Flow Cytometric Analysis of the Influence of Myosmine on the Cell Cycle[†]

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Abstract. Myosmine (3-(1-pyrrolin-2-yl)pyridine) is an alkaloid found in tobacco as well as various staple foods, fruits and vegetables. Myosmine has recently been suspected to be a tobacco-independent carcinogenic source. Using cell flow cytometry, we have examined the influence of myosmine on the cell cycle of murine erythroleukemia (MEL) cells *in vitro* and have compared this with its effects on murine bone marrow cells *in vivo*. Myosmine at low concentrations inhibited cell proliferation dose dependently; while at concentrations close to 300 µmol dm^{-3} it acted in a cytostatic fashion, that is, it increased the percentage of cells in the S and G2/M phases. At doses of 350–400 µmol dm^{-3} myosmine induced apoptosis and the hypoploid fraction. *In vivo* intraperitoneal injection of mice with 100 mg/kg of myosmine resulted in a statistically significant increase in the percentage of cells in S phase; *i.e.* from 13.75 to 18.22 %. The percentage of bone marrow cells in the G2/M phase increased from 6.79 to 8.93 % in treated mice compared to controls. All of these results are in agreement with the hypothesis that myosmine possesses genotoxic potential. (doi: 10.5562/cca1767)

Keywords: myosmine, cytotoxicity, cell cycle, apoptosis, bone marrow

INTRODUCTION

Myosmine, 3-(1-pyrrolin-2-yl)pyridine, as well as nicotine, is among the fully structurally characterized tobacco alkaloids.[1] Unlike nicotine, myosmine is found in various commonly consumed foods such as nuts, vegetables, and fruits.[2,3] It is not surprising therefore, that myosmine has also been detected in human toenail, plasma and saliva from normal controls.[4] After nitrosation and/or peroxidation, myosmine gives rise to reactive pyridyloxobutylating species which are capable of forming pyridyloxobutylated DNA adducts.[5–8] Nitrosation of myosmine also leads to the formation of the esophageal carcinogen *N*'-nitrosonornicotine.[5] Originally, nitrosonornicotine and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB)-releasing adducts were thought to be highly tobacco-specific.

A number of scientists connect human intake of myosmine to the increase in incidence of esophageal adenocarcinoma in the Western world.[9] In support of this hypothesis, there is data which shows that in sudden death victims HPB-releasing DNA adducts found in

esophageal mucosa taken from close to the esophagogastric junction, were similar in quantity in smokers and nonsmokers.[10] It was therefore hypothesized that a tobacco-independent source for these adducts might have been myosmine uptake from food.[2,3,5,6]

The genotoxicity of myosmine has been studied in a number of *in vitro* models. Comet assay studies have shown that myosmine induces DNA damage in nasal mucosa cells, human lymphocytes and in cell lines derived from human esophageal adenocarcinoma.[11,12] In another investigation, the mutagenic potential of myosmine was demonstrated in human lymphocytes using the human lymphocyte hypoxanthine guanine phosphoribosyl transferase gene mutation assay.[13]

It has been shown that eukaryotic cells respond to DNA damage by inducing temporary cell cycle arrest at the G1/S and G2/M transition points;[14] these transient delays in cell cycle progression presumably allow time for repair and prevent the replication and segregation of damaged DNA templates. That is why it is interesting to investigate whether or not myosmine is also able to influence the cell cycle both *in vitro* and *in vivo*. As our

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in vivo model we have chosen to study bone marrow cells because the generally accepted method for determining the carcinogenic potential of a substance is the mouse micronucleus assay,[15] an assay which uses bone marrow polychromatic erythrocytes. In order to correctly interpret our *in vivo* results, it was logical to study the effect of myosmine in the closest possible *in vitro* model, and for this we chose Friend murine erythroleukemia cells, virally transformed erythroid precursors that are held in an immature and proliferating state.[16] The metabolism of these cells is similar to that of the majority of proliferating hematopoietic cells in the bone marrow.[17]

MATERIALS AND METHODS

Materials

Myosmine was synthesized in the Institute of Molecular Biology, Bulgarian Academy Science (BAS), in accordance with the published method of Brandänge and Lindblom (1976).[18] The product was found to be 99.9 % pure; it's identity with commercial myosmine was confirmed by ultraviolet and infrared spectral analysis, gas chromatography and high pressure liquid chromatography. All other reagents were purchased from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany and were of analytical grade. The solutions needed for flow cytometric analysis were obtained from BD Bioscience.

Cell Culture

Murine erythroleukemia cells, clone F4N (virus-transformed erythroid precursor cells)[16] were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10 % calf serum, and cultured in a 5 % CO_2 atmosphere at 37 °C, and passed every day at a concentration of 5×10^5 cells/ml.

Incubation with Myosmine and *In Vitro* Cytotoxicity Test

An aliquot of 5×10^5 cells/ml in the exponential phase of growth was incubated with myosmine in 24-well microtiter plates. After 24 or 48 hours of treatment with myosmine, the cells were counted hemocytometrically and prepared for cell cycle analysis. The number of dead cells was determined by staining with trypan blue. The mean ± SD of triplicate determinations of viable cells was calculated. The fraction of cells undergoing apoptosis was determined using fluorescent microscopy with propidium iodide.

Animals

30–40 day-old BALB/c mice weighing 25–30 g were obtained from the National Center of Public Health Protection. They received proper care and were pro-

vided with food and water ad libitum. Each group of 6 mice was treated with an intraperitoneal injection of various concentrations of myosmine diluted in sterile water and housed in a separate compartment. Experiments were performed in accordance with the guidelines for Care and Use of Laboratory Animals under work permit №11130007 of the Institute of Molecular Biology, Bulgarian Academy of Sciences.

Bone Marrow

Twenty four hours after their last treatment with myosmine, mice were killed by cervical dislocation. The left femur was removed and the bone marrow cavity was flushed with 1 ml Hanks Balanced salt solution (HBSS). The extracted bone marrow was suspended in cold HBSS, and the cells were suspended in the solution by passing them through a 70 μm nylon mesh (Cell Strainer, BD Falcon). Five mls of 70 % ethanol was added dropwise to the cells while vortexing. Cells were stored at −20 °C for 24 hours to fix.

Cell Cycle Analysis

After 24 h incubation with different concentrations of myosmine, the cells were harvested by centrifugation and resuspended in 0.5 ml cold PBS. A quantity of 70 % ethanol was added dropwise to the cells while vortexing. Cells were stored at −20 °C 24 hours to fix. Just before flow cytometric analysis (FACS) cells were spun down, washed with PBS and resuspended in propidium iodide (PI) staining solution containing 100 μg/ml RNase and 50 μg/ml PI. FACS analysis was performed on a FACSCalibur (Beckton Dikinson) using the FL3-A channel. A minimum of 10 000 cells were analyzed from each cell sample and the resulting histograms were evaluated using WinMDI 2.9 software.

Statistical Analysis

The percentage of bone marrow cells in each phase of the cell cycle was expressed as the mean ± SD. Differences between control and treated samples were analyzed for significance by Student's *t* test. A P value of <0.05 indicated statistical significance.

RESULTS AND DISCUSSION

In Vitro Cytotoxicity of Myosmine

For this study we used mouse erythroleukemia cells, clone F4N, in which several antitumor drugs have already been shown to readily trigger apoptosis.[19,20] The growth inhibitory and cytotoxic effect of incubation of MEL cells with different concentrations of myosmine have been shown in Figure 1. The presence in the cell culture of concentrations of myosmine lower than
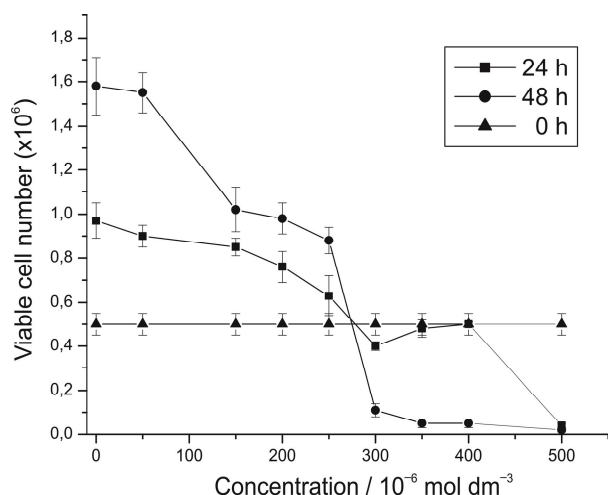
Figure 1. The dependence of the number of viable murine leukemia cells on myosmine concentration. Cells were treated with different concentrations of myosmine for 24 or 48 hours.

50 µmol dm^{-3} did not influence cell proliferaton, whereas higher concentrations of myosmine were found to inhibit cell growth. Twenty four hours after incubation with either 300 and 400 µmol dm^{-3} myosmine a cytostatic effect was observed, and by 48 hours post treatment all cells were dead. This time course suggests programmed cell death rather than necrosis. To further clarify the mode of cell death, samples were examined by fluoroscent microscopy. Using this method as well as the comet assay, we have previously shown[20] that apoptotic MEL cells exhibited changed morphology with fragmented condensed nuclei, while untreated control cells displayed uniform size and morphology. Myosmine, when added to cells in cytotoxic concentrations, induced apoptosis, and cells showed typical fragmented condensed nuclei, ghosts, and apoptotic bodies.

At concentrations of myosmine higher than 500 µmol dm^{-3} all cells appeared dead by 24 hours; some with typical apoptotic morphology, while others appeared necrotic. All of the cells were trypan blue positive, reflecting the fact that all had a defective external cell membrane. We have not presented photos of the apoptotic cells in this publication as they will published in a future article devoted to pro and anti apoptogenic action of myosmine.

In Vitro **Effect of Myosmine on Cell Cycle Progression**

To further analyze the nature of cell death, flow cytometric analysis was performed. If myosmine forms DNA adducts and the damage is significant enough, the cell cycle with be temporarily arrested, allowing the cells time for repair. In this study, MEL cells were incubated for 48 hours with different doses of myosmine (Figure 2). At a concentration of 100 µmol dm^{-3} myosmine
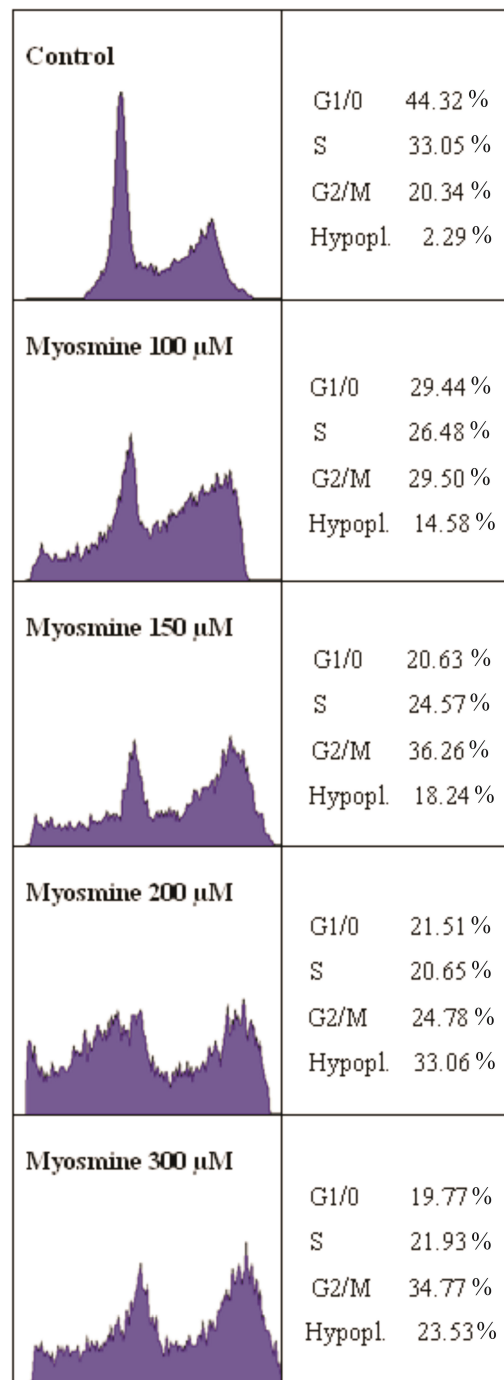
Figure 2. The influence of myosmine on cell cycle kinetics of murine leukemia cells. Cells were treated with different concentrations of myosmine for 48 hours. The histograms are from one typical experiment.

there was a decrease in the cell fraction in the G1/0 phase from 44 to 29 % and an increase in G2/M from 20 to 29 %. After treatment with 150 µmol dm^{-3} myosmine, the fraction of cells in G2/M had increased significantly to 36 %. At 200 µmol dm^{-3} myosmine the most characteristic change was a dramatic increase in the

fraction of hypoploid cells to 33 % compared to 2 % in the control cells. According to the morphological analysis these were apoptotic cells. At 300 µmol dm^{-3} there was an additional increase in the fraction of cells in G2/M and a decrease in the number of hypoploid cells, probably a result of increased toxicity of higher concentrations of myosmine.

These results demonstrate that MEL cells responds to myosmine in the same way as they do to other genotoxic stressors, that is, by activating cell cycle checkpoints and by altering progression to the G2/M stage of the cell cycle, thus allowing the cells time to repair damage or move into apoptosis.

In Vivo Effect of Myosmine on the Cell Cycle

Former studies have shown that the LD$_{50}$ for myosmine in rats is 190 mg/kg (i.p.) and 1875 mg/kg (p.o.).[21] There is no available data on the acute toxicity of myosmine in mice. Therefore our first task was to find out the maximal tolerable dose of myosmine when given intraperitoneally to mice. Balb/C mice of 25–30 g were divided into groups of 2 animals and injected with myosmine in doses of 60, 125, 250, 500 mg/kg. Mice injected with either 500 or 250 mg/kg died within two minutes. When two mice were given 125 mg/kg, one lived and the other died. At 60 mg/kg, both animals lived. An additional two animals were then injected with 100 mg/kg, and both lived. Therefore we concluded that the maximum tolerable dose was approximately 100 mg/kg. This does was then used in the *in vivo* experiments.

The cell cycle distribution in bone marrow was studied 24 h after a single injection of 100 mg/kg myosmine (Table 1). Control mice were injected with a similar quantity of sterile water. There was a statistically significant increase in the percentage of cells in S phase (*i.e.* from 13.75 to 18.22 %) and in the G2/M phase from 6.79 to 8.93 % in the bone marrow isolated from treated mice compared to that of controls. It is likely that the *in vivo* effects were much less pronounced than the *in vitro* because only a portion of the bone marrow cells would have been in a proliferating state, while the remainder of the cells would have been in the G0/G1 phase. There are no studies providing evidence for the accumulation of myosmine or myosmine's carcinogenic intermediates in bone marrow. In this study we have therefore acquired additional confirmation that myosmine or its metabolites reaches the bone marrow and influences cell division.

CONCLUSION

Our investigation has shown the ability of myosmine to inhibit cell proliferation and to alter cell cycle kinetics. The mode of cytotoxic action depends on the concentration of myosmine: at low concentrations it inhibited cell proliferation dose dependently; at concentrations close to 300 µmol dm^{-3} myosmine acts in a cytostatic fashion, that is, increasing the percentage of cells in the S and G2/M phases. At 350–400 µmol dm^{-3}, myosmine induces apoptosis and the hypoploid fraction. *In vivo* treatment of mice with myosmine resulted in an increased percentage of bone marrow cells in S and G2/M phases, the same effect as was found *in vitro*. All of these results are in agreement with the hypothesis that myosmine possesses genotoxic potential.

Table 1. Cell cycle phase distribution in bone marrow of control and myosmine treated mice.

BONE MARROW	G0/G1	S	G2/M
control	79.76 ± 2.88	13.75 ± 1.35	6.79 ± 1.86
100 mg/kg	73.45 ± 5.99	18.22 ± 4.03	8.93 ± 2.98

Data are expressed as means ± SD of the proportion of cells in each cell-cycle phase.

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