Purvalanol A induces apoptosis and downregulation of antiapoptotic proteins through abrogation of phosphorylation of JAK2/STAT3 and RNA polymerase II

Daisuke Iizuka, Aki Ogura, Mikinori Kuwabara and Osamu Inanami

To clarify the mechanisms of purvalanol A in the induction of apoptosis, we investigated whether purvalanol A influenced the RNA synthesis and expression of RNA polymerase II and signal transducer and activator of transcription 3 (STAT3). When MKN45 cells were treated with 30 μmol/l purvalanol A, mitochondrial dysfunction occurred before the induction of the apoptosis and the expression of antiapoptotic proteins survivin, Bcl-X_L, and Bcl-2 was reduced. The treatment with parvalanol A was also shown to reduce not only mRNA for these proteins but also global RNA synthesis. The phosphorylation of the carboxy-terminal domain of RNA polymerase II, which was involved in transcriptional regulation, was strongly inhibited by purvalanol A, followed by the partial inhibition of the expression of RNA polymerase II. Furthermore, the phosphorylation at Tyr705 of STAT3, which is known to be a phosphorylation site for Janus kinase 2 (JAK2), was completely inhibited by purvalanol A early (3 h) after drug treatment, although the phosphorylation of STAT3 at Ser727, which is a phosphorylation site for Ras/Raf/MEK and extracellular signal-regulated protein kinase 1/2, was still detectable until late (12 h) after treatment. In addition, the tyrosine phosphorylation of JAK2 was efficiently inhibited by purvalanol A. These results suggest that the inhibition of JAK2/STAT3 and RNA polymerase II is crucial in the downregulation of antiapoptotic proteins leading to the apoptotic cell death induced by parvalanol A.
Anti-Cancer Drugs **19:565–572** © **2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2008, **19**:565–572

Keywords: carboxy-terminal domain, Janus kinase 2, purvalanol A, RNA polymerase II, signal transducer and activator of transcription 3

Department of Environmental Veterinary Sciences, Laboratory of Radiation Biology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

Correspondence to Osamu Inanami, Laboratory of Radiation Biology, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, 060-0818, Japan
Tel: +81 11 706 5235; fax: +81 11 706 7373;
e-mail: inanami@vetmed.hokudai.ac.jp

Received 3 November 2007 **Revised form accepted** 22 February 2008

Introduction

Recently, various cyclin-dependent kinase (Cdk) inhibitors have been developed and some have been tested under preclinical trials. Purvalanol A was recently developed as a protein kinase inhibitor for cell division cycle 2 (Cdc2) (Cdk1) through competitive inhibition of ATP binding. It inhibits both G_1 and G_2 phase progression and the phosphorylation of Rb, resulting in the lasting inhibition of cell proliferation and cell death in both mouse fibroblasts and human cancer cell lines [1]. Purvalanol A also enhances taxol-induced and radiation-induced cell death through the inhibition of Cdc2 kinase activity [2,3]. In our earlier study, we found that treatment with purvalanol A inhibited not only Cdc2 kinase activity but also expression of the antiapoptotic proteins survivin, XIAP, Bcl-2, and Bcl-X_L. These facts led us to speculate that purvalanol A could inhibit not only Cdc2 kinase activity but also unknown kinase-dependent protein synthesis or RNA synthesis, causing degradation of these antiapoptotic proteins, because this compound was designed as a purine analogue to bind with ATP-binding sites of the other kinases.

It is well known that some transcription factors regulate the expression of antiapoptotic proteins through their phosphorylation. Signal transducer and activator of transcription 3 (STAT3) is known to be constitutively activated in various human tumors [4] and to play an important role in the expression of various proteins related to oncogenic potential [5] and antiapoptotic functions [6]. Aberrant STAT3 activation in tumors is reported to promote tumor growth and survival in several human cancers. STAT3 is phosphorylated at a single tyrosine residue (Tyr705). This process is initiated by cytokines, hormones, and growth factors, which interact with the extracellular domains of specific transmembrane receptors. This binding triggers receptor dimerization and association with protein tyrosine kinases, for example, Janus kinase (JAK)1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2). Alternatively, growth factor receptors (e.g. epidermal growth factor receptor) have an intrinsic tyrosine kinase domain and ligand-induced receptor stimulation results in kinase activation. After phosphorylation at the Tyr residue, STAT3 forms a homodimer, then translocates from the cytoplasm to the nucleus. Transcriptional activation seems to be regulated by phosphorylation at serine residue 727 (Ser727), which is induced by Ras/Raf/mitogen-activated protein kinase–ERK kinase (MEK) or extracellular signal-regulated protein kinase (ERK) 1/2 [7,8]. The activated form of

0959-4973

STAT3 plays a critical role as a transcription factor that initiates increases in the transcription of a number of genes, including the apoptotic regulatory genes Bcl-X_L, survivin, and MCL-1 [9]. Thus, it seems that the STAT3-related signal transduction pathway is a candidate target for purvalanol A.

The purpose of this study was to demonstrate the mechanism of downregulation of antiapoptotic proteins by purvalanol A. We, therefore, investigated the inhibition of RNA synthesis and suppression of the regulatory system in STAT3 by purvalanol A. Furthermore, we examined the effect of parvalanol A on the phosphorylation status and expression of RNA polymerase II, which plays a central role in transcriptional regulation.

Materials and methods

Chemicals

RPMI 1640 was purchased from Invitrogen (Carlsbad, California, USA). Purvalanol A was obtained from CALBIOCHEM (San Diego, California, USA) and dissolved in dimethylsulfoxide at 4 mmol/l. Propidium iodide (PI) was purchased from Sigma Chemical Company (St Louis, Missouri, USA). Anti-RNA polymerase II (A-10, sc-17798), anti-JAK2 (HR-758, sc-278), antiphosphotyrosine (PY20, sc-508), antiactin (I-19, sc-1616), donkey antigoat IgG-HRP (sc-2020), goat antimouse IgG-HRP (sc-2005), goat antirabbit IgG-HRP (sc-2004), and normal rabbit IgG (sc-2027) were from Santa Cruz Biotechnology (Santa Cruz, California, USA). Anti-Bcl-X_L (010-16851) and anti-Bcl-2 (013-16101) were from Wako (Osaka, Japan). Antisurvivin (2808), anti-p53 (9282), anti-Rbp1 carboxy-terminal domain (CTD) (Ser2/5) (4735S), anti-STAT3 (9132), anti-phospho-STAT3 (Tyr705) (9131S), anti-phospho-STAT3 (Ser727) (9134S), and anti-phospho-JAK2 (Tyr1007/1008) (3771) were from Cell Signaling Technology (Beverly, Massachusetts, USA). The chemiluminescence detection kit, Western Lightning Chemiluminescence Reagent Plus, was from Perkin Elmer (Boston, Massachusetts, USA).

Cell culture

Human gastric adenocarcinoma MKN45 cells (wild-type p53) were grown in RPMI 1640 containing 10% fetal calf serum at 37°C in 5% CO_2–air. Cells were cultured for 24 h before starting experiments. All samples except for those treated with purvalanol A were treated with dimethylsulfoxide only.

Fluorescence microscopic observation of apoptotic cells

Cells incubated for the indicated periods of time after treatment with and without purvalanol A were collected by centrifugation at 1000 rpm for 5 min at 4°C. The pellet was washed in phosphate-buffered saline [PBS(−)] and fixed with 70% ethanol. For fluorescence microscopy, the fixed cells were washed and resuspended in PBS(−). An aliquot containing 2×10^5 cells was stained with 40 μg/ml PI in 20 μl of PBS(−) for 15 min in the dark. Fluorescence microscopic observation was performed using an Olympus BX50 microscope (Olympus Optical Co., Tokyo, Japan) with reflected-light fluorescence. Apoptosis was defined by apoptotic morphological changes such as nuclear fragmentation and condensation. The apoptotic fraction was calculated as the percentage of apoptotic cells against total cells microscopically observed. Three independent experiments were performed.

Detection of apoptotic cells by annexin V-fluorescein isothiocyanate

After cells were treated with and without purvalanol A, they were collected by centrifugation at 1000 rpm for 5 min at 4°C. The pellet was washed with PBS(−) and stained with binding buffer [10 mmol/l HEPES (pH 7.4), 140 mmol/l NaCl, and 2.5 mmol/l $CaCl_2$] containing a fluorescein isothiocyanate conjugate of annexin V for 10 min in the dark at 4°C. Then 10 000 cells were analyzed using an EPICS XL flow cytometer (Beckman Coulter, Fullerton, California, USA). Three independent experiments were performed, combined to generate a mean value and a representative profile was obtained.

Mitochondrial viability assay

The cell viability was determined using Dojindo Cell Counting kit including 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-1) (Dojindo, Kumamoto, Japan). First, 3×10^3 cells were seeded 24 h before starting experiments. Cells incubated for the indicated periods of time after treatment with and without purvalanol A were collected by trypsinization and centrifugation at 1000 rpm for 5 min at 4°C. The pellet was washed in PBS(−), resuspended with 100 μl of RPMI 1640 medium, and transferred into a 96-well microplate (Corning Incorporated, Corning, New York, USA). A 10 μl aliquot [0.3% WST-1/20 mmol/l HEPES (pH 7.4), 0.2 mmol/l 1-methoxy PMS] was added to each well. The cells were then incubated for 2 h at 37°C. The absorbance was read at 450 nm using a microplate reader (Model 680; Bio-Rad, Hercules, California, USA). The cytotoxicity induced by purvalanol A was calculated as the rate of viability of each sample compared with the untreated cells. Three independent experiments were performed and combined to generate a mean value.

Measurement of RNA synthesis

RNA synthesis was measured by the cellular incorporation of ^{3}H-uridine according to the method described by Wang *et al.* [10]. First, 2×10^5 cells were seeded into 6-well plates 24 h before starting experiments and incubated with medium with and without purvalanol A. After 1 h, the supernatant was discarded and all the samples were washed with PBS(−) and again incubated with medium containing 1 μCi/ml uridine [5-^{3}H] (Moravek Biochemicals, Inc., Brea, California, USA) with and

without purvalanol A. After incorporation of ^{3}H-uridine for 3 h, the reaction was stopped by adding 10% trichloroacetic acid and the mixture was incubated for 20 min at 4°C. The plates were washed with 70% ethanol and dried. The residues were dissolved in 1% SDS in 0.3 N NaOH at 60°C for 30 min. The solutions were then placed in 10 ml of a liquid scintillator (AQUASOL-2; Perkin Elmer, Boston, Massachusetts, USA), and the radioactivity of each sample was measured with a liquid scintillation counter (TRI-CARB 2100TR; Perkin Elmer). Three independent experiments were performed.

Semiquantitative reverse transcription-PCR

RNA isolation was performed with an RNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription from cellular RNA was performed with an RNA PCR kit (AMV) Ver.2.1. Cellular RNA (1 μg) as a template, 2.5 μmol/l random 9 mers, 0.25 units/μl AMV reverse transcriptase, 0.1 volume of 10X RNA PCR buffer, 1 mmol/l deoxynucleoside triphosphate mixture, 5 mmol/l $MgCl_2$, and 1 unit/μl RNase inhibitor were used for the first strand cDNA synthesis at 30°C for 10 min, 45°C for 30 min, and 99°C for 5 min. For all amplifications, 2 μl of a 1 : 5 cDNA dilution was used. The total 10 μl reaction mixture contained 0.1 U of Taq DNA polymerase (Promega, Madison, Wisconsin, USA), 0.2 mmol/l deoxynucleoside triphosphate mixture, 1X PCR buffer (containing 1.5 mmol/l $MgCl_2$), 1 μmol/l sense primer, and 1 μmol/l antisense primer. Cycling conditions were as follows for each primer set. Survivin and glyceraldehyde-3P-dehydrogenase (GAPDH): initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. Bcl-2: initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Bcl-X_L: 94°C for 2 min, followed by 26 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s. The primer pairs of survivin (forward primer, 5′-AGAACTGGCCCTTCTTG-3′, reverse primer, 5′-TTCCTCTATGGGGTCGTC-3′), Bcl-2 (forward primer, 5′-CCTGTGGATGACTGAGTACC-3′, reverse primer, 5′-GAGACAGCCAGGAGAAAT -3′), Bcl-XL (forward primer, 5′-GGCCTTTTTCTCCTTCGGTG-3′, reverse primer, 5′-CTCTCGGCTGCTGCATTGTT-3′), and GAPDH (forward primer, 5′-GAAGGTGAAGGTCG GAGTC-3′, reverse primer, 5′-GAAGATGGTGATGG GATTTC-3′) were used for amplification. PCR-amplified products were analyzed on 2% agarose gels after ethidium bromide staining. Two independent experiments were performed and a representative result was obtained.

Immunoprecipitation

After 1×10^7 cells had been treated with and without purvalanol A, they were collected by centrifugation at 1000 rpm for 5 min at 4°C. The pellet was washed with PBS(−) and was counted to ascertain the same number of cells. Then the cells were lysed in lysis buffer [1% Triton X-100, 20 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 10% glycerol, 5 mmol/l sodium fluoride, 2 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A] for 30 min at 4°C. The cell lysate was centrifuged at 13 000 rpm for 30 min at 4°C, and was added to protein G PLUS Agarose (Santa Cruz, sc-2002), preassociated with the anti-JAK2 antibody or normal rabbit IgG, and mixed gently overnight at 4°C. The immunoprecipitate was washed twice with lysis buffer, then mixed with 30 μl of Laemmli's sample buffer [62.5 mmol/l Tris–HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 1% SDS and 0.004% bromophenol blue], heated for 5 min at 95°C, and subjected to SDS–PAGE and western blotting as described in the following section.

SDS–PAGE and western blotting

Cells were collected at the indicated periods of time after X irradiation and 5×10^5 cells were suspended in 100 μl of lysis buffer [20 mmol/l HEPES (pH 7.4), 20 mmol/l ethylene glycol abis(β-aminoethyl ester)-N,N,N,N-tetra-acetic acid (EGTA), 50 mmol/l glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin], kept on ice for 30 min, and sonicated for 30 s at ice-cold temperature. After centrifugation at 12 000 rpm for 30 min at 4°C, a three-fold volume of Laemmli's sample buffer was added to the supernatant and it was boiled for 3 min. Proteins were separated by SDS–PAGE and transferred onto nitrocellulose membranes (Advantec Toyo, Tokyo, Japan). The membranes were probed with anti-RNA polymerase II, anti-STAT3, antisurvivin, anti-p53, anti-JAK2, or antiactin in Tris-buffered saline/Tween 20 (TBST) buffer (10 mmol/l Tris–HCl, 0.1 mol/l NaCl, 0.1% Tween-20, pH 7.4) containing 5% nonfat skim milk, anti-Rpb1 CTD, anti-phospho-STAT3 (Tyr705) antiphosphotyrosine, or anti-phospho-STAT3 (Ser727) in TBST buffer containing 5% bovine serum albumin and anti-Bcl-X_L or anti-Bcl-2 in TBST buffer containing 3% nonfat skim milk. These antibodies were detected by a method using HRP-conjugated antirabbit, antimouse, and antigoat IgG antibodies with Perkin Elmer Western Lightning, Chemiluminescence Reagent Plus. Two independent experiments were performed and a representative result was obtained.

Statistics

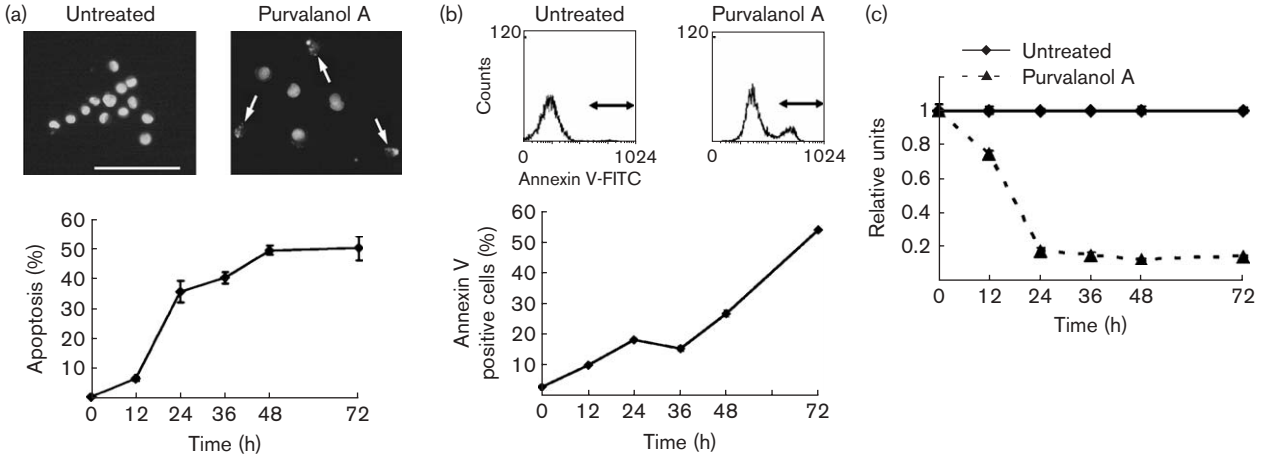
The results are given as means ± SE. The statistical significance of differences between the groups was tested with Student's *t*-test. $P < 0.05$ was accepted as statistically significant.

Results

Purvalanol A induces mitochondrial dysfunction before apoptosis

First, we examined the effects of purvalanol A on apoptotic cell death by morphological evaluation. Figure 1a shows fluorescence microscopic observation of apoptotic

Fig. 1



Purvalanol A-induced mitochondria dysfunction before apoptotic cell death. (a) (Upper panel) Fluorescence microscopy photographs of nuclei of untreated cells stained with propidium iodide, and of those treated with purvalanol A (30 μmol/l) at 48 h. White arrows indicate apoptotic morphological changes in nuclei. White bar represent picture scale (100 μm). (Lower panel) Induction of apoptosis (%) in cells treated with 30 μmol/l purvalanol A. In each case, at least 300 cells were evaluated for apoptosis. Data are expressed as means ± SE for three experiments. (b) Detection of apoptosis by flow cytometry using fluorescein isothiocyanate (FITC)-annexin V staining. The *x*-axis and *y*-axis indicate the fluorescence intensity of annexin V-FITC and the number of cells, respectively. Frequency of apoptotic induction indicated in upper panels by bars is shown as a graph. Data are expressed as means ± SE for three experiments. (c) The WST-1 assay was used to determine mitochondrial viability. Cells treated with and without purvalanol A (30 μmol/l) were incubated for 0, 12, 24, 36, 48, and 72 h. Data are expressed as means ± SE for three experiments.

morphological changes in nuclei stained with PI, including DNA fragmentation and chromatin condensation. When cells were treated with 30 μmol/l purvalanol A, typical morphological alterations characteristic of apoptosis were observed (white arrows in the second panel of Fig. 1a). Quantitative measurements of apopotic cells done with fluorescence microscopy revealed that apoptosis was induced in a time-dependent manner (Fig. 1a). To confirm the induction of apoptosis by parvalanol A, annexin V staining was used. Although the amount of apoptotic cell death detected by annexin V at 24, 36, and 48 h after the drug treatment was slightly lower than that detected by morphological changes, the annexin V-positive cells increased time dependently and the annexin V-positive population at 72 h reached 54% of total cells, similar to that detected by morphological changes (Fig. 1b). Next, to evaluate whether purvalanol A induced mitochondrial dysfunction, WST-1 assay was used (Fig. 1c). The data showed that mitochondrial dysfunction was induced in 80% of the cells within 24 h after purvalanol A treatment, although apoptotic cell death was detected in only 37% (detection by morphological changes) and 20% (detection by annexin V) of the cells at 24 h after the treatment. These facts suggested that purvalanol A induced mitochondrial dysfunction before the induction of apoptosis.

Purvalanol A inhibits the expression of antiapoptotic proteins

To examine whether parvalanol A-induced apoptosis was associated with downregulation of expression of antiapoptotic proteins, western blot analysis of apoptosis regulatory proteins was performed. As shown in Fig. 2, ;the treatment with purvalanol A gradually suppressed the expression of survivin, Bcl-X_L, Bcl-2, and Bax with different time courses. The expression of antiapoptotic proteins such as survivin, Bcl-X_L, and Bcl-2 was strongly suppressed from 24 to 36 h after the treatment with purvalanol A, whereas a significant expression of Bax, which is known as a proapoptotic protein, was still observed at 48 h after the treatment with purvalanol A.

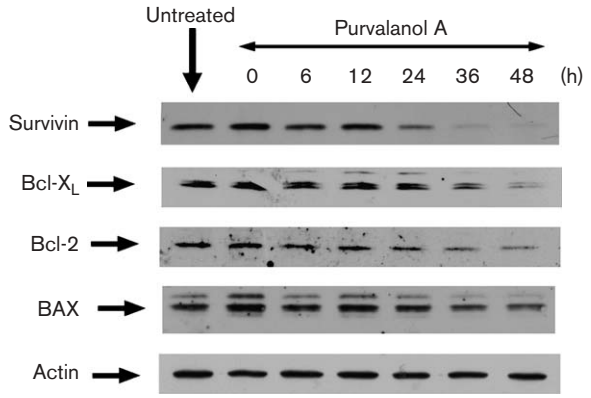
Purvalanol A inhibits RNA synthesis

As the Cdk inhibitor roscovitine, which is structurally similar to purvalanol A, has been reported to suppress RNA synthesis by the downregulation of RNA polymerase II through the inhibition of kinase activities of Cdk7 and Cdk9 [11], we investigated the inhibitory effect of purvalanol A on RNA synthesis. For this purpose, cells were pretreated with parvalanol A for 1 h and then incubated with ^{3}H-uridine for 3 h in the presence of purvalanol A. As shown in Fig. 3a, the treatment with purvalanol A significantly inhibited the incorporation of ^{3}H-uridine ($P < 0.01$, Student's *t*-test), indicating that it suppressed global RNA synthesis. Next, to test the effects of purvalanol A on the expression of mRNA of antiapoptotic proteins, semiquantitative RT-PCR was performed. Figure 3b shows the expression of mRNAs of survivin, Bcl-2, and Bcl-X_L. Purvalanol A inhibited their mRNA expression in a time-dependent manner.

Purvalanol A inhibits the expression of RNA polymerase II and the phosphorylation of signal transducer and activator of transcription 3

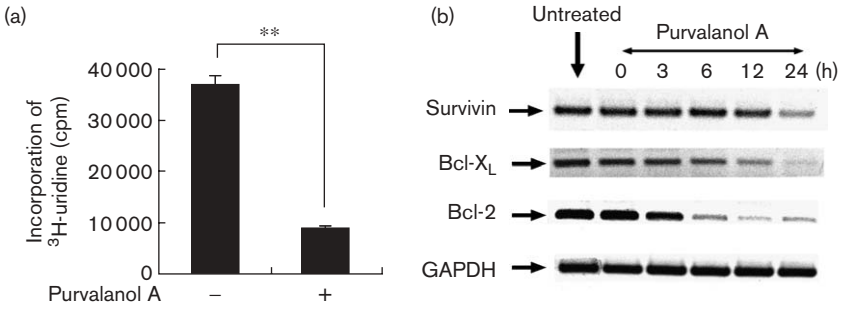
As the treatment with purvalanol A inhibited global RNA synthesis and downregulation of mRNA for specific antiapoptotic proteins as shown in Fig. 3, we explored the phosphorylation status and expression of polymerase II to regulate synthesis of mRNA and those of STAT3 to act as a transcription factor for several antiapoptotic proteins such as survivin and Bcl-X_L. Figure 4a and b show western blot analysis of the expression and phosphorylation status of RNA polymerase II and STAT3, respectively. The purvalanol A treatment slightly inhibited the expression of RNA polymerase II and completely diminished upper-shifted bands, indicating the phosphorylation of RNA polymerase II within 1 h after the drug treatment (Fig. 4a, asterisk). To confirm that this upper-shifted band was owing to the phosphorylated RNA polymerase II, an antibody-recognizing phosphorylation of Ser2 and Ser5 of the RNA polymerase II CTD region was used. The band owing to the phosphorylated RNA polymerase II was observed in the same position as the band indicated by the asterisk in the upper panel in Fig. 4a and was suddenly diminished within 1 h after the drug treatment. Figure 4b shows the effects of purvalanol A on the phosphorylation status and expression of STAT3. The phosphorylation of STAT3 at Tyr705 was inhibited by purvalanol A from 3 h after the cells were treated with it, but phosphorylation of STAT3 at Ser727 started to decrease from 12 h, though the expression of total STAT3 did not change. These results suggested that purvalanol A inhibited the activation of STAT3 through a decrease in STAT3 phosphorylated at Tyr705. As Tyr705 of STAT3 is known to be phosphorylated by active JAK2 (the phosphorylated form of JAK2), we investigated the phosphorylation status of JAK2. Fig. 4c (I) showed the western blotting patterns of immunoprecipitate with a specific antibody against JAK2 and a normal IgG. When the cell lysate was immunoprecipitated by a specific antibody against JAK2 but not normal IgG, a phosphorylated protein of 125 kDa, which corresponded to the size of JAK2, was observed as indicated by arrow heads in Fig. 4c. This result indicated that the active form (phosphorylated form) of JAK2 was constitutively present in our culture condition. In Fig. 4c (II), it is shown that the amount of phosphorylated JAK2 remarkably decreased at 5 and 10 h after purvalanol treatment. To obtain precise data for time course of phosphorylation status of JAK2 after treatment of purvalanol A, we used a phospho-JAK2 antibody to detect levels of JAK2 phosphorylated at Tyr1007/1008. As shown in Fig. 4d, the inhibition of phosphorylation of JAK2 started within 1 h after purvalanol A treatment and was sustained at least for

Fig. 2



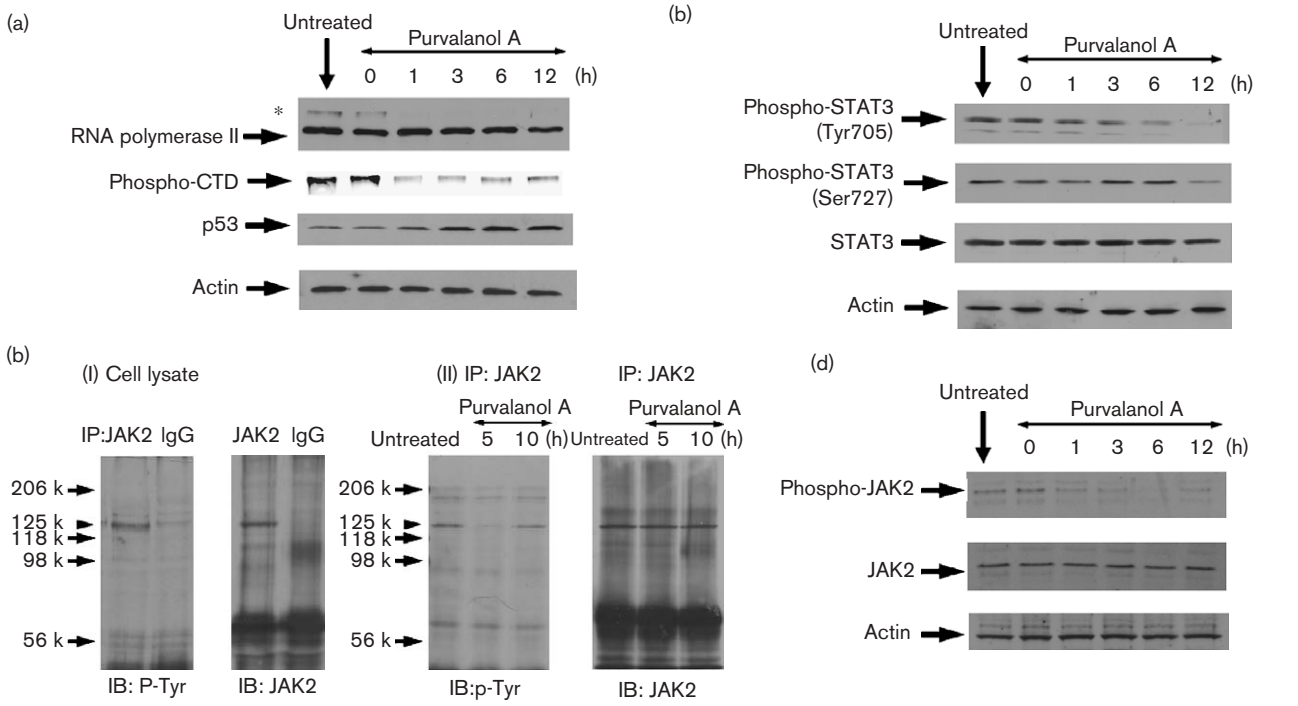
Western blot analysis of the expression of survivin, Bcl-X_L, Bcl-2, and Bax using corresponding antibodies. MKN45 cells treated with or without 30 μmol/l purvalanol A were incubated for 0, 6, 12, 24, 36, and 48 h. Actin in each sample was used as a standard.

Fig. 3



(a) The incorporation of ^{3}H-uridine into MKN45 cells. The cells were pretreated with purvalanol A (30 μmol/l) for 1 h and with medium containing 1 μCi/ml uridine [5-^{3}H] for 1 h in the presence of 30 μmol/l purvalanol A. The procedures for collection of cells and measurement of radioactivity were described in Materials and methods. Each point represents the mean of triplicate samples (mean ± SE). $^{**}P < 0.01$ by Student's *t*-test. (b) mRNA expression of survivin, Bcl-X_L, and Bcl-2 was detected by semiquantitative reverse transcription-PCR analysis using specific primers for each. MKN45 cells treated with or without 30 μmol/l purvalanol A were incubated for 0, 3, 6, 12, and 24 h. GAPDH in each sample was used as a standard.

Fig. 4



(a) Western blot analysis of the expression of RNA polymerase II, phospho-Rpb1 carboxy-terminal domain, and p53 using corresponding antibodies. MKN45 cells treated with 30 μmol/l purvalanol A were incubated for 0, 1, 3, 6, and 12 h. Actin in each sample was used as a standard. (b) Western blot analysis of the expression of phospho-signal transducer and activator of transcription 3 (STAT3) (Tyr705), phospho-STAT3 (Ser727), and STAT3 using corresponding antibodies. MKN45 cells treated with 30 μmol/l purvalanol A were incubated for 0, 1, 3, 6, and 12 h. Actin in each sample was used as a standard. (c) Detection of phosphorylation of Janus kinase 2 (JAK2). (I): equal amounts of cell lysate obtained from exponential growing MKN45 cells were immunoprecipitated with a JAK2 antibody (first lane) and a normal IgG (second lane), respectively, and western blotting was performed with an antiphosphotyrosine antibody [left panel in (I)] and an anti-JAK2 antibody [right panel in (I)]. (II): cells were treated with 30 μmol/l purvalanol A for 5 and 10 h, and equal amounts of total cell lysate were immunoprecipitated with a JAK2 antibody, and western blotting was performed with an antiphosphotyrosine antibody [left panel in (II)] and an anti-JAK2 antibody [left panel in (II)]. Arrows indicated the molecular weight (kDa) estimated by standard proteins (prestained SDS–PAGE standards, broad range; Bio-Rad). (d) Western blot analysis of the expression of phospho-JAK2 (Tyr1007/1008) and non-phospho-JAK2 using corresponding antibodies. MKN45 cells treated with 30 μmol/l purvalanol A were incubated for 0, 1, 3, 6, and 12 h. Actin in each sample was used as a standard.

12 h. Furthermore, as MKN45 cells have wild-type p53, which is widely accepted to be an apoptotic protein, the accumulation of p53 proteins was measured. Interestingly, the expression of p53 increased within 1 h after the drug treatment as shown in the bottom panel of Fig. 4a.

Discussion

STAT3 is constitutively upregulated in a variety of tumor cells and possesses oncogenic potential and antiapoptotic functions. It is phosphorylated at a Tyr705 by the receptor kinase, or by JAK2 through cytokines, hormones, and growth factors. Following phosphorylation of this site, STAT3 forms a homodimer, then translocates from the cytoplasm to the nucleus where it plays a critical role as a transcription factor that initiates an increase in the transcription of a number of genes, including the antiapoptotic regulatory genes Bcl-X_L, survivin, and MCL-1 [9]. Transcriptional activation seems to be regulated by phosphorylation at Ser727. This phosphorylation at Ser727 was reported to be involved in the ERK pathway [8].

In this study, transcription factor STAT3 was inhibited in the early stage (3 h) after addition of purvalanol A and phosphorylation of JAK2 was also inhibited within 1 h after purvalanol A addition as shown in Fig. 4b–d, whereas phosphorylation of STAT3 at Ser727 was not inhibited until 12 h after addition of the drug. In our earlier experiments, the treatment of gastric adenocarcinoma MKN45 cells with purvalanol A did not result in any inhibition of the constitutive phosphorylation state of ERK1/2 [3]. Based on these results, treatment with purvalanol A seems to directly inhibit the phosphorylation mechanism of JAK2, followed by the abrogation of the JAK2/STAT3-related transcriptional machinery. As the expression of Bcl-X_L and survivin is known to be tightly associated with the activation of STAT3 in tumor cells, the downregulation of mRNA expression of these antiapoptotic proteins was at least partly owing to the inhibition of the JAK2/STAT3 pathway by purvalanol A.

Recently, as structural analogues 2, 6, 9-trisubstituted purine Cdk inhibitors, olomoucine and roscovitine, were

developed. Roscovitine was reported to preferentially inhibit not only Cdk1, 2, 5 activities but also the activities of Cdk7, 9 and RNA polymerase II associated with the expression of the antiapoptotic protein Mcl-1 [11]. As shown in Fig. 4, purvalanol A completely inhibited the phosphorylation of Ser2 and Ser5 of the RNA polymerase II CTD region, which was recently reported to play an important role in transcriptional regulation from the viewpoint of mRNA processing and chromatin remodeling [12], and it also partially inhibited expression of RNA polymerase II. These results suggest that purvalanol A inhibits transcription via the suppression of RNA polymerase II activity, because cyclin T/Cdk9 and cyclin H/Cdk7 complexes are reported to be able to phosphorylate the residues at Ser2 and Ser5 of the RNA polymerase II CTD region and it is possible that purvalanol A treatment inhibits the activities of these cyclin/Cdk complex [13]. In addition, the expression of p53 started to increase within 1 h after the drug treatment as shown in the bottom panel of Fig. 4a. This may be explained by p53 stabilization owing to a purvalanol A-induced decrease in mdm-2 mRNA synthesis, because inhibition of transcription of Mdm-2 by flavopiridol, actinomycin D, and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole was reported to prevent degradation of p53, thus inducing p53 [14–16]. These observations may mean that parvalanol A promotes p53-dependent apoptosis signaling in MKN45 cells with wild-type p53. As MKN45 cells are known to have wild-type p53, this accumulation of p53 may also, at least partially, contribute to parvalanol A-induced apoptosis.

Recently, downregulation of STAT3 has been well investigated for anticancer therapy. Several approaches have been used to block STAT3 in cancer cells, resulting in reduced proliferation and apoptosis [9]. Xi *et al.* [17] have also demonstrated that blocking STAT3 activation using a transcription factor decoy approach decreases tumor growth and STAT3 target gene expression in a xenograft model of squamous cell carcinoma of the head and the neck. This study indicates that purvalanol A has antitumor activity via the downregulation of transcription for antiapoptotic genes. Furthermore, treatment combining the inhibition of STAT3 and other genotoxic reagents is reported to have a high efficacy in apoptotic cell death in various tumors. Duan *et al.* [18] have reported that SD-1029, an inhibitor of IL-6-induced and oncostatin-induced STAT3 nuclear translocation, enhanced apoptosis induced by paclitaxel in ovarian cancer cells. Alas *et al.* [19] have reported that piceatannol (JAK1/STAT3 inhibitor) and tyrphostin AG490 (JAK2/STAT3 inhibitor) enhance apoptosis in 2F7 and U266 cells, respectively, exposed to therapeutic drugs, including cisplatin, fludarabine, adriamycin, and vinblastine. Kim *et al.* [20] have reported that dominant-negative STAT3 results in the greatest radiosensitization of MDA-MB-231, decreasing angiogenesis and cell survival. Our earlier reports showed that X-ray-induced apoptosis and reproductive cell death were enhanced by combination with 1-(3-C-ethynyl-β-D-*ribo*-pentofuranosyl) cytosine (ECyd), which was synthesized as an antitumor drug targeting RNA synthesis, or purvalanol A in various tumor cells [3,21–23]. When these drugs enhanced radiation-induced apoptosis, the downregulation of G_2 checkpoint-related proteins and antiapoptotic proteins was found to be important in radiation-induced cell death in various cell lines.

In summary, the Cdk inhibitor purvalanol A was shown to efficiently inhibit the expression of antiapoptotic proteins such as survivin, Bcl-X_L, and Bcl-2 through the inhibition of RNA synthesis by downregulation of the phosphorylated forms of JAK2/STAT3 and RNA polymerase II and to produce apoptotic cell death through mitochondrial pathways.

Acknowledgements

This work was supported in part by Grants-in-Aid for Basic Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan [No. 17658126 (M.K.) and No. 17380178 (O.I.)].

References

1 Villerbu N, Gaben AM, Redeuilh G, Mester J. Cellular effects of purvalanol A: a specific inhibitor of cyclin-dependent kinase activities. *Int J Cancer* 2002; **97**:761–769.
2 O'Connor DS, Wall NR, Porter AC, Altieri DC. A p34(cdc2) survival checkpoint in cancer. *Cancer Cell* 2002; **2**:43–54.
3 Iizuka D, Inanami O, Kashiwakura I, Kuwabara M. Purvalanol A enhances cell killing by inhibiting up-regulation of CDC2 kinase activity in tumor cells irradiated with high doses of X rays. *Radiat Res* 2007; **167**:563–571.
4 Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin Cancer Res* 2002; **8**:945–954.
5 Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, *et al.* Stat3 as an oncogene. *Cell* 1999; **98**:295–303.
6 Barre B, Vigneron A, Perkins N, Roninson IB, Gamelin E, Coqueret O. The STAT3 oncogene as a predictive marker of drug resistance. *Trends Mol Med* 2007; **13**:4–11.
7 Ceresa BP, Horvath CM, Pessin JE. Signal transducer and activator of transcription-3 serine phosphorylation by insulin is mediated by a Ras/Raf/MEK-dependent pathway. *Endocrinology* 1997; **138**:4131–4137.
8 Chung J, Uchida E, Grammer TC, Blenis J. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol Cell Biol* 1997; **17**:6508–6516.
9 Bhardwaj A, Sethi G, Vadhan-Raj S, Bueso-Ramos C, Takada Y, Gaur U, *et al.* Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-κB-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells. *Blood* 2007; **109**:2293–2302.
10 Wang CC, Wu CH, Hsieh KJ, Yen KY, Yang LL. Cytotoxic effects of cantharidin on the growth of normal and carcinoma cells. *Toxicology* 2000; **147**:77–87.
11 MacCallum DE, Melville J, Frame S, Watt K, Anderson S, Gianella-Borradori A, *et al.* Seliciclib (CYC202, R-Roscovitine) induces cell death in multiple myeloma cells by inhibition of RNA polymerase II-dependent transcription and down-regulation of Mcl-1. *Cancer Res* 2005; **65**:5399–5407.
12 Cho EJ. RNA polymerase II carboxy-terminal domain with multiple connections. *Exp Mol Med* 2007; **39**:247–254.
13 Shapiro GI. Cyclin-dependent kinase pathways as targets for cancer treatment. *J Clin Oncol* 2006; **24**:1770–1783.
14 Demidenko ZN, Blagosklonny MV. Flavopiridol induces p53 via initial inhibition of Mdm2 and p21 and, independently of p53, sensitizes

apoptosis-reluctant cells to tumor necrosis factor. *Cancer Res* 2004; **64**:3653–3660.
15 Poele RHT, Okorokov AL, Joel SP. RNA synthesis block by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) triggers p53-dependent apoptosis in human colon carcinoma cells. *Oncogene* 1999; **18**:5765–5772.
16 Blagosklonny MV, Demidenko ZN, Fojo T. Inhibition of transcription results in accumulation of Wt p53 followed by delayed outburst of p53-inducible proteins: p53 as a sensor of transcriptional integrity. *Cell Cycle* 2002; **1**:67–74.
17 Xi S, Gooding WE, Grandis JR. In vivo antitumor efficacy of STAT3 blockade using a transcription factor decoy approach: implications for cancer therapy. *Oncogene* 2005; **24**:970–979.
18 Duan Z, Bradner JE, Greenberg E, Levine R, Foster R, Mahoney J, *et al*. SD-1029 inhibits signal transducer and activator of transcription 3 nuclear translocation. *Clin Cancer Res* 2006; **12**:6844–6852.
19 Alas S, Bonavida B. Inhibition of constitutive STAT3 activity sensitizes resistant non-Hodgkin's lymphoma and multiple myeloma to chemotherapeutic drug-mediated apoptosis. *Clin Cancer Res* 2003; **9**:316–326.
20 Kim KW, Mutter RW, Cao C, Albert JM, Shinohara ET, Sekhar KR, *et al*. Inhibition of signal transducer and activator of transcription 3 activity results in down-regulation of survivin following irradiation. *Mol Cancer Ther* 2006; **5**:2659–2665.
21 Iizuka D, Inanami O, Matsuda A, Kashiwakura I, Asanuma T, Kuwabara M. X irradiation induces the proapoptotic state independent of the loss of clonogenic ability in Chinese hamster V79 cells. *Radiat Res* 2005; **164**:36–44.
22 Inanami O, Iizuka D, Iwahara A, Yamamori T, Kon Y, Asanuma T, *et al*. A novel anticancer ribonucleoside, 1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl) cytosine, enhances radiation-induced cell death in tumor cells. *Radiat Res* 2004; **162**:635–645.
23 Yasui H, Inanami O, Asanuma T, Iizuka D, Nakajima T, Kon Y, *et al*. Treatment combining X-irradiation and a ribonucleoside anticancer drug, TAS106, effectively suppresses the growth of tumor cells transplanted in mice. *Int J Radiat Oncol Biol Phys* 2007; **68**:218–228.