. Therefore, BB-PAC inhibited the formation of the HSP90/AHA1 complex and promoted the degradation of JAK protein due to HSP90 dysfunction. These results

**Abbreviations:** AHA1, ATPase homolog 1; ATL, adult T-cell leukemia; BB, blueberry; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; HTLV-1, Human T-cell leukemia virus type 1; JAK/STAT, Janus kinase/signal transducer and activator of transcription; mDP, mean degree of polymerization; PAC, proanthocyanidin.

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suggest that the highly polymerized PAC component from blueberry leaves has great potential as a preventive and therapeutic agent against HTLV-1-infected and ATL cells.

#### KEYWORDS

ATL, blueberry leaf, HSP90, JAK/STAT, proanthocyanidin

## 1 | INTRODUCTION

Human T-cell leukemia virus type 1 is an oncogenic retrovirus associated with an aggressive type of T-cell leukemia termed TL and chronic inflammatory disorders, such as HAM/TSP. Human T-cell leukemia virus type 1 is endemic to various regions, including Japan, Africa, the Caribbean Islands, Central America, and South America; approximately 10–20 million people worldwide are infected with HTLV-1. Human T-cell leukemia virus type 1 retroviruses maintain persistent infection in CD4<sup>+</sup> T lymphocytes, and over 90% of HTLV-1-infected carriers remain asymptomatic throughout their lives. Adult T-cell leukemia and HAM/TSP develop in approximately 5% of HTLV-1 carriers after a long latency period of 20–50 years. Although the incidence of new HTLV-1 carriers is decreasing due to the prevention of mother-to-child transmission, treatment of ATL remains challenging, and there is an urgent need to identify additional therapeutic targets for HTLV-1-infected diseases.<sup>1</sup>

We previously reported that the aberrant activation of several signal transduction pathways in HTLV-1-infected T cells plays an important role in the development of HTLV-1-associated diseases.<sup>2–5</sup> Moreover, the constitutive activation of the JAK/STAT signaling pathway is involved in the progression and development of ATL- and HTLV-1-infected diseases. Although the molecular mechanism of the activated JAK/STAT signaling pathway has not yet been completely elucidated in ATL, the persistent phosphorylation of JAK1, JAK2, and JAK3 through the autocrine pathways of IL-2/IL-2R and the collapse of the dephosphorylation system lead to the subsequent entry of phosphorylated STAT1, STAT3, and STAT5 into the nucleus, resulting in the upregulation of downstream target genes that are involved in processes such as cell proliferation (Cyclin D), inhibition of apoptosis (Bcl-2, Bcl-xL), and inflammation (IL-1 $\beta$ , IL-6, and IFN- $\gamma$ ). Therefore, suppression of the JAK/STAT signaling pathway is crucial for the prevention and treatment of HTLV-1-associated diseases.<sup>6–9</sup>

It has been reported that agricultural products have numerous compounds and extracts with beneficial health effects, such as antioxidant, anti-inflammatory, and antitumor properties, which reduce the risk of developing chronic diseases by eating more of agricultural products.<sup>10–13</sup> Among our research project involving 52 agricultural products, rabbit-eye blueberry leaves (*Vaccinium virgatum* Aiton), which are cultivated in the southern areas of Japan, contain high levels of polyphenols, including flavonoids, tannins, phenolic acids, and PACs, and exert more effective functions for the inhibition of tumor growth and HCV viral replication.<sup>14–16</sup> Although the extracts of blueberry leaves have attracted the attention of academia and the food industry as therapeutic agents and health products to cure or reduce

the risk of chronic diseases such as tumors and diabetes, the details of the major chemical constituents and mechanism of the antitumor effects in blueberry leaves are not yet understood.

Proanthocyanidin is included in a wide variety of agricultural foods, such as fruits, nuts, beans, plants, and leaves, and consists of condensed flavan-3-ols, (+)-catenin, and (-)-epicatechin units that link doubly A-type linkages, including C4→C8, an additional ether bond between O7→C2, and a single B-type linkage of C4→C8 through interflavonoid bonds.<sup>17</sup> A previous study showed that a diverse number of food product extracts exist, such as cacao (B-type), apple (B-type), grape seed (B-type), and cranberry (A-type), and they contain a high degree of polymerization and concentration of PAC. They also have biological activities, including antimicrobial, antiallergy, anticancer, and antiobesity activities.<sup>18</sup> Furthermore, a higher degree of polymerization, especially with the pentamers in apple PAC, significantly inhibited the proliferation of cancer cells compared with the lower polymer samples,<sup>19</sup> suggesting that the inhibitory effects of tumor growth were dependent on the degree of polymerization and constituent unit. Proanthocyanidins in blueberry leaves are characterized by the presence of A-type bonds and cinchonaine units, and it has been speculated that oligomeric PACs contribute to biological functions.<sup>15</sup>

Although previous studies indicated that the extract of blueberry leaves suppresses the proliferation of ATL cells,<sup>16</sup> it is unclear what kinds of structures are contained in the extract of blueberry leaves and how mechanisms suppressed cell proliferation following treatment with blueberry leaves in HTLV-1-infected and ATL cells. To identify the components in the extract of blueberry leaves with the function of inhibiting HTLV-1-infected and ATL cell proliferation, hot water extraction was followed by fractionation with Sephadex LH-20 columns (Sigma-Aldrich), followed by thiolysis cleavage and MALDI-TOF-MS. The results showed that PACs consisting mainly of polyflavan-3-ol units linked with B-type bonds were abundant, and the strongest antitumor effect was observed, especially in fractions with high degrees of polymerized PAC. Furthermore, we examined the intracellular function of this highly polymerized PAC and found that it degraded JAK protein in a proteasome-dependent manner; the degradation of JAK protein caused inhibition of HTLV-1 infected and ATL cell proliferation and apoptosis. Furthermore, PAC directly binds to HSP90, which is essential for the stabilization of oncogene products in cancer cells, and inhibits the binding of HSP90 to the cochaperone AHA1, which is essential for its activation. Therefore, we found that PAC acts as an HSP90 inhibitor and promotes the degradation of JAK proteins in HTLV-1-infected and ATL cells, resulting in the inhibition of cell proliferation and induction of

apoptosis. Therefore, polymerized PAC in blueberry leaves has been determined to be a novel therapeutic agent for ATL- and HTLV-1-associated diseases.

## 2 | MATERIALS AND METHODS

### 2.1 | Fraction of blueberry leaf extraction

The fractionation of blueberry leaf extract has been described elsewhere.<sup>16</sup> A lyophilized powder of fresh blueberry leaves was kindly supplied by Unkai Shuzo Co., Ltd. Lyophilized blueberry leaf powder (300 g) was extracted for 30 min with boiling water (15 L) and then cooled in an ice bath. After centrifugation (10 min at 1700 g), the supernatant was lyophilized and stored at  $-20^{\circ}\text{C}$  until use. The lyophilized powder (57.7 g) of blueberry leaf hot water extracts was suspended in distilled water (3 L), followed by sonication at  $50^{\circ}\text{C}$  for 30 min. The insoluble material was removed by filtration. The filtrate was directly subjected to Sephadex LH-20 column chromatography (4.5 cm i.d.  $\times$  50 cm) (Sigma-Aldrich), eluted with water containing increasing proportions of methanol (100% water, 20%, 40%, 60%, 80%, and 100% methanol), and finally eluted with 60% acetone. The elution was collected in an Erlenmeyer flask (1000 ml in each) to yield seven fractions (BBFr1–BBFr7), concentrated with a vacuum evaporator, and completely freeze-dried.

For the quantification of PAC, the fractions were measured using the butanol/HCl method.<sup>14</sup> Each fraction was quantitated from 0.5 mg/ml (for Fr0–Fr4) or 0.2 mg/ml (for Fr5–Fr7) in methanol. Briefly, 400  $\mu\text{l}$  solution was mixed with 3.5 ml *n*-butanol/HCl (95:5, v/v) and 100  $\mu\text{l}$  of 2% (w/v)  $\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  in 2 M HCl and heated in an oil bath at  $105^{\circ}\text{C}$  for 30 min. After heating, the reaction mixture was cooled in water for 15 min, and the absorbance was measured at 550 nm. The PAC content was expressed in milligrams of cyanidin chloride equivalent/g dry weight.

The structure and polymerization of PAC were determined by MALDI-TOF-MS and thiolysis.<sup>15</sup> The mDP of PAC was calculated as follows:  $\text{mDP} = [\text{sum of (2-hydroxyethylthio adducts} \cdot n) + \text{sum of (free flavan-3-ol} \cdot n)] / [\text{total free flavan-3-ol}]$ , where *n* represents the degree of polymerization of the detected flavan-3-ol by thiolysis. Average percentage of A-type bonds =  $[\text{sum of (thiolysis compounds containing A-type bonds} \cdot n)] / [\text{total free flavan-3-ol}] / \text{mDP} \times 100$ . Average percentage of cinchonain I unit =  $[\text{sum of (thiolysis compounds containing cinchonain I unit} \cdot n)] / [\text{total free flavan-3-ol}] / \text{mDP} \times 100$ .

### 2.2 | Reagents

Chemical samples consisted of ApplePhenon (Asahi Food and Healthcare), oligonol (OLG-F; Amino Up Chemical), dimeric PAC (ChemFaces), EGCG, malvidin (Sigma Aldrich), kaempferol, and quercetin (Tokyo Chemical Industry). The powder was dissolved in DMSO. MG132 was obtained from LifeSensor. The CCK-8 and

HilyMax transfection reagents were purchased from Dojindo. Most of the Abs used in this experiment were purchased from the companies listed in Table S1. The mouse mAb against HTLV-1/Tax (MI73) was a kind gift from Dr M. Matsuoka (Kumamoto University).<sup>20</sup> The HA-tagged HSP90A was a kind gift from Dr H. Iha (Ohita University). The Flag-tagged AHA1 expression vector (Flag-AHA1) has been described elsewhere.<sup>21</sup> The primer sequences used are listed in Table S2.

### 2.3 | Patient samples

HCT-5 cells were derived from the cerebrospinal fluid of HAM/TSP patients and were a kind gift from Dr T. Nakamura (Nagasaki International University).<sup>22</sup> The HCT-5 cells were maintained in RPMI-1640 medium supplemented with 20% FBS and 0.75  $\mu\text{g/ml}$  recombinant human IL-2 (PeproTech). Blood samples were obtained with informed consent and with the approval of the Institutional Review Board of the Faculty of Medicine, University of Miyazaki. Acute T-cell leukemia cells were collected from patients at the time of hospital admission before the start of chemotherapy.  $\text{CD4}^+$  lymphocytes were isolated from the blood samples of healthy volunteers by AutoMACS negative selection using a  $\text{CD4}^+$  T-cell isolation kit (Miltenyi Biotech). The ATL cells were maintained in AIM-V medium (Thermo Fisher Scientific) supplemented with 20% FBS, 10 mM 2-mercaptoethanol, and 0.75  $\mu\text{g/ml}$  recombinant human IL-2.<sup>23</sup>

### 2.4 | Isolation and detection of PAC binding proteins

Commercially available PAC was immobilized with magnetic FG beads with an epoxy linker (Tamagawa Seiki) according to the manufacturer's instructions.<sup>24</sup>

The remaining materials and methods are presented in Supplementary materials and methods.

## 3 | RESULTS

### 3.1 | High degree of polymerization of proanthocyanidin in blueberry leaf extract results in a high anticancer effect in HTLV-1-infected and ATL cells

Oligomeric PACs from rabbiteye blueberry leaves have been reported to inhibit the proliferation of HTLV-1-positive ATL cell lines.<sup>16</sup> Therefore, we initially examined which components and structures of blueberry leaf PAC have antileukemic properties against HTLV-1-infected and ATL cells. We first separated the hot water extraction fraction of blueberry leaves and then further separated them into eight fractions (see Materials and Methods). The content and degree of PAC polymerization identified in the different fractions of blueberry leaf extract are shown

in Table 1. As we quantified the amount of PAC in the fractions by the butanol/HCl method, the content of PAC started to increase from Fr3 and peaked at Fr7. The blueberry leaf PAC was characterized by the presence of dodecamers with A-type linkages and cinchonain units<sup>15</sup>; therefore, we further determined the mDP and structure of PAC in fractions by MALDI-TOF-MS after thiolysis cleavage. The MALDI spectrum of fractions showed the presence of oligomers containing cinchonain I units, and the mDP was 2.47 in Fr4 and then reached a peak value of 14.33 in Fr7 (Table 1). The highly polymerized PAC of Fr7 contains 7.1% A-type bonds and 86.8% B-type bonds with 6.1% cinchonain I units; therefore, the structure of PAC from blueberry leaf extracts has mainly B-type linkage bonds and cinchonain I units (Table 1 and Figure S1A,B).

To confirm which fraction of blueberry leaf extract showed anti-tumor effects, the HTLV-1-infected and ATL cell lines MT2 and SO4 were treated with various amounts of each fraction. Increasing the dose to 20 µg/ml for fractions 0 to 5 with low PAC concentrations did not produce any cytotoxic effect on HTLV-1-infected and ATL cell lines; however, treatment of Fr6 and Fr7 with high concentrations of PAC significantly inhibited the viability of the HTLV-1-infected and

ATL cell lines in a dose-dependent manner (Figure 1A). Moreover, the high degree of polymerization of PAC in blueberry leaf extracts (BBFr7) could have the strongest inhibitory effect on cell growth in HTLV-1-infected and ATL cells.

### 3.2 | Tumor growth inhibitory effect of BBFr7 in HTLV-1-infected and ATL cells accompanied by suppression of the JAK/STAT signaling pathway

In the next experiment, we undertook a comparative study of the effects of different types of PACs available in the market, such as ApplePhenon (apple, oligomeric procyanidin) and OLG-F (lychee, low-molecular-weight procyanidin). The inhibition of T-ALL cell proliferation by BBFr7 at different concentrations did not reach 50% inhibition, even at high concentrations. However, when we examined HTLV-1-infected and ATL cell lines (MT2, SU9T-01, KK1, SO4, and ED), they were found to be highly sensitive to BBFr7, with IC<sub>50</sub> values ranging from 1.91 to 11.31 µg/ml (Figure 2A and Table S3), indicating

TABLE 1 Fractionation of blueberry leaf extract

|     | PAC content (mg/g) | MT2/IC <sub>50</sub> (µg/ml) | SO4/IC <sub>50</sub> (µg/ml) | mDP   | A-type bond (%) | B-type bond (%) | Cinchonain I unit (%) |
|-----|--------------------|------------------------------|------------------------------|-------|-----------------|-----------------|-----------------------|
| Fr0 | 3.97               | >20                          | NT                           | NT    | NT              | NT              | NT                    |
| Fr1 | 2.82               | >20                          | NT                           | NT    | NT              | NT              | NT                    |
| Fr2 | 2.85               | >20                          | NT                           | NT    | NT              | NT              | NT                    |
| Fr3 | 23.13              | >20                          | NT                           | NT    | NT              | NT              | NT                    |
| Fr4 | 109.43             | >20                          | >20                          | 2.49  | 7.2             | 85.7            | 7.1                   |
| Fr5 | 296.23             | >20                          | >20                          | 5.14  | 10.6            | 77.1            | 12.3                  |
| Fr6 | 384.62             | 18.73                        | 4.9                          | 8.88  | 8.8             | 81.3            | 9.9                   |
| Fr7 | 396.52             | 11.92                        | 4.0                          | 14.33 | 7.1             | 86.8            | 6.1                   |

Note: A-type bond, B-type bond, and Cinchonain I unit represent average percentage of bond/%. Abbreviations: Fr, fraction; mDP, mean degree of polymerization; NT, not tested; PAC, proanthocyanidin.

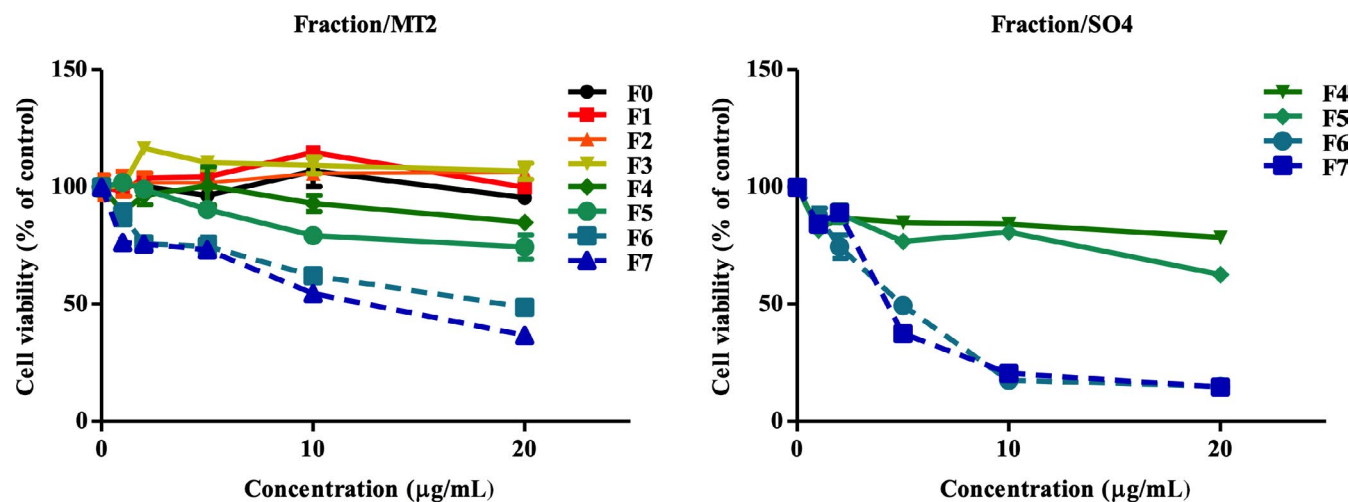
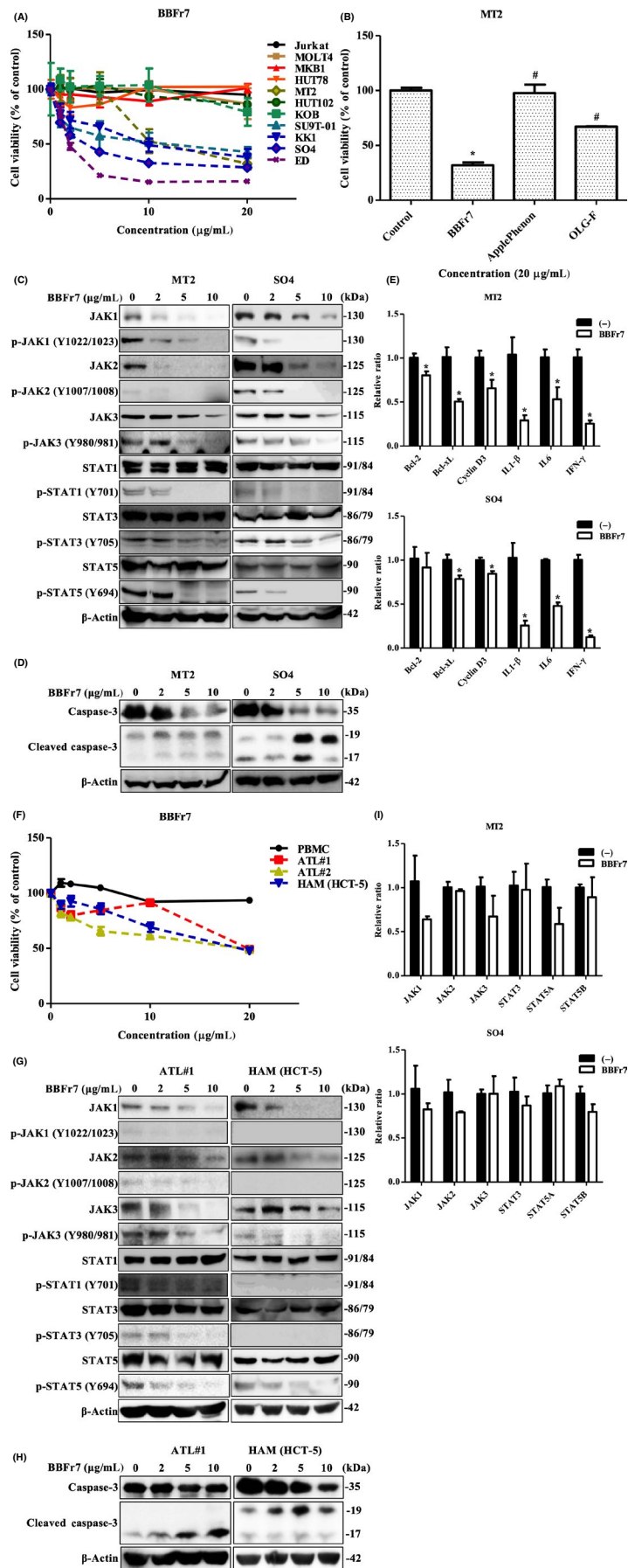


FIGURE 1 High degree of polymerization of proanthocyanidin in blueberry leaf extract indicates a low IC<sub>50</sub> value in HTLV-1-infected and adult T-cell leukemia (ATL) cells. Cell viability and IC<sub>50</sub> were determined by CCK-8 after treatment with 0–20 µg/ml of different fractions of blueberry leaf extract for 72 h in HTLV-1-infected and ATL cell lines MT2 and SO4





**FIGURE 2** Treatment with blueberry fraction 7 (BBFr7) inhibits cell proliferation through suppression of the JAK/STAT signaling pathway in HTLV-1-infected and adult T-cell leukemia (ATL) cells. A, Cell viability and IC<sub>50</sub> were determined by CCK-8 after treatment with 0–20 µg/ml BBFr7 for 72 h in T-cell acute lymphoblastic leukemia, HTLV-1-infected, and ATL cell lines. B, Cell viability was determined by CCK-8 after treatment with 20 µg/ml ApplePhenon and oligonol (OLG-F) for 72 h in MT2 cells. Mean and SD are shown (n = 4). \*p < 0.05 compared with control; #p < 0.05 compared with BBFr7. C, Expression and phosphorylation of the JAK/STAT signaling pathway in MT2 and SO4 cells after treatment with the indicated doses of BBFr7 for 24 h were determined by immunoblot analysis using each specific Ab. D, Cell apoptosis under the same experimental conditions as in (C) was determined by immunoblot assay using anticaspase 3 or anticleaved caspase 3 Abs. E, Quantitative RT-PCR analysis of JAK/STAT target mRNAs after treatment with 2 µg/ml BBFr7 for 24 h in MT2 and SO4 cells. Mean and SD are shown (n = 3). \*p < 0.05 compared with (-). F, Cell viability and IC<sub>50</sub> were determined by CCK-8 after treatment with 0–20 µg/ml BBFr7 at 72 h in PBMCs, ATL patients, and HTLV-1-associated myelopathy (HAM) cells. G, Expression and phosphorylation of the JAK/STAT signaling pathway in ATL patients and HAM cells after treatment with indicated doses of BBFr7 for 24 h were determined by immunoblot analysis using specific Abs. H, Cell apoptosis under the same experimental conditions as in (G) was determined by immunoblot assay using anticaspase-3 or anticleaved caspase-3 Abs. I, Quantitative RT-PCR analysis of JAK/STAT mRNA was carried out after treatment with 2 µg/ml BBFr7 for 24 h in MT2 and SO4 cells. Mean and SD are shown (n = 3)

that HTLV-1-infected and ATL cell lines were more effective in suppressing the growth of BBFr7 than HTLV-1-negative T-ALL cell lines, such as Jurkat and MOLT4. We compared BBFr7 with other types of PAC products, including ApplePhenon from apples and OLG-F from lychee. ApplePhenon and OLG-F showed no inhibitory effect in HTLV-1-infected and ATL cell lines, even the dose was increased up to 20 µg/ml (Figures 2B and S2A,B and Table S3). Furthermore, previous reports have shown that EGCG, kaempferol, and quercetin, which are flavonoids, and malvidin, which is also found in blueberry fruit, show antitumor effects through the inhibition of STAT phosphorylation rather than the inhibition of JAK protein.<sup>25</sup> We purchased pure flavanol compounds and tested their proliferative effects on HTLV-1-infected and ATL cells using each compound. The results showed that the effects of these compounds were weaker than those of BBFr7 (Table S3), indicating that the highly polymerized PAC of BBFr7 can inhibit the proliferation of HTLV-1-infected and ATL cells more effectively than other plant-derived PACs.

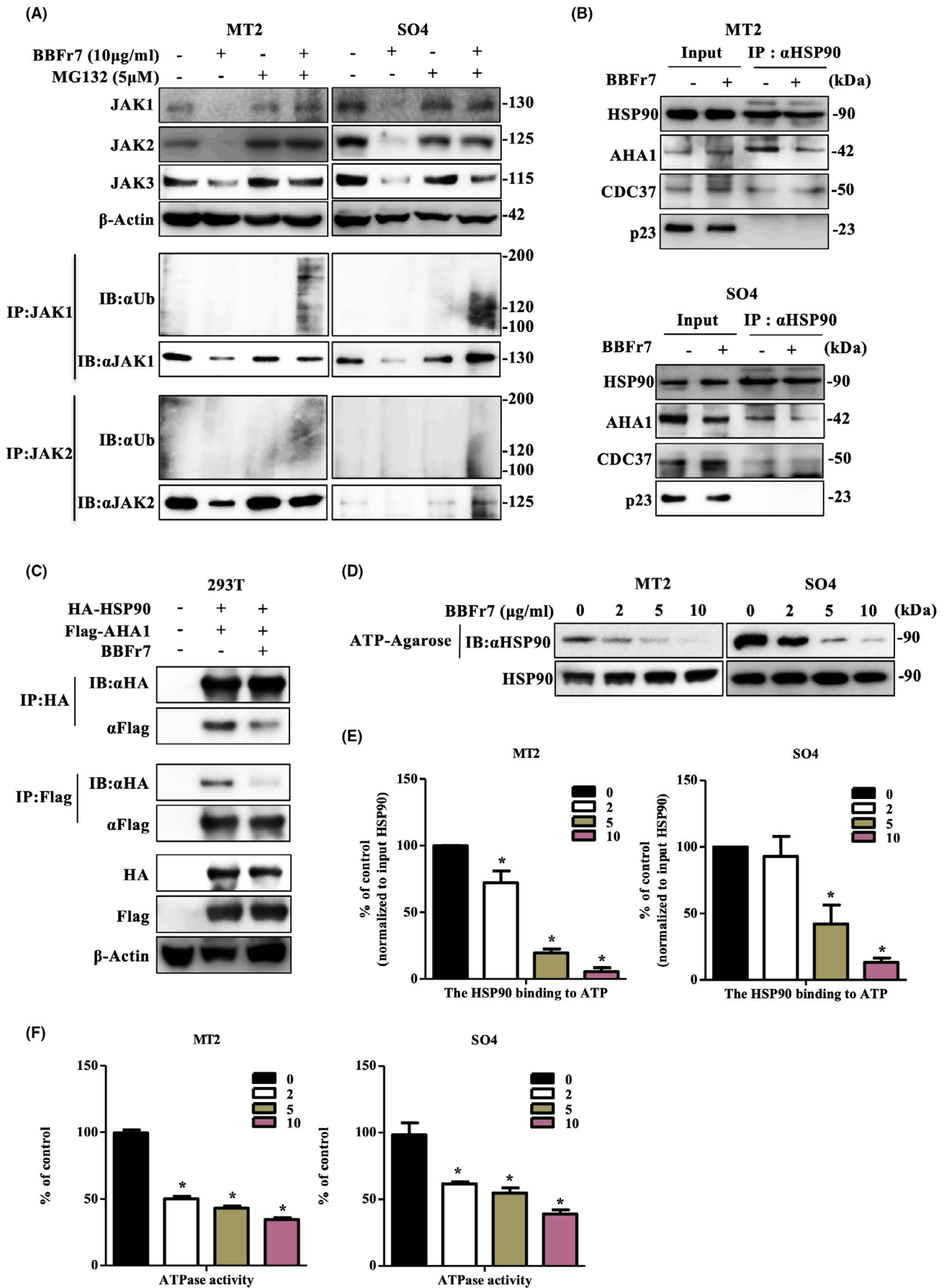
It is well known that abnormal activation of intracellular signaling pathways such as PI3K/AKT, NF-κB, and JAK/STAT is important for the development and progression of ATL.<sup>2,3,6,7</sup> Therefore, we investigated whether BBFr7 affects these intracellular signaling pathways by inhibiting HTLV-1 infection and ATL cell proliferation. When BBFr7 was administered to HTLV-1-infected and ATL cells, the phosphorylation status of JAK1 (Tyr1022/1023), JAK2 (Tyr1007/1008), and JAK3 (Tyr980/981) decreased in a dose-dependent manner, and their respective proteins were also significantly decreased. At the same time, the phosphorylation status of STAT1 (Tyr701), STAT3 (Tyr705), and STAT5 (Tyr694) also decreased, although the quantity of STAT proteins did not change (Figures 2C and S2C). However, there was little change in the protein levels of Tax and HBZ, the phosphorylation status of AKT (Ser473) in the PI3K/AKT signaling pathway, and the phosphorylation status of IκBα (Ser32/36) in the NF-κB signaling pathway (Figure S2D). After treatment with BBFr7, cleaved caspase-3 appeared in HTLV-1-infected and ATL cells due to the induction of cell apoptosis, and the expression of target genes downstream of JAK/STAT (Bcl-2, Bcl-xL, Cyclin D3, IL-1β, IL-6, and IFN-γ) was significantly decreased (Figure 2D,E). To further investigate the cytotoxic effects of BBFr7 in a wider range of HTLV-1-infected cells, we examined the cell proliferation and activation status of the JAK/STAT signaling pathway in primary ATL cells and

HTLV-1-infected T cell lines derived from patients with HAM. When BBFr7 was added to PBMCs derived from healthy individuals as a control, almost no cell death was observed. However, BBFr7 was effective in inhibiting the proliferation of ATL cells derived from ATL patients and the HAM-derived T cell line (HCT-5) with IC<sub>50</sub> values of 17.79–19.72 µg/ml (Figure 2F and Table S4). At the same time, the expression of JAK proteins decreased with a reduction in the phosphorylation state of the JAK/STAT signaling pathway, and cleaved caspase-3 was increased with the induction of apoptosis (Figures 2G,H and S2E). In contrast, BBFr7 treatment did not induce a decrease in JAK proteins in the T-ALL cell lines Jurkat and MOLT4 (Figure S2F). To test whether the decrease in JAK proteins was dependent on the reduction in JAK transcription, we examined the mRNA expression of JAK/STAT after treating HTLV-1-infected and ATL cell lines (MT2 and SO4) with BBFr7. The results showed that the expression levels of JAK and STAT mRNAs did not change significantly before or after BBFr7 treatment (Figure 2I). Therefore, the decrease in JAK proteins by BBFr7 treatment might be due to the enhancement of the proteasomal degradation system.

### 3.3 | Downregulation of JAK proteins by treatment with BBFr7 in HTLV-1-infected and ATL cells dependent on the proteasomal degradation pathway through dysregulation of HSP90

The SOCS family is a classical negative feedback regulator of the JAK/STAT signaling pathway that targets JAK for degradation through the proteasome.<sup>26,27</sup> Therefore, we determined the expression of the SOCS family before and after treatment with BBFr7 in HTLV-1-infected and ATL cell lines. The expression of SOCS1, SOCS3, and the JAK-specific E3 ligase RNF125 were not significantly altered (Figure S3A). Next, we examined protein degradation by BBFr7 treatment using MG132, a proteasome inhibitor, to determine whether the ubiquitin-proteasome pathway is involved in the JAK protein degradation system. The degradation of JAK proteins by BBFr7 was recovered by MG132 treatment, and the recovered JAK proteins were highly ubiquitinated (Figure ).

Next, we used commercially available, purified dimeric PACs to comprehensively analyze their binding proteins by mass



**FIGURE 3** Treatment with blueberry fraction 7 (BBFr7) enhances the downregulation of JAK proteins through the proteasomal degradation pathway by disrupting heat shock protein 90 (HSP90) in HTLV-1-infected and adult T-cell leukemia (ATL) cells. (A) To identify proteasomal degradation of JAK1 and JAK2 protein in MT2 and SO4 cells after treatment with 10  $\mu\text{g}/\text{ml}$  BBFr7 for 24 h, the protein levels of JAK1 and JAK2 were determined by immunoblot (IB) analysis with or without treatment with 5  $\mu\text{M}$  MG132. The ubiquitination of JAK1 and JAK2 was identified by immunoblot analysis using an anti-ubiquitin Ab after immunoprecipitation (IP) with anti-JAK1 or anti-JAK2 Abs. B, HSP90 immunoprecipitated from MT2 and SO4 cells treated with 10  $\mu\text{g}/\text{ml}$  BBFr7 for 24 h was analyzed by immunoblotting using the indicated Abs. C, HA-tagged HSP90A was cotransfected with FLAG-tagged AHA1 into HEK293T cells. After the cell lysates were immunoprecipitated with either an anti-HA (HSP90A) or anti-Flag Ab (AHA1), the precipitated lysates were immunoblotted with each specific Ab. D, HSP90 binding to ATP-Agarose in the lysates of MT2 and SO4 cells after treatment with the indicated doses of BBFr7 for 24 h was determined by immunoblot analysis using HSP90 Ab. E, Bar graphs show the quantification of the relative band intensity of bound HSP90 to ATP-Agarose normalized by input HSP90 in the lysates of MT2 and SO4 cells treated with or without BBFr7. Mean and SD are shown ( $n = 3$ ). \* $p < 0.05$  compared with 0. F, Immunoprecipitated HSP90 in the lysates of MT2 and SO4 cells after treatment with indicated doses of BBFr7 for 24 h examined the ATPase activity of HSP90. Mean and SD are shown ( $n = 3$ ). \* $p < 0.05$  compared with 0

spectrometry. Proanthocyanidin was immobilized on magnetic FG beads with an epoxy linker, and the immobilized FG beads were incubated with cell lysates from the MT2 cell line. The proteins binding to the FG beads were separated by SDS-PAGE, and the specific bands were subjected to peptide mapping by liquid chromatography-tandem mass spectrometry analysis. The majority of the protein groups bound to PAC were HSPs such as HSP90A/B, HSPA8, and HSPA2. We suggest that HSP90A is included in this group of PAC-binding proteins by western blotting with an anti-HSP90A Ab (Figure S3B and Table S5). To investigate the mechanism by which BBFr7 treatment enhances JAK protein degradation through HSP90 function, we examined the cochaperone binding ability of HSP90 in HTLV-1-infected and ATL cells, which regulates the ATPase activity of HSP90 through conformational changes. The binding of CDC37 was not changed to HSP90A, but the binding of AHA1, which is one of the major HSP90 ATPase stimulators, was clearly decreased (Figures 3B and S3C). Furthermore, using an experimental system in which 293T cells were forced to express tagged HSP90 and AHA1, the specimens after BBFr7 treatment showed a clear decrease in HSP90A and AHA1 binding (Figure 3C). Because the cochaperone AHA1 promotes the HSP90 chaperone function to client proteins through the stimulation of ATPase activity, we examined whether BBFr7 inhibited HSP90 binding to ATP and ATPase activity in HTLV-1-infected and ATL cells. ATP-Agarose successfully bound to HSP90 in the lysates of MT2 and SO4 cells, and HSP90 binding to ATP-Agarose was significantly inhibited by treatment with BBFr7 compared with that of the control lysates without treatment with BBFr7 (Figure 3D,E). Furthermore, treatment with BBFr7 suppressed the ATPase activity of HSP90 in these two cell lines (Figure 3F). Therefore, the proteasomal degradation of JAK proteins by treatment with BBFr7 in HTLV-1-infected and ATL cells might be dependent on the suppression of HSP90 chaperone activity through the disassociation of HSP90 and AHA1.

### 3.4 | Blueberry Fr7 by oral gavage inhibits tumor growth in vivo

To investigate the effect of BBFr7 on tumor growth in ATL cells in vivo, we used a mouse model in which MT2 cells were subcutaneously

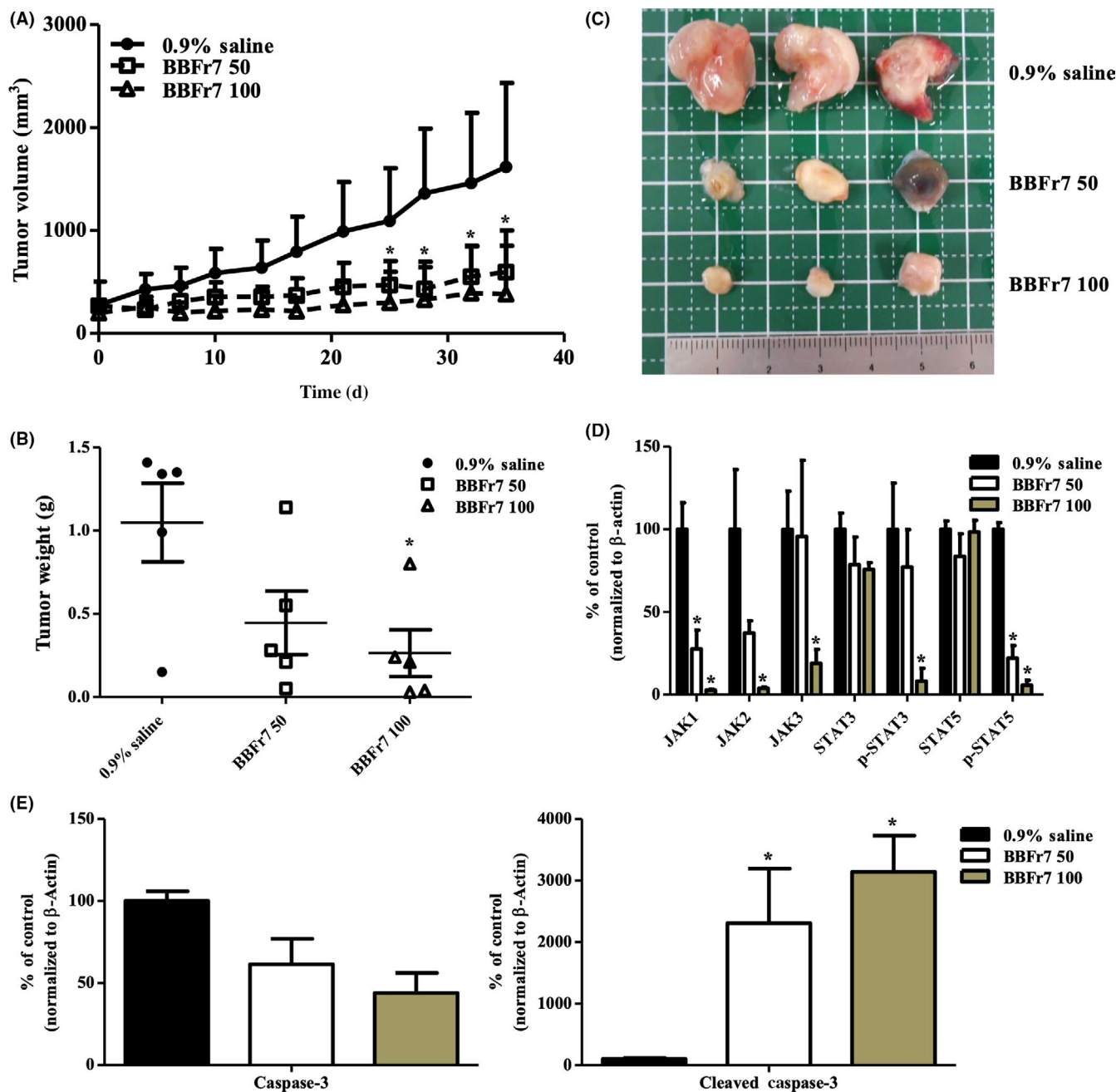
implanted into immunodeficient mice (NSG). The mice were randomly divided into three groups for each dose: control (0.9% saline)-treated mice and 50 or 100 mg BBFr7/kg body weight-treated mice. Blueberry Fr7 was given by oral gavage twice a week, and tumor growth was monitored for 35 days. During the experiment, BBFr7 treatment did not cause serious harm to the mice, as the average body weight was similar between the control and BBFr7-treated mice (Figure S4A). Treating mice with 50 or 100 mg/kg BBFr7 significantly suppressed their tumor volumes, sizes, and weights compared to those of the control group (Figure 4A–C). In addition, we examined the JAK/STAT signaling pathway in MT2 tumors used in this transplantation experiment using western blot analysis. The xenografts treated with BBFr7 showed decreased levels of JAK1, JAK2, and JAK3 proteins, as well as significantly suppressed levels of phosphorylated STAT3 and STAT5, and cleaved caspase-3, a marker of apoptosis, increased in BBFr7-treated xenografts compared to the control group (Figures 4D,E and S4B). These results showed that BBFr7 suppressed the JAK/STAT signaling pathway and exerted a marked inhibitory effect on ATL cells in vitro and in vivo. Therefore, BBFr7 could be a promising therapeutic agent for HTLV-1-infected and ATL cells.

## 4 | DISCUSSION

In this study, we show that highly polymerized PAC in blueberry leaves is crucial for antitumor functions and has better performance than other agricultural products in HTLV-1-infected and ATL cells. This study is the first to identify the proteasomal degradation of JAK proteins through the suppression of HSP90 chaperone activity by PAC treatment, which leads to the suppression of the JAK/STAT signaling pathway in vitro and in vivo, suggesting that polymerized PAC in blueberry leaves could be a novel therapeutic agent for HTLV-1-infected and ATL cells.

Aberrant activation of the JAK/STAT signaling pathway has been frequently implicated in the development and progression of many types of malignancies, and the molecular mechanism of tumorigenesis by JAK/STAT has been well identified; therefore, JAK/STAT signaling molecules have attracted attention as suitable therapeutic targets for inhibitors. Several kinds of JAK inhibitors are currently available and are used in the treatment of rheumatoid arthritis,





**FIGURE 4** Blueberry fraction 7 (BBFr7) by oral gavage inhibits tumor growth in vivo. **A**, MT2 adult T-cell leukemia cells were injected subcutaneously into NSG mice ( $n = 5$ ) with or without BBFr7 treatment by oral gavage, and the tumors were measured on the indicated days. Mean and SD are shown ( $n = 5$ ). \* $p < 0.05$  compared with the control 0.9% saline. **B**, Weight of tumors was measured after the mice were killed. Mean and SD are shown ( $n = 5$ ). \* $p < 0.05$  compared with the control 0.9% saline. **C**, Pictures of tumors are shown with a ruler. **D**, Bar graphs show the quantification of the relative band intensity of the JAK/STAT signaling pathway normalized by  $\beta$ -actin in the lysate of tumors developed in NSG. Mean and SD are shown ( $n = 4$ ). \* $p < 0.05$  compared with the control 0.9% saline. **E**, Bar graphs show the quantification of the relative band intensity of caspase-3 or cleaved caspase-3 normalized to  $\beta$ -actin in the lysate of tumors developed in NSG. Data are expressed as mean and SD ( $n = 4$ ). \* $p < 0.05$  compared with control 0.9% saline

myelofibrosis, and tumors. Ruxolitinib and tofacitinib are JAK1/2 and JAK3 inhibitors, respectively, which inhibit the phosphorylation of JAK and STAT3/5 without changing the protein level, which leads to a reduction in the expression of oncogenic target genes and cell proliferation in ATL and solid tumors.<sup>8,9</sup> Furthermore, malvidin in blueberry, EGCG in green tea, and procyanidins in grape seeds induce

apoptosis through the suppression of phosphorylated JAK/STAT in many types of tumors.<sup>25,28,29</sup> However, some malignancies can become insensitive to ruxolitinib and other JAK inhibitors through reactivation and phosphorylation of JAK by tyrosine kinases,<sup>30</sup> suggesting that therapeutic targets that induce the degradation of JAK proteins should be effective.

We have previously reported that posttranslational modifications of HSP90, such as arginine methylation, are made by cochaperones of AHA1, p23, and CDC37 to enhance HSP90 chaperone activity, which in turn stabilizes and matures oncogenic client proteins such as AKT, NEMO, and JAK1/2.<sup>21</sup> JAK1/2 is thus an HSP90 client, and HSP90 inhibitors have been reported to be therapeutic agents in myeloproliferative disease, where JAK2 point mutations cause homeostatic activation of JAK2.<sup>30</sup> Similarly, in ATL, HSP90 is a major contributor to leukemogenesis, and treatment with 17-AAG, an HSP90 inhibitor that binds directly to the ATP-binding pocket, induces suppression of cell proliferation accompanied by degradation of client proteins.<sup>21,31</sup> Because HSP90 inhibitors PU-H71 and AUY922 induced the degradation of mutated (V617F) and phosphorylated JAK2 protein but not WT JAK2 in myeloproliferative neoplasm cells,<sup>32,33</sup> and HTLV-1/Tax expression in HTLV-1-infected T cells homeostatically phosphorylates JAK proteins,<sup>34</sup> we suggest that the highly phosphorylated JAK proteins by HTLV-1 infection might be more sensitive to the highly polymerized PACs than non-phosphorylated JAKs in T-ALL.

ATPase homolog 1 is a cochaperone that induces HSP90 ATPase activity by binding to the N-terminal and middle domains of HSP90. ATPase homolog 1 facilitates conformational changes to establish ATPase-driven HSP90 chaperone cycles. Although the ATPase activity of HSP90 remains relatively low in normal cells, overexpression and activation of AHA1 has been found in many types of cancers, and it has been reported that increased HSP90 chaperone activity is induced through the interaction of AHA1 with HSP90. Furthermore, suppressing the activity or expression of AHA1 promotes the degradation of HSP90 client proteins in tumors, which in turn inactivates signaling pathways, suppresses metabolic activity, and inhibits tumor development.<sup>35,36</sup> The suppression of AHA1 function could result in disruption of the correct folding environment of chaperones and aggregation of misfolded proteins.<sup>37</sup> Therefore, treatment of ATL cells with highly polymerized PACs could inhibit the binding of HSP90 to AHA1, which in turn could inhibit the growth of leukemia through misfolding and further degradation of JAK proteins. Therefore, suppression of HSP90 by BB-PAC could be an important function in the treatment of ATL. In this study, we showed that BB-PAC is a potent inhibitor of HSP90, which inhibits the binding of HSP90 to the HSP90 cochaperone protein AHA1, thereby suppressing the function of HSP90 by promoting the degradation of JAK protein (Figure S5). Thus, the highly polymerized PACs in blueberry leaves showed more potent antitumor effects due to the sustained degradation of JAK proteins, but how the polymerized PACs regulate the interaction of HSP90/AHA1 needs to be further elucidated.

It is difficult to remove HTLV-1-infected cells because of persistent lifelong infections. Individuals with HTLV-1 infection have a high rate of depression and anxiety at the onset of illness and in the asymptomatic phase,<sup>38</sup> and therefore, consuming functional foods and their compounds might reduce HTLV-1-infected lymphocytes, delay the onset of HTLV-1-related diseases, maintain the mental health of HTLV-1 carriers, and prevent ATL development through

monotherapy or a combination of therapeutic options such as allogeneic hematopoietic stem-cell transplantation, IFN- $\alpha$  plus zidovudine, and the anti-CCR4 mAb.

In conclusion, our results indicate that polymerized PAC in blueberry leaves has more antitumor effects in HTLV-1-infected and ATL cells than other agricultural products and is a potent JAK/HSP90 inhibitor through the proteasomal degradation of JAK proteins in vitro and in vivo. Suppression of the phosphorylated JAK/STAT signaling pathway by treatment with polymerized PAC prevents its target genes from playing important roles in cell proliferation and inflammation. Polymerized PAC could become a promising therapeutic agent for ATL and might lead to the development of novel combinations of compounds. Clinical trials using polymerized PAC in blueberry leaves could offer important advances in the treatment of HTLV-1-infected diseases.

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## DISCLOSURE

The authors declare no competing interests in this paper.

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