

Benzoxathiol derivative BOT-4-one suppresses L540 lymphoma cell survival and proliferation *via* inhibition of JAK3/STAT3 signaling

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Abbreviations: BOT-4-one, 2-cyclohexylimino-6-methyl-6,7-dihydro-5H-benzo[1,3]oxathiol-4-one; JAK, janus kinase; STAT3, signal transducer and activator of transcription 3; upd, unpaired

Abstract

Persistently activated JAK/STAT3 signaling pathway plays a pivotal role in various human cancers including major carcinomas and hematologic tumors, and is implicated in cancer cell survival and proliferation. Therefore, inhibition of JAK/STAT3 signaling may be

a clinical application in cancer therapy. Here, we report that 2-cyclohexylimino-6-methyl-6,7-dihydro-5H-benzo [1,3]oxathiol-4-one (BOT-4-one), a small molecule inhibitor of JAK/STAT3 signaling, induces apoptosis through inhibition of STAT3 activation. BOT-4-one suppressed cytokine (upd)-induced tyrosine phosphorylation and transcriptional activity of STAT92E, the sole *Drosophila* STAT homolog. Consequently, BOT-4-one significantly inhibited STAT3 tyrosine phosphorylation and expression of STAT3 downstream target gene SOCS3 in various human cancer cell lines, and its effect was more potent in JAK3-activated Hodgkin's lymphoma cell line than in JAK2-activated breast cancer and prostate cancer cell lines. In addition, BOT-4-one-treated Hodgkin's lymphoma cells showed decreased cell survival and proliferation by inducing apoptosis through down-regulation of STAT3 downstream target anti-apoptotic gene expression. These results suggest that BOT-4-one is a novel small molecule inhibitor of JAK3/STAT3 signaling and may have therapeutic potential in the treatment of human cancers harboring aberrant JAK3/STAT3 signaling, specifically Hodgkin's lymphoma.

Keywords: BOT-4-one; cancer; JAK; small molecule inhibitor, apoptosis; STAT3

Introduction

The JAK/STAT signaling cascade was originally characterized during interferon-mediated signal transduction studies in early 1990s (Schindler *et al.*, 1992; Shuai *et al.*, 1992; Müller *et al.*, 1993; Watling *et al.*, 1993). JAKs belong to a family of non-receptor tyrosine kinases and STATs are latent cytosolic transcription factors that activate signals from the cell membrane to the nucleus. The JAK and STAT protein families are composed of four and seven members in mammals, respectively and JAK/STAT pathways are up-regulated by more than fifty different cytokines and growth factors (Schindler and Plumlee, 2008). The binding of cytokines and growth factors to their corresponding transmembrane receptors subsequently activates membrane-associated JAK and STAT proteins by phosphorylation of specific tyrosine residues. STAT

proteins are a family of cytosolic transcription factors that have dual functions - they transduce signals through the cytoplasm and have a function as transcription factors in the nucleus (Takeda and Akira, 2000).

The JAK/STAT-mediated signaling cascade represents essential roles for proliferation or differentiation, development, hematopoiesis, and immune responses (Park *et al.*, 1995; Meraz *et al.*, 1996; Darnell, 1997; Neubauer *et al.*, 1998). However, recent studies showed that persistently activated JAK/STAT signaling correlates with tumorigenesis and cancer progression through its intimate connection to growth factor signaling and observed high frequency in human cancers. Numerous studies have shown that constitutively activated JAK kinases are found in a variety of cancer patients with lymphoblastic leukemia, myeloproliferative diseases, acute megakaryoblastic leukemia, and acute lymphoblastic leukemia (James *et al.*, 2005; Walters *et al.*, 2006; Bercovich *et al.*, 2008; Flex *et al.*, 2008; Mullighan *et al.*, 2009; Oh *et al.*, 2010). In addition, STAT3, in part STAT5 and STAT6, is also constitutively activated in multiple human cancers as well as in various hematopoietic malignancies (Klampfer, 2006; Yu *et al.*, 2009; Haftchenary *et al.*, 2011). Therefore, regulation of inappropriately activated JAK and/or STAT signaling is valuable therapeutic

targets for the treatment of human cancers. Several JAK/STAT inhibitors have been developed and are used on clinical trials for the cancer treatments (O'Shea *et al.*, 2004; Atallah and Verstovsek, 2009; Fletcher *et al.*, 2009; Haftchenary *et al.*, 2011).

Benzoxathiol derivatives, especially 6-hydroxy-1,3-benzoxathiol-2-one (called also tioxelone) have been used in the local therapy of psoriasis vulgaris and acne, and also reported to have anti-bacterial, anti-mycotic, and cytostatic properties (Goeth and Wildfeuer, 1969; Wildfeuer, 1970; Lius and Sennerfeldt, 1979). Recent report showed that benzoxathiol derivatives have anti-inflammatory and anti-tumorigenic effects through inhibition of NF- κ B and STAT3 activation (Kim *et al.*, 2008a, 2008c). We herein identified 2-cyclohexylimino-6-methyl-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (BOT-4-one) has a potent anti-cancer activity via inhibition of JAK/STAT3 signaling in both *Drosophila* and human cancer cells. BOT-4-one inhibited persistently activated cancer cell proliferation and survival through induction of apoptosis by down-regulation of anti-apoptotic gene expressions, which are known to STAT3 downstream target molecules. BOT-4-one predominantly induced cell death in Hodgkin's lymphoma L540 cells that are aberrantly activated JAK3/STAT3 signaling.

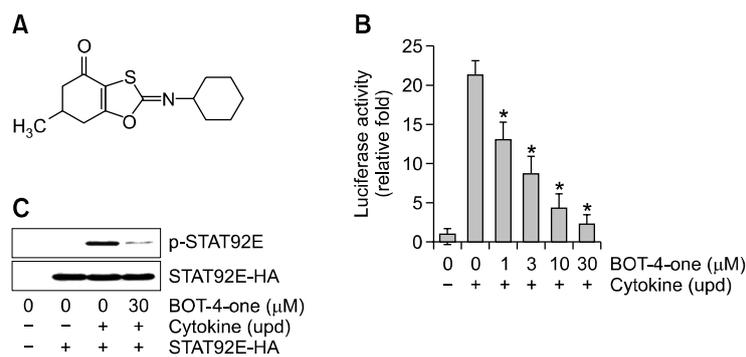


Figure 1. BOT-4-one inhibits phosphorylation and transcription of STAT92E in *Drosophila* cells. (A) The chemical structure of BOT-4-one ($C_{14}H_{19}NO_2S$; M.W., 265.4). (B) BOT-4-one inhibited cytokine (upd)-induced tyrosine phosphorylation of STAT92E. S2-NP cells transiently transfected with an expression plasmid for STAT92E-HA were co-cultured with upd-producing cells for 24 h in the presence of either vehicle (DMSO) alone or BOT-4-one. Immunoblot analysis was performed with phospho-STAT92E and HA antibodies. STAT92E-HA served as a loading control. (C) BOT-4-one inhibited STAT92E transcriptional activity. Cultured *Drosophila* S2-NP-STAT92E cells expressing a STAT92E reporter gene were co-cultured with upd-producing cells for 24 h in the presence of BOT-4-one. The STAT92E luciferase activity was measured and the firefly luciferase activity was normalized to *Renilla* luciferase activity. Results are shown as the mean of three independent experiments (\pm SD indicated by error bar). * $P < 0.001$, significant difference when the value of treatment was compared to that of the control.

Results

BOT-4-one inhibits STAT92E activation in *Drosophila* cells

Drosophila cells have one JAK and one STAT protein called Hop and STAT92E compared with those of mammalian cells, respectively (Hou and Perrimon, 1997). To identify small molecules that are potential inhibitors of JAK/STAT signaling, we performed a cell-based high throughput screening using *Drosophila* cell line as previously described (Kim *et al.*, 2008b, 2010a) and identified 2-cyclohexylimino-6-methyl-6,7-dihydro-5H-benzo[1,3]oxa thiol-4-one (BOT-4-one; Figure 1A) as a potential inhibitor of STAT92E signaling. Cytokine (upd)-induced STAT92E transcriptional activity was increased more than 21-fold compared to that of vehicle treatment and BOT-4-one was found to inhibit STAT92E transcriptional activity in a dose-dependent manner (Figure 1B). Cytokine-induced phosphorylation of tyrosine residues is a key step in STAT activation. To determine whether BOT-4-one could affect tyrosine phosphorylation, we examined tyrosine phosphorylation levels of STAT92E followed cytokine treatment. Treatment with 30 μ M BOT-4-one almost completely suppressed STAT92E phosphorylation (Figure 1C). These results indicate that BOT-4-one is a small molecule inhibitor of STAT92E signaling in *Drosophila* cells.

BOT-4-one inhibits STAT3 activation in human cancer cell lines

We next examined the effect of BOT-4-one on the expression levels of STATs in various human cancer cell lines. Treatment with 30 μ M BOT-4-one showed reduction of STAT3 expression in all cancer cell lines, and the effect was much stronger in L540 cells compared to MDA-MB-468 and DU145 cells (Figure 2A). STAT1 and STAT5 expression levels were also decreased in L540 cells by BOT-4-one, however, their expressions were not affected in MDA-MB-468 and DU145 cells. We therefore examined the dose effect of BOT-4-one on STATs phosphorylation in L540 cells. BOT-4-one significantly decreased STAT3 and STAT5 phosphorylation compared to that of STAT1 (Figure 2B), indicating that BOT-4-one selectively inhibits STAT3 and STAT5 phosphorylation in L540 cells. Phosphorylated STAT proteins on tyrosine residues undergo dimerization and translocation to the nucleus, where they initiate transcription and translocated STAT3 proteins to the nucleus undergoes dephosphorylation and exports to the cytosolic region through the nuclear pore complex by nucleocytoplasmic shuttling (Herrmann *et al.*, 2007). We next examined whether BOT-4-one could reduce tyrosine-phosphorylation status of STAT3. BOT-4-one inhibits phosphorylation of STAT3 in the cytosolic and nuclear regions, but its expression is not altered in the both regions (Figure 2C). In L540 cells, JAK3 is

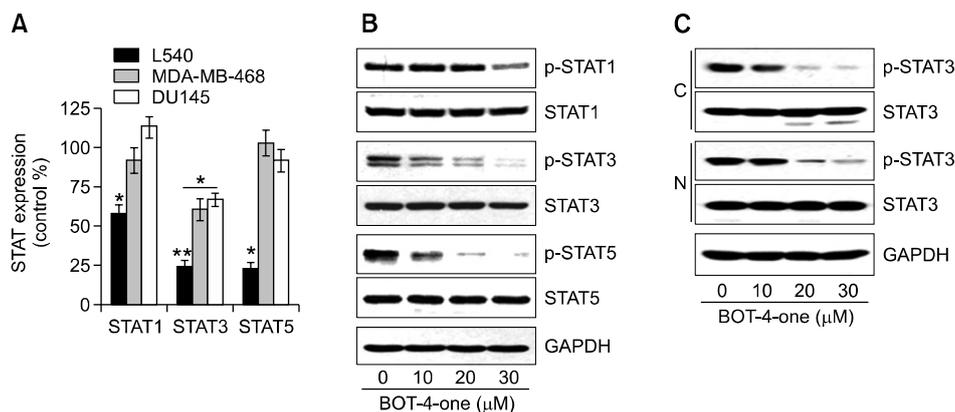


Figure 2. BOT-4-one inhibits expression and phosphorylation of STAT3 in human cancer cell lines. (A) Human cancer cell lines that express constitutively-active STAT3 were incubated with either vehicle (DMSO) alone or BOT-4-one (30 μ M) for 24 h. Total RNA was extracted and subjected to quantitative real-time PCR. BOT-4-one inhibited STAT3 expression in all cell lines, but inhibition of STAT1 and STAT5 expression was cell type-dependent manner. Results are shown as the mean of three independent experiments (\pm SD indicated by error bar). * $P < 0.001$, significant difference when the value of treatment was compared to that of the control. (B) Whole cell extracts were prepared from L540 cells after treatment for 24 h with either vehicle (DMSO) alone or BOT-4-one, and immunoblot analysis was performed with antibodies specific for the molecules indicated. BOT-4-one inhibited tyrosine phosphorylation of STATs, and its inhibitory effect on STAT3 and STAT5 was much higher than those of STAT1. GAPDH served as a loading control. (C) Cytosolic and nuclear fractions were extracted from L540 cells after treatment for 24 h with either vehicle (DMSO) alone or BOT-4-one, and immunoblot analysis was performed with phospho-STAT3 and STAT3 antibodies. BOT-4-one inhibited STAT3 activation. C, cytosolic, N, nucleus.

constitutively activated and JAK family kinases are upstream regulator of STATs activation. These results therefore suggest that BOT-4-one inhibits STAT3 activation, but not STAT3 expression and BOT-4-one may suppose the inhibition of JAK3 activity in L540 cells.

BOT-4-one predominantly inhibits JAK3/STAT3 signaling

L540 cells are persistently activated JAK3/STAT3 pathway, whereas MDA-MB-468 and DU145 cells are persistently activated JAK1/STAT3 and JAK2/STAT3 pathways (Kim *et al.*, 2010b). BOT-4-one strongly decreased STAT3 phosphorylation, in part STAT5, in L540 cells than in MDA-MB-468 and DU145 cells. In order to identify the effect of BOT-4-one on specificity of JAK3, we examined the effect of BOT-4-one on phosphorylation of JAK2, JAK3 and Src family kinases as well as ERK signaling. Reduction of JAK3/STAT3 activation by BOT-4-one in L540 cells was stronger than that of JAK2/STAT3-activated MDA-MB-468 and DU145 cells (Figures 3A-C). In addition, expression of the STAT3 target protein SOCS3 also inhibited and the effect was parallel as JAK/STAT3 inhibition in the cells. However, phosphorylation of Src family tyrosine kinases such as Lyn and Src was weakly affected upon 30 μ M BOT-4-one in all cell lines and ERK phosphorylation was inhibited only in MDA-

MB-468 and DU145 cells, but not in L540 cells. These results suggest that BOT-4-one inhibits STAT3 activation through a little different pathways in various cancer cell lines.

BOT-4-one inhibits cancer cell survival

A number of studies reported that inhibition of STAT3 signaling reduce cancer cell survival (Al Zaid Siddiquee and Turkson, 2008). We next examined whether BOT-4-one reduces cancer cell survival by down-regulation of STAT3 activation. For the assay, L540 or DG-75 cells were treated with either vehicle alone or various concentrations of BOT-4-one. We found that viability and proliferation of L540 cells were significantly decreased by BOT-4-one in a dose- and time-dependent manner (Figures 4A and C). However, viability and proliferation of DG-75 cells were not affected by BOT-4-one, where STAT3 pathway was not activated (Kim *et al.*, 2008b). IL-6 activates JAK/STAT signaling pathway by binding with IL-6R/gp130, and increases cancer cell survival and proliferation. To know that BOT-4-one could affect exogenous cytokine-induced cancer cell survival, we cultured L540 cells with IL-6 and measured cell viability. BOT-4-one inhibited IL-6 induced cancer cell survival in a dose-dependent manner and the effect was a little weaker than without IL-6 treatment (Supplementary Figure S1). Together, these results suggest that

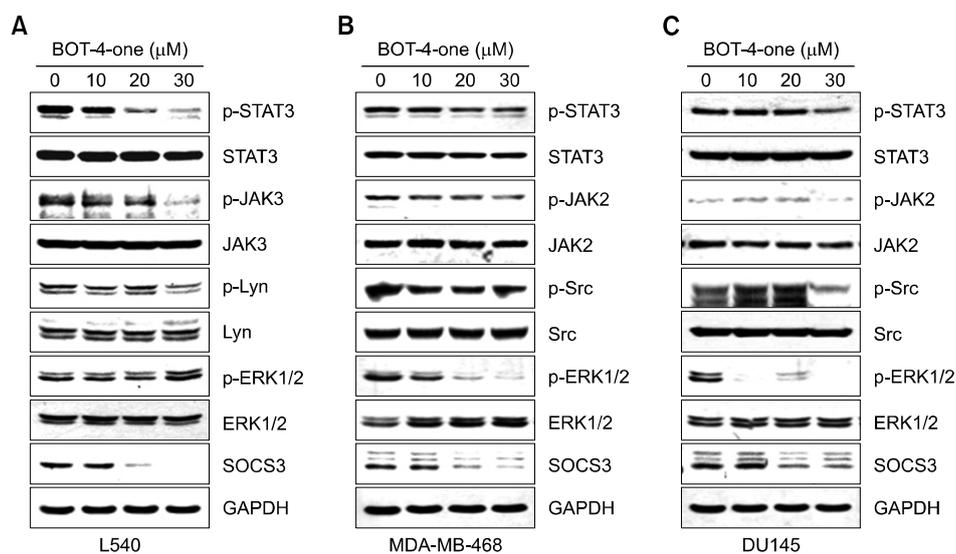


Figure 3. BOT-4-one inhibits JAK/STAT3 signaling. L540 (A), MDA-MB-468 (B), and DU145 (C) cells were incubated with either vehicle (DMSO) alone or BOT-4-one for 24 h. Whole cell extracts were proceeded for immunoblot analysis using antibodies specific for the molecules indicated. BOT-4-one inhibited constitutively-active STAT3 in all cell lines. Interestingly, BOT-4-one predominantly inhibited JAK3-mediated STAT3 phosphorylation and SOCS3 expression known to STAT3 downstream target molecule than those of JAK2-mediated. However, BOT-4-one inhibited ERK1/2 phosphorylation in a cell type-dependent manner, and has weak effect on Src family kinases such as Src and Lyn in all cell lines. GAPDH served as a loading control.

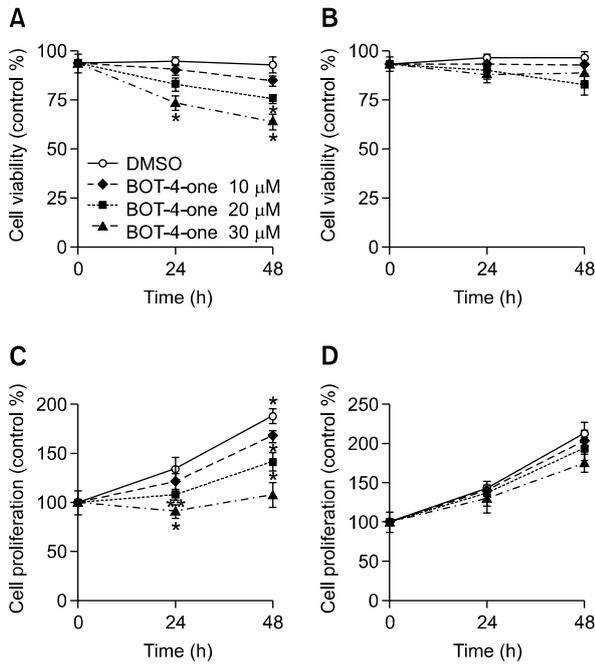


Figure 4. BOT-4-one affects cancer cell survival and proliferation. L540 (A and C) and DG-75 (B and D) cells were treated with either vehicle (DMSO) alone or BOT-4-one and incubated for the indicated time periods. Total and viable cell numbers were counted by trypan blue exclusion assay. The cell viability and proliferation of L540 cells were decreased by BOT-4-one in a dose- and time-dependent manner, but DG-75 cells were not affected, where JAK/STAT3 signaling was not activated. Results are shown as the mean of three independent experiments (\pm SD indicated by error bar). * $P < 0.001$; ** $P < 0.05$, significant difference when the value of treatment was compared to that of the control.

BOT-4-one affects cancer cell survival by down-regulation of JAK/STAT3 signaling.

BOT-4-one induces apoptosis through down-regulation of anti-apoptotic gene expression

To identify whether the inhibition of cell survival in BOT-4-one-treated L540 cells resulted in induction of apoptosis, we performed a TUNEL assay. Treatment with BOT-4-one increased TUNEL-positive cells about 6-fold compared to that of control cells (Figure 5A). Cleavage of Poly (ADP-ribose) polymerase (PARP) and caspase-3 are known to hallmarks of apoptosis (Decker *et al.*, 2000). BOT-4-one increased cleaved fragments of both PARP and caspase-3 in a dose-dependent manner (Figure 5B). The inhibition of STAT3 signaling also reported to induce apoptosis by down-regulation of anti-apoptotic gene expression (Iwamaru *et al.*, 2007; Al Zaid Siddiquee and Turkson, 2008). To elucidate the molecular mechanism of BOT-4-one on apoptosis, we examined the expression levels of anti-apoptotic proteins known to STAT3 targets. BOT-

4-one decreased the expression levels of anti-apoptotic mRNA and proteins such as Bcl-2, Bcl-xL, Mcl-1, and survivin in a dose-dependent manner (Figures 5C-E). These results indicate that BOT-4-one decreases cancer cell survival by inducing apoptosis through down-regulation of anti-apoptotic genes.

Discussion

Although benzoxathiol derivatives have been used in the treatment of psoriasis and acne, and reported to have anti-bacterial and cytostatic properties (Goeth and Wildfeuer, 1969; Wildfeuer, 1970; Lius and Sennerfeldt, 1979), the molecular basis of the pharmacological properties has not been defined yet. Psoriasis and acne are common inflammatory skin diseases that involved in immune responses. Recent reports showed that an anti-inflammatory effect of benzoxathiol derivatives was due to inhibition of NF- κ B activation by targeting IKK as well as inhibition of STAT1 phosphorylation (Kim *et al.*, 2008a, 2008d; Chung *et al.*, 2009). NF- κ B is one of the transcription factors implicated in inflammatory diseases. Therefore, blocking of NF- κ B activation by benzoxathiol derivatives for the treatment of psoriasis was expected. JAK/STAT3 signaling is also activated in psoriasis and inhibition of this signaling may have therapeutic target for the treatment of the diseases (Chang *et al.*, 2009; Miyoshi *et al.*, 2011).

Persistent activation of JAK/STAT signaling, especially JAK/STAT3, is observed in various types of human cancers and contributes to tumorigenesis and cancer progression. The activation of STAT3 proteins in cancers is implicated to phosphorylation of JAK and Src family kinases (Niu *et al.*, 2002; Klampfer, 2006; Yu *et al.*, 2009; Hazan-Halevy *et al.*, 2010). Accumulated results imply that development of new drugs to regulation of constitutively activated JAK/STAT3 is valuable therapeutic targets for cancer treatment. We found small molecule BOT-4-one as a potential inhibitor of JAK/STAT signaling using a cell-based high throughput screening in *Drosophila* cell line (Figure 1). The fruit fly *Drosophila* consists of only one JAK and one STAT (Hou and Perrimon, 1997). Despite the simplicity of the *Drosophila* JAK/STAT pathway, the mode of action of the JAK/STAT pathway in *Drosophila* is similar to that of mammals (Bach *et al.*, 2003). Therefore, *Drosophila* to identify small molecule inhibitors of JAK/STAT signaling can serve as an excellent model organism (Arbouzova and Zeidler, 2006). BOT-4-one effectively inhibited cytokine-induced transcriptional activity and phos-

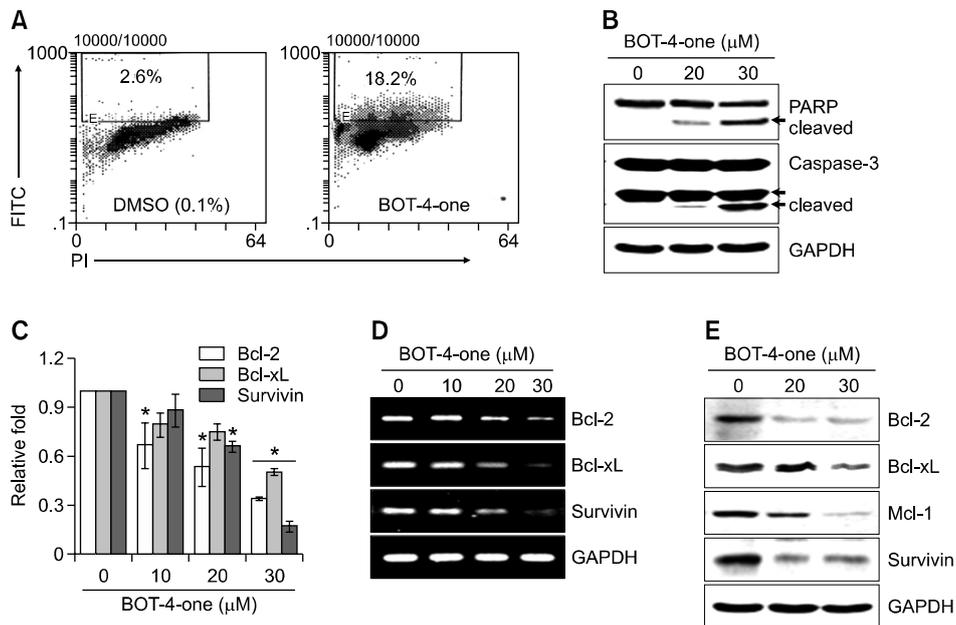


Figure 5. BOT-4-one induces apoptosis by down-regulation of anti-apoptotic gene expression. (A) L540 cells treated with either vehicle (DMSO) alone or BOT-4-one (30 μ M) for 48 h were harvested, stained with fluorescein isothiocyanate-conjugated BrdU antibody and propidium iodide (PI). The cells were subsequently subjected to flow cytometry. A representative flow cytometric analysis was shown and the percentages of TUNEL-positive cells were indicated. (B and E) L540 cells were treated with either vehicle (DMSO) alone or BOT-4-one for 48 h, whole cell extracts were prepared and immunoblot analysis was performed with antibodies specific for the molecules indicated. (C and D) Total RNA was extracted from L540 cells treated with BOT-4-one for 8 h, and real-time PCR was performed. Anti-apoptotic gene expression was represented with relative fold (C) or stained with EtBr after electrophoresis (D). Treatment with BOT-4-one increased cleaved fragments of PARP and caspase-3, an apoptotic hallmarks (arrows in B), and decreased the expression of anti-apoptotic genes such as Bcl-2, Bcl-xL, Mcl-1, and survivin (C-E). GAPDH served as a loading control.

phorylation of STAT92E in *Drosophila* cells (Figures 1B and C).

Our previous results showed that identified small molecule inhibitors of STAT92E activity using *Drosophila* model were well-fitted in human cell lines (Kim *et al.*, 2008b, 2010a). In fact, the benzoxathiol derivatives were synthesized for the development of anti-cancer drugs by targeting NF- κ B signaling pathway, and BOT-4-one has anti-cancer and anti-inflammatory effects by inhibition of the pathways (not published yet). BOT-4-one decreased mRNA expression level of STAT3 in different types of human cancer cell lines, but the effect was stronger in Hodgkin's lymphoma cell line L540 compare to breast cancer cell line MDA-MB-468 and prostate cancer cell line DU145 (Figure 2A). In addition, the compound strongly inhibited mRNA expression and phosphorylation of STAT3 and STAT5 rather than those of STAT1 in L540 cells (Figure 2). However, mRNA expression levels of STAT1 and STAT5 in MDA-MB-468 and DU145 cells were not affected by BOT-4-one (Figure 2A). These results reveal that BOT-4-one has differential effect on the inhibition of STAT activation. As

evidence for this hypothesis, BOT-4-one showed differential inhibition against JAK2 and JAK3 phosphorylation, and the effect was parallel compared to inhibition of STAT3 phosphorylation and STAT3 target protein SOCS3 expression. Non-receptor Src family kinases and ERK pathway can also regulate STAT3 phosphorylation (Garcia *et al.*, 2001; Steelman *et al.*, 2004). BOT-4-one strongly inhibited ERK1/2 phosphorylation in MDA-MB-468 and DU145 cells, but not in L540 cells. However, BOT-4-one showed weak effect on the activation of Src family kinases (Figure 3). We previously showed that JAK3 is important for STAT3-mediated signaling in L540 cells and JAK1 and JAK2 are important in MDA-MB-468 and DU145 cells (Kim *et al.*, 2008b, 2010a, 2010b). Together, our results suggest that BOT-4-one has more selectivity for the regulation of JAK3/STAT3 signaling in L540 cells than that of JAK1/STAT3 and JAK2/STAT3 signaling in MDA-MB-468 and DU145 cells. This conclusion was further supported by reducing cell survival and inducing apoptosis through down-regulation of the expression of anti-apoptotic genes such as Bcl-2, Bcl-xL, Mcl-1, and survivin that are known to

STAT3 downstream targets (Figures 4 and 5).

In summary, we identified a small molecule inhibitor of JAK/STAT signaling, especially JAK3/STAT3 signaling using *Drosophila* and human cancer cell lines. Inhibition of JAK3/STAT3 signaling by BOT-4-one decreased cancer cell survival, and induced apoptosis by down-regulation of anti-apoptotic gene expression in L540 cells. Therefore, BOT-4-one can be used as a lead compound to develop new group of anti-cancer drugs to target cancer cells harboring aberrant JAK3/STAT3 signaling.

Methods

Drosophila cell line, transfection and reporter assay

Maintenance of parental macrophage-like *Drosophila* Schneider (S2-NP) cells and reporter assay were conducted as previously described (Kim *et al.*, 2008b, 2010a). Briefly, cells were cultured in Schneider's *Drosophila* medium containing 10% FBS and antibiotics (Invitrogen, Calsbad, CA) in an incubator at 25°C. S2-NP-STAT92E cells that stably express both the 10×STAT92E-firefly luciferase reporter gene and the PolIII-*Renilla* luciferase gene were also grown in the same medium supplemented with 500 µg/ml G418. For experiment STAT92E transcriptional activity, parental S2-NP cells were transiently transfected with Actin promoter-driven upd using Effectene Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol and the cells were co-cultured with S2-NP-STAT92E cells for 24 h in the presence of BOT-4-one at various concentrations. The reporter activity was quantified by measuring relative luciferase units (RLU) and the firefly luciferase activity was normalized to *Renilla* luciferase activity.

Human cancer cell lines

The Hodgkin's lymphoma cell line L540 and the Burkitt's lymphoma cell line DG-75 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and cultured in RPMI 1640 supplemented with 20% FBS and antibiotics. The breast cancer cell line MDA-MB-468 and the prostate cancer cell line DU145 were purchased from the American Type Culture Collection (Manassas, VA), and cultured in DMEM supplemented with 10% FBS and antibiotics. Cells were cultured in a 37°C humidified incubator containing a mixture of 95% air and 5% CO₂. DMEM, RPMI 1640, fetal bovine serum (FBS), and antibiotics (penicillin/streptomycin) were obtained from Invitrogen (Carlsbad, CA).

Reagents and antibodies

All reagents used in this experiment were obtained from Sigma-Aldrich (Saint Louis, MO), unless otherwise specified. Antibodies specific for phospho-STAT92E, phospho-STAT1 (Tyr701), phospho-STAT3 (Tyr705), phospho-STAT5 (Tyr694), phospho-JAK3 (Tyr980/981), phospho-JAK2 (Tyr1007/

1008), JAK2, phospho-Src (Tyr416), Src, phospho-Lyn (Tyr507), phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, PARP, caspase-3, Bcl-2, Bcl-xL, Mcl-1, survivin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Danvers, MA) and used at a dilution of 1:1000-1:2500. Antibodies specific for STAT1, STAT3, STAT5, JAK3, Lyn and SOCS3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a dilution of 1:500-1:2000. Mouse anti-HA antibody was purchased from Roche Applied Science (Indianapolis, IN). Secondary antibodies against from goat anti-rabbit and anti-mouse IgG horseradish peroxidase conjugate were purchased from Invitrogen (Calsbad, CA).

Immunoblot analysis

To analyze the effect of BOT-4-one on upd-induced STAT92E phosphorylation, S2-NP cells were transiently transfected with an expression plasmid for HA-tagged STAT92E and the cells were co-cultured with upd-producing cells in the presence of BOT-4-one (30 µM) for 24 h. Cell pellets were lysed with a lysis buffer (50 mM Tris-HCl, pH 7.4, 350 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 10% glycerol, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 1 mM phenylmethylsulphonyl fluoride (PMSF) and phosphatase inhibitor cocktails) on ice. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane. Immunoblot analysis was performed with the phospho-STAT92E (1:1000 dilution) or HA antibody (1:1000 dilution). Human cancer cell pellets were lysed with a lysis buffer and membranes were prepared as described above. Immunoblot analysis was performed with appropriate primary antibody (1:1000 or 1:2500 dilution), horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution), and an Enhanced Chemiluminescence Reagent System (iNtRON Biotechnology, Korea).

Cell viability, proliferation and FACS analysis

L540 cells (5×10^4 cells/ml) were treated with either vehicle (DMSO) alone or various concentrations of BOT-4-one in the presence or absence of IL-6 and incubated for the indicated time periods. Trypan blue exclusion assay was performed to count total and viable cells. Apoptosis assay was conducted using Terminal Transferase dUTP Nick End Labeling (TUNEL) assay system as previously described (Kim *et al.*, 2008b, 2010a). Briefly, L540 cells (1.0×10^6 cells/ml) were treated with either vehicle (DMSO) alone or BOT-4-one (30 µM) for 48 h. Cells were harvested, stained using an APO-BRDU kit (Phoenix Flow Systems, Inc., San Diego, CA), and subsequently subjected to Elite ESP flow cytometry (Coulter Inc., Miami, FL).

RNA isolation and quantitative real-time PCR

Total RNA was isolated from human cancer cell lines treated with either vehicle (DMSO) alone or BOT-4-one for 24 or 48 h. For real-time PCR analysis, cDNA was synthesized from 1 µg of total RNA by reverse transcription using QuantiTect Reverse Transcription Kit (Qiagen) and performed real time-PCR using the KAPA SYBR fast qPCR Kit (KAPA biosystems, Woburn, MA). Primers were purchased

from Qiagen.

Statistical analysis

Data obtained from independent experiments are represented as means \pm SD. Statistical analysis was performed using a two-tailed Student's *t* test. *P* values were considered to be statistically significant at **P* < 0.001 or ***P* < 0.05.

Supplemental data

Supplemental data include a figure and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-43-5-07.pdf.

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