

Antitumor activity of asukamycin, a secondary metabolite from the actinomycete bacterium *Streptomyces nodosus* subspecies *asukaensis*

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Received June 3, 2009; Accepted July 23, 2009

DOI: 10.3892/ijmm_00000283

Abstract. Asukamycin, a manumycin-type metabolite, was isolated by a rapid and easily scalable purification scheme. Thus far, studies on the biological activity of asukamycin have been limited to its role as an antibacterial and antifungal agent. By using five different tumor cell lines we demonstrate antineoplastic activity of asukamycin. It inhibited cell growth at concentrations similar to other members of the manumycin family (IC₅₀ 1-5 μ M). Cytotoxicity of asukamycin was accompanied by activation of caspases 8 and 3 and was diminished by SB 202190, a specific p38 mitogen-activated protein kinase (MAPK) inhibitor. These data, in combination with earlier observations showing its low *in vivo* toxicity, indicate that further studies on the potential antitumor activity of asukamycin are warranted.

Introduction

In recent years, intensive research has been focused on identification of new natural antitumor agents derived from various plants, marine organisms, animals and microorganisms. The manumycin-group metabolites are a small and distinct class of secondary metabolites produced by actinomycete bacteria. Most of the metabolites from this group inhibit the growth of Gram-positive bacteria, but are ineffective against Gram-negative bacteria (1). This group includes manumycin itself (Fig. 1A) as well as several other related molecules, all varying in the construction of the upper polyketide chain (Fig. 1). Thus this structural element is responsible for the variance in activities displayed by the family. Asukamycin is a manumycin-group metabolite that is produced by

Streptomyces nodosus subspecies *asukaensis* (Fig. 1B). In asukamycin the upper polyketide chain is primed by cyclohexane carboxylic acid and extended with three equivalents of acetate.

A number of different biological properties of manumycin-group metabolites have been reported. They include not only antibacterial activity but also other effects as diverse as antifungal, anticoccidial and insecticidal activity, inhibition of farnesyltransferase and interleukin 1 β converting enzyme (ICE), prevention of atherosclerosis, regulation of I κ B kinase signalling, as well as cytotoxic effects towards various tumor cells (1-4). The antitumor activity of this group of metabolites has been studied, for example, by using manumycins A-D, which are primarily produced by *Streptomyces parvulus*. They have shown cytotoxic effects against a variety of tumor cells including murine and human leukemia cells (5,6) as well as human colon, thyroid, hepatic and ovarian cancer cells (3,4,7-10). Only a very limited number of biological activities have been reported for asukamycin thus far. It has been shown to possess antibacterial, weak antifungal and anticoccidial properties (11).

In view of the structural similarity of asukamycin and various manumycins, we hypothesized that asukamycin, like some of the other manumycins, possesses antitumor activity. In order to test this hypothesis, we first developed a novel method of rapid isolation of asukamycin from bacterial cultures, which we subsequently used to obtain milligram quantities of the compound. We show that asukamycin is toxic against several human leukemia and astrocytoma cell lines. Furthermore, we demonstrate that, as with manumycins, the observed toxicity is dependent on the p38 mitogen-activated protein kinase (MAPK) pathway and is associated with activation of caspases 8 and 3.

Materials and methods

Materials. Intracellular caspase activity detection kits were purchased from MBL International (Woburn, MA, USA). SB 202190, a highly selective, cell permeable inhibitor of p38 MAPK, the formazan dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Bradford reagent were purchased from Sigma

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Key words: asukamycin, antitumor agents, manumycin metabolites, leukemia, astrocytomas, cancer

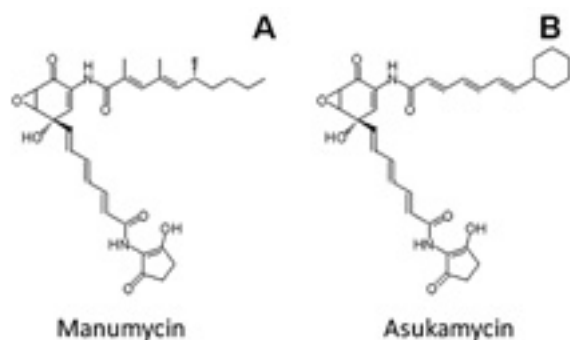


Figure 1. Chemical structures of manumycin (A) and asukamycin (B).

(St. Louis, MO, USA). Cell culture reagents were supplied by Thermo Scientific HyClone (Logan, UT, USA). Reagents used for bacterial growth and purification of asukamycin were from Fisher Scientific (Ottawa, ON, Canada) unless stated otherwise.

Growth of *Streptomyces nodosus*. *Streptomyces nodosus* subspecies *asukaensis* (Sno) cultures [American Type Culture Collection (ATCC) catalogue #29757] were kindly provided by Dr Heinz G. Floss, University of Washington, Seattle, USA. Asukamycin production medium (100 ml; glucose 20 g/l, peptone 5 g/l, K_2HPO_4 0.25 g/l, $MgSO_4$ 0.25 g/l, Hopwood trace elements solution 20 ml/l, pH 7.0) in a 500 ml baffled Erlenmeyer flask was inoculated with 100 μ l of a Sno aerial mycelium stock (12). Three of these vegetative cultures were incubated in a New Brunswick Innova (Edison, NJ, USA) shaking incubator at 28°C, 200 rpm for 48 h. Aliquots of the cultures (10 ml) were then used to inoculate each of twenty 500 ml baffled Erlenmeyer flasks containing 100 ml asukamycin production medium. These production cultures were incubated at 28°C, 200 rpm, for 72 h before harvesting.

Isolation of asukamycin. The production cultures were combined and the solids were separated by centrifugation at 16,000 \times g for 1 h. The supernatant and pellets were extracted separately. The supernatant was extracted three times with an equal volume of ethyl acetate. The ethyl acetate fractions were combined, dried with an excess of anhydrous $MgSO_4$, and the solvent was removed *in vacuo*.

Pellets were combined and suspended in a total of 200 ml of acetone. The resulting suspension was separated by centrifugation at 16,000 \times g for 1 h and the acetone decanted off of the solids. The acetone was removed *in vacuo*, and the resulting paste extracted three times with an equal volume of ethyl acetate. The ethyl acetate fractions were combined, dried with an excess of anhydrous $MgSO_4$, and the solvent was removed *in vacuo*.

The residue from the extractions of the supernatant and pellets were dissolved in a total volume of 10 ml of methylene chloride and combined. Fine solids were then removed using a 0.45- μ m syringe filter. Hexane was added in 1 ml aliquots to a final volume of 50 ml, causing the asukamycin to precipitate from solution. The asukamycin was collected on a Whatman #3 filter then washed three times with water followed by three hexane washes. The asukamycin was liberated from the paper with acetonitrile, which was removed *in vacuo* resulting

in the purified product. The above procedure produced up to 140 mg of asukamycin per liter of the initial bacterial culture. Asukamycin was found to be stable for at least 3 weeks as a 20 mg/ml solution in DMSO at -20°C.

Determination of asukamycin purity and its quantification. Ultraviolet/visible (UV/Vis) spectroscopic analysis was performed using a Cary 100 double beam spectrophotometer with matched quartz 1-cm cuvettes. The quantity of asukamycin was determined by serial dilution in acetonitrile and Beer's law analysis using the literature λ_{max} at 313 nm ($\epsilon=49,400$) (13).

Identity and purity of asukamycin were confirmed by separation on an Acquity Ultra Performance Liquid Chromatography coupled with a Micromass LCT Premier High Resolution Time of Flight Mass Spectrometry (UPLC/(ToF)MS) (Waters Inc., Milford, MA, USA). The purified asukamycin was dissolved in a 50:50 (v/v) solution of methanol in 0.1% aqueous trifluoroacetic acid (TFA) and separated on a Waters Acquity BEC C18 reversed phase UPLC column (2.1 \times 100 mm, 1.7 μ m particle size) using a gradient from 5% acetonitrile in 0.1% aqueous TFA to 95% acetonitrile in aqueous TFA over 10 min at 250 μ l/min. Asukamycin was eluted in 9.2 min, with a minor contaminant eluting just before it at 8.8 min. Comparison of the measured mass to the mass calculated using Beer's law was also used to confirm purity. The above analyses yielded a purity of >85%. High resolution time of flight mass spectrometry using positive mode electrospray ionization showed a molecular ion ($M+H^+$) at 547.2441 g/mol (calculated 547.2444 g/mol).

Nuclear magnetic resonance (NMR) spectrometry was performed using a Varian Mercury Plus spectrometer equipped with a gradient field capable probe operating at 400 MHz for 1H . Proton spectra were acquired in chloroform-*d* and compared with literature values (13).

Human cell cultures. The human myeloid leukemia THP-1, HL-60 and U-937 cell lines, as well as the human astrocytoma U-118 MG and U-87 MG cells, were obtained from the ATCC (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium-nutrient mixture F12 ham (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml)/streptomycin (100 μ g/ml).

Cytotoxicity assay. Cell growth inhibition was measured by using the MTT assay to estimate the relative number of viable cells (14). Human myeloid THP-1, HL-60 and U-937 cells were seeded into 24-well plates (BD Falcon, Mississauga, ON, Canada) at a concentration of 3×10^5 cells per well in 0.6 ml of DMEM-F12 medium containing 5% FBS. Human U-118 MG and U-87 MG astrocytoma cells were seeded at 1×10^5 cells per well in 0.4 ml of culture medium and allowed to adhere for 24 h before asukamycin treatment was initiated. Various concentrations of asukamycin (1-50 μ M) or its vehicle solution (DMSO) were used. Concentration of DMSO in the cell culture medium did not exceed 0.5%. After 24 h of incubation, the number of surviving cells was measured by the MTT assay as described before (15). This method is based on the ability of viable cells to convert the tetrazolium salt (MTT) to colored formazan. The viability of cells was determined by adding MTT to the cell cultures to reach a final concentration

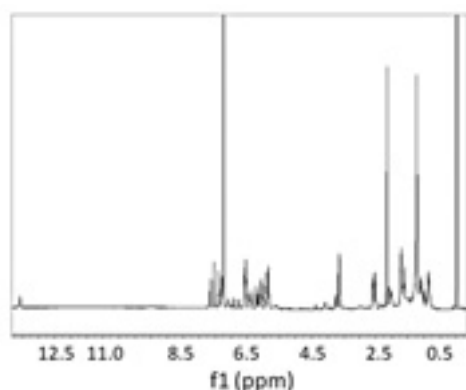


Figure 2. A typical ^1H NMR spectrum obtained from the samples of purified asukamycin dissolved in chloroform- d . Spectrum is representative of NMR analyses of four independent asukamycin samples.

of 0.5 mg/ml. Following a 1 h incubation at 37°C , the dark crystals formed were dissolved by adding to the wells an equal volume of SDS/DMF extraction buffer (20% sodium dodecyl sulfate, 50% N,N -dimethylformamide, pH 4.7). Subsequently, plates were placed overnight at 37°C in order to dissolve aggregates of lysed cells. Optical densities (OD) at 570 nm were measured by transferring 100 μl aliquots to 96-well plates and the values were recorded using a microplate reader with the appropriate filter. Viability values in wells containing cells exposed to asukamycin were compared with the viability values from cells exposed to DMSO only. The latter values were set at 100%. The baseline value for 0% cell survival was determined by lysing cells with 1% Triton X-100. Effects of the MAPK inhibitor SB 202190 were studied by adding this drug (10–50 μM) to tumor cell cultures 15 min before asukamycin treatment.

Caspase assays. THP-1 cells were seeded into 60 mm tissue culture dishes (BD Falcon) at a concentration of 5×10^5 cells per ml in 6 ml of DMEM-F12 medium containing 5% FBS. Asukamycin (50 μM) or DMSO aliquots were added and cells incubated for 24 or 72 h. The enzymatic activity of caspases 3 and 8 was measured colorimetrically (16) using assay kits purchased from MBL International. Assays were carried out according to the instructions supplied by the manufacturer and caspase activity in cell lysates was expressed in mmol p-nitroaniline (pNA) per h per mg protein. Protein concentrations in the samples were measured by using the Bradford reagent and bovine serum albumin solutions to generate a standard curve (17).

Statistical analysis. Data are presented as means \pm standard error of the mean (SEM). The concentration-dependent effects of asukamycin and SB 202190 were evaluated statistically by the randomized blocks design analysis of variance (ANOVA). $P < 0.05$ was defined as statistically significant.

Results

Fig. 2 shows a typical NMR spectrum obtained from the samples of purified asukamycin dissolved in chloroform- d . The obtained NMR spectral characteristics were identical to

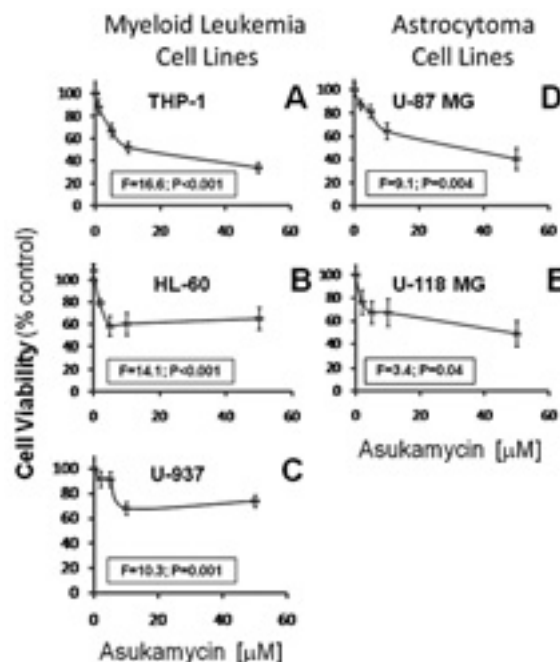


Figure 3. Asukamycin in a concentration-dependent manner reduces viability of five different human cell lines. Three myeloid leukemia cell lines THP-1 (A); HL-60 (B) and U-937 (C), and two astrocytoma cell lines U-87 MG (D) and U-118 MG (E) were exposed to various concentrations of asukamycin (1–50 μM , shown on the abscissa). Cell viability was assessed after 24 h by the MTT assay. Data (means \pm SEM) from 4 to 10 independent experiments are expressed as % control. Values obtained from cells exposed to DMSO only were set at 100%. The concentration-dependent effects of asukamycin were evaluated by the randomized blocks design ANOVA. F- and P-values shown in the boxes are all significant.

the previously reported values (13). Beer's law and NMR analyses confirmed $>85\%$ content of asukamycin in the purified samples.

Fig. 3 shows that asukamycin reduced viability of five different cell lines in a concentration-dependent manner (1–50 μM). Although in all cases the effects were statistically significant, each of the cell lines tested had different sensitivity towards the drug. The lowest level of viability after exposure to 50 μM asukamycin for 24 h was observed in THP-1 myelomonocytic cell cultures (Fig. 3A; $34 \pm 5\%$). Similar low levels of viability were registered in the case of U-87 MG (Fig. 3D; $41 \pm 2\%$) and U-118 MG (Fig. 3E; $49 \pm 11\%$) astrocytoma cells. The other two cell lines were inhibited less effectively with cell viability values of $65 \pm 10\%$ in the case of HL-60 (Fig. 3B) and $74 \pm 6\%$ in the case of U-937 cells (Fig. 3C). Asukamycin inhibited cell growth with IC_{50} values of $\sim 5 \mu\text{M}$ for all cell lines tested except HL-60 cells where this value was closer to 1 μM (Fig. 3B).

Next, we studied the mechanism of asukamycin-induced cell death in cultured human myelomonocytic THP-1 cells. Exposure of these cells to asukamycin (50 μM) caused a significant increase in intracellular caspase 8 (Fig. 4A) and caspase 3 (Fig. 4B) activity when compared to the vehicle solution (DMSO)-treated cell cultures. Preliminary data showed that this upregulation was maximal when measured between 24 and 72 h after asukamycin administration. Longer incubation time did not cause the differential caspase activity to increase further (data not shown).

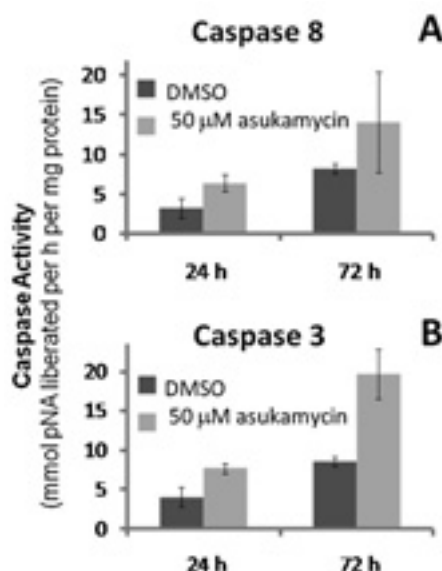


Figure 4. Asukamycin causes significant activation of caspases 3 and 8 in human THP-1 myelomonocytic cell culture. THP-1 cells (3×10^6) were exposed to 50 μ M asukamycin or DMSO for 24 or 72 h. Caspase 8 (A) and caspase 3 (B) activities were measured by colorimetric assays. Data (means \pm SEM) from 3 to 6 independent experiments are expressed as mmol pNA liberated per h per mg protein. Statistical evaluation of the data by the randomized blocks design ANOVA indicates that the enzymatic activities of caspase 3 (A-F=11.8; $P=0.01$) and caspase 8 (B-F=8.7; $P=0.03$) are significantly elevated in asukamycin-treated THP-1 cells when compared to cells exposed to the vehicle solution (DMSO) only.

Fig. 5 illustrates that SB 202190 (10–50 μ M), a selective, cell-permeable inhibitor of p38 MAPK, partially reversed the toxic action of asukamycin. In this series of experiments, incubation of THP-1 cells with 50 μ M asukamycin caused reduction of cell viability to $30 \pm 11\%$ after 24 h. Pretreatment of cells with 50 μ M SB 202190 reversed this value to $70 \pm 24\%$.

Discussion

Very few studies on asukamycin have been published thus far. Most of them describe the biosynthetic pathways of this metabolite (13). Lack of a simple purification procedure and the fact that asukamycin is not available commercially could be blamed for this shortfall. Here, we describe a rapid method of isolation that uses a selective precipitation instead of the lengthy and relatively expensive chromatographic methods described earlier (11,18), giving a dual advantage of decreasing the amount of time required, and making it much simpler and less expensive to scale the purification for larger fermentations. We were able to readily confirm the identity of the asukamycin by its mass, UV/Vis, and proton NMR spectra. Purity of the sample was shown to be at least 85% by chromatographic, spectrophotometric and spectrometric analyses. We tested the biological activity of four different batches of purified asukamycin. They were applied to five different human tumor cell lines including three leukemia and two astrocytoma cell lines. Asukamycin effectively reduced viability of all tumor cells tested with IC_{50} of 1–5 μ M. This value is within the range of minimal inhibitory concentration (MIC) values originally reported by Omura *et al* (11) for the effects of asukamycin on

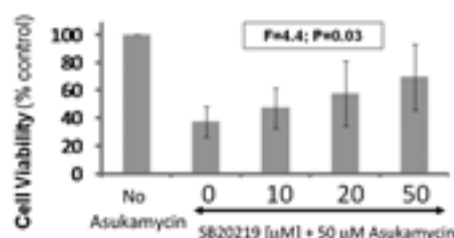


Figure 5. Asukamycin-induced toxicity in THP-1 myelomonocytic cell cultures is reduced by a specific inhibitor of p38 MAPK. THP-1 cells were pre-incubated with various concentrations (0–50 μ M, shown on the abscissa) of SB 202190 before their exposure to 50 μ M asukamycin. Cell viability was assessed after 24 h by the MTT assay. Data (means \pm SEM) from five independent experiments are expressed as % control. Values obtained from cells exposed to DMSO only were set at 100%. The concentration-dependent effect of SB 202190 was evaluated by the randomized blocks design ANOVA. F- and P-values are shown in the box.

various Gram-positive bacteria (1.5–45 μ M range). It also corresponds well with, for example, the cytotoxic effects of manumycin A on U-937 and HL-60 myeloid leukemia cells (6) and COLO320-DM human colon tumor cells (9). Therefore, the cytotoxic activity of asukamycin, similar to that of manumycin A, is somewhat modest. Nonetheless asukamycin appears to be among the most effective antitumor agents within the manumycin-group metabolites, which generally exhibit antineoplastic activity with the IC_{50} values ranging from 0.5 to 50 μ M (1,4). It is also important to note that asukamycin has been demonstrated to have low toxicity *in vivo*. The LD_{50} for acute toxicity in mice after intraperitoneal injection was reported to be 48.5 mg/kg, while up to 450 mg/kg of asukamycin caused no toxicity when administered *per os* (11).

While no reports on the antitumor activity of asukamycin have been published thus far, the cytotoxicity of various other manumycins has been studied in detail. Several reports indicated that this activity was due to inhibition of farnesyltransferase. Initially, Hara *et al* (19) demonstrated that manumycins A, B and C inhibit farnesyltransferase with IC_{50} ranging from 5 to 13 μ M. Since farnesylation of *ras* proteins is crucial for development of different cancers, various inhibitors of farnesyltransferase have been studied as potential anticancer agents. Some of these drugs are already undergoing clinical trials in various solid and hematological malignancies (20).

Even though inhibition of farnesyltransferase may account for the antiproliferative effects of manumycins, recent data indicate that manumycins induce tumor cell death independently of farnesyltransferase and inhibition of *ras* signal transduction pathways (21). Manumycin-induced apoptosis in tumor cells has been shown by measuring various parameters including release of mitochondrial cytochrome c into the cytosol, activation of caspases 8, 9 and 3, as well as increased DNA fragmentation (6,7,9,21,22). She *et al* (7) suggested that the cellular death induced by manumycin in human thyroid cancer cells fits the general pattern of xenobiotic-induced apoptosis, which is characterized by induction of oxidative stress, MAPK signaling (p38 MAPK in particular), release of cytochrome c and subsequent activation of the apoptotic pathways (reviewed in ref. 23). Activation of caspases 8, 9 and 3 occurs further downstream in the manumycin-induced apoptotic pathway (22).

In this study we used the human THP-1 myelomonocytic cell line to investigate the effects of asukamycin. We demonstrated that the cytotoxic effect of asukamycin can be inhibited by SB 202190, a highly selective, cell permeable inhibitor of p38 MAPK. This kinase has already been implicated in manumycin-induced cell toxicity (7) and was shown to be a good target for the development of drugs for various cancers and leukemias (24,25). Furthermore, we showed that asukamycin treatment caused significant activation of caspases 8 and 3. These caspases have previously been shown to mediate apoptosis induced by manumycin (7,26) as well as a number of other anticancer drugs (22,25,27). Involvement of p38 MAPK as well as caspases 8 and 3 indicates that the cytotoxic action of asukamycin is mediated by pathways that are very similar to those described for the effect of manumycin, and a combination of manumycin with paclitaxel, on thyroid cancer cells (7,22) as well as for the effects of auranofin on HL-60 leukemia cells (25). In the latter study, p38 MAPK was shown to activate both the extrinsic and intrinsic apoptotic pathways (28) that resulted in parallel processes of caspase 8 activation and cytochrome c release, both of which lead to downstream activation of caspase 3.

In summary, we developed a rapid and scalable new technique that can be used to obtain large quantities of asukamycin from cultures of *Streptomyces nodosus* subspecies *asukaensis*. We demonstrate that this manumycin-group metabolite possesses moderate antitumor activity, which is associated with activation of caspases 8 and 3 and involves p38 MAPK signalling. Similar pathways have already been implicated in the case of cytotoxic activity of manumycins and other antineoplastic agents. Due to the low *in vivo* toxicity of asukamycin reported earlier, further studies of asukamycin as a potential antitumor agent are warranted.

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada, Irving K. Barber School of Arts and Sciences (UBC Okanagan) and the Jack Brown and Family AD Research Fund.

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