

DNA Methylation Changes Caused by Lawsone

Zuzana Poborilova, Anna B. Ohlsson, Torkel Berglund, Anna Vildova, Petr Babula

Abstract—Lawsone is a pigment that occurs naturally in plants. It has been used as a skin and hair dye for a long time. Moreover, its different biological activities have been reported. The present study focused on the effect of lawsone on a plant cell model represented by tobacco BY-2 cell suspension culture, which is used as a model comparable with the HeLa cells. It has been shown that lawsone inhibits the cell growth in the concentration-dependent manner. In addition, changes in DNA methylation level have been determined. We observed decreasing level of DNA methylation in the presence of increasing concentrations of lawsone. These results were accompanied with overproduction of reactive oxygen species (ROS). Since epigenetic modifications can be caused by different stress factors, there could be a connection between the changes in the level of DNA methylation and ROS production caused by lawsone.

Keywords—DNA methylation, Lawsone, Naphthoquinone, Reactive Oxygen Species.

I. INTRODUCTION

NAPHTHOQUINONES represent a group of naturally occurring secondary metabolites. They were found in some actinomycetes, fungi, lichens, and algae as well as in higher plants [1]. Naphthoquinones are interesting due to their broad range of biological effects including antibacterial, antiviral, antipyretic, anti-inflammatory, antiproliferative, and cytotoxic properties. Especially their cytotoxic effect awakes an interest as promising anticancer drugs. They are able to interfere with various biochemical processes. Important is their ability to interact with electron transport, creation of reactive oxygen species (ROS) that are responsible for interactions with other molecules, mainly proteins, membrane lipids, and nucleic acids [2]. All above-mentioned mechanisms can result in programmed cell death [3]. Lawsone as a pigment in the leaves of *Lawsonia inermis* has been used as a skin and hair dye [4]. Nevertheless, it possesses cytotoxic properties that deal with its ability to create ROS [5].

Epigenetic modifications can be induced by different stress factors and can further influence regulation of gene expression. It is suggested that DNA methylation status can be changed by environmental stresses. The level of DNA

methylation can be both increased and decreased by stress factors [6]. Correlation between DNA methylation and gene inactivity has already been documented [7]. Hypomethylation of DNA is associated with gene activation whereas extra methylated genes become silenced [8].

The aim of our study was to investigate lawsone-induced DNA methylation changes in tobacco BY-2 cells.

II. MATERIALS AND METHODS

A. Plant Material and Lawsone Treatment

Nicotiana tabacum L. cv. Bright Yellow-2-suspension-cultured cells (BY-2) were grown in liquid medium according to Murashige and Skoog [9], modified by Nagata et al. [10] with constant shaking (125 rpm) at 26,5°C at a photoperiod of 12 h light and 12 h dark in 250-ml Erlenmeyer flasks.

Cells in the exponential growth phase were treated by lawsone for 24 h and 48 h. Lawsone was dissolved in DMSO and added to the suspension at final concentrations 0, 60, 80, 100 µM in triplicates. The control cells were exposed to dimethylsulfoxide. Both treated and control cells were harvested by vacuum filtration after 24 h and 48 h, respectively.

B. DNA Extraction

BY-2 cells were homogenized under liquid nitrogen using a pre-chilled mortar and pestle. Total DNA was extracted from the tobacco cells by using DNeasy® Plant Mini Kit from QIAGEN (Sollentuna, Sweden).

C. DNA Methylation

DNA methylation was analysed by the Luminometric Methylation Assay (LUMA) [11] in a PyroMark Q24 instrument (QIAGEN) using Pyro Gold Reagents (QIAGEN). DNA was cleaved in two parallel setups with restriction enzymes *HpaII* and *MspI* from Fisher Scientific (Gothenburg, Sweden). Height peaks were calculated using the PyroMark Q24 Analysis Software (QIAGEN).

D. TBARS Assay

Thiobarbituric acid (TBA) reactants were analysed as a measure of lipid peroxidation [12]. Malondialdehyde (MDA), a product of lipid peroxidation, reacts with TBA giving thiobarbituric acid reactive substances (TBARS), measured by spectrophotometer at 532 and 600 nm.

III. RESULTS

The influence of lawsone on the cell suspension culture BY-2 was determined after 24 h and 48 h treatment. Lawsone was added at the exponential growth phase of BY-2 cell culture. The presence of lawsone caused a concentration

Z. Poborilova is with Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno CZ-612 42, Czech Republic (corresponding author: zuzka.poborilova@centrum.cz).

A. B. Ohlsson and T. Berglund are with KTH, Royal Institute of Technology, School of Biotechnology, Department of Industrial Biotechnology, AlbaNova University Center, Stockholm SE-106 91, Sweden (e-mail: annao@biotech.kth.se, torkelb@kth.se).

A. Vildova is with Charles University Prague, Faculty of Pharmacy in Hradec Králové, Department of Pharmacognosy, Hradec Králové CZ-500 05, Czech Republic (e-mail: vildovaa@faf.cuni.cz).

P. Babula is with Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno CZ-612 42, Czech Republic (e-mail: petr-babula@email.cz).

dependent decrease of growth after 24 h and 48 h compared to BY-2 control cells. A stronger influence was determined in concentration 80 μM and maximum decline in the cell growth was observed at the lawsone concentration 100 μM after both 24 h and 48 h exposure (Fig. 1).

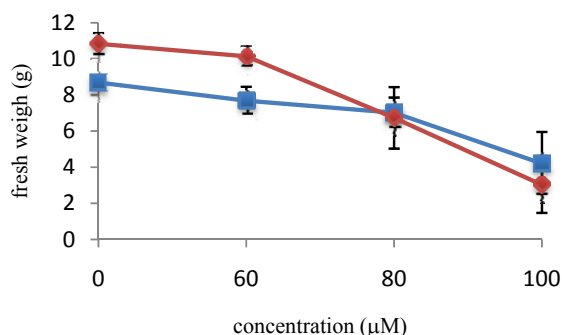


Fig. 1 Growth response of tobacco BY-2 cells treated with lawsone. BY-2 cells treated for 24 h (□); BY-2 cells treated for 48 h (◇). Each point represents the mean of three independent experiments

The changes of DNA methylation were detected after lawsone treatment for 24 h and 48 h. Two restriction enzymes, *HpaII* and *MspI* were chosen to determine DNA methylation level. For these endonucleases is characteristic the recognition of the same DNA sequence 5'-CCGG-3', but they have different sensitivity to cytosine methylation. DNA is cleaved according to the position of methyl groups on cytosine moieties. If there is the methylation of external and internal cytosine ($^{\text{m5}}\text{C}^{\text{m5}}\text{CGG}$) or external cytosine ($^{\text{m5}}\text{CCGG}$), none of the enzymes is able to cut the sequence. On the other hand, unmethylated DNA strands are cut by both enzymes. DNA sequence with methylated internal cytosine ($\text{C}^{\text{m5}}\text{CGG}$) is recognized and digested by *MspI* whereas *HpaII* is not able to cut DNA in the presence of methyl group at the internal cytosine [13]. Since the growth of BY-2 cells was strongly inhibited at the concentration 100 μM , the level of DNA methylation changes was determined for concentrations 0, 60, 80 μM . Fig. 2 shows the changes in DNA methylation after 24 h lawsone exposure. DNA treated by lawsone in 80 μM concentration is more cleaved by *HpaII* (Fig. 2 (a)) compared to the control cells. The same cleavage pattern was observed for *MspI* cleavage (Fig. 2 (b)). This points out on DNA hypomethylation caused by lawsone. As shown in Fig. 3 (a) and (b), the levels of DNA methylation were decreased as well after 48 h treatment. DNA sequence was cleaved by restriction endonucleases with the highest effect observed for 80 μM lawsone. DNA methylation changes were determined already after 24 h exposure of cell suspension culture BY-2 to lawsone. The experiment provided the lawsone ability to induce DNA hypomethylation in concentration- and time-dependent manners.

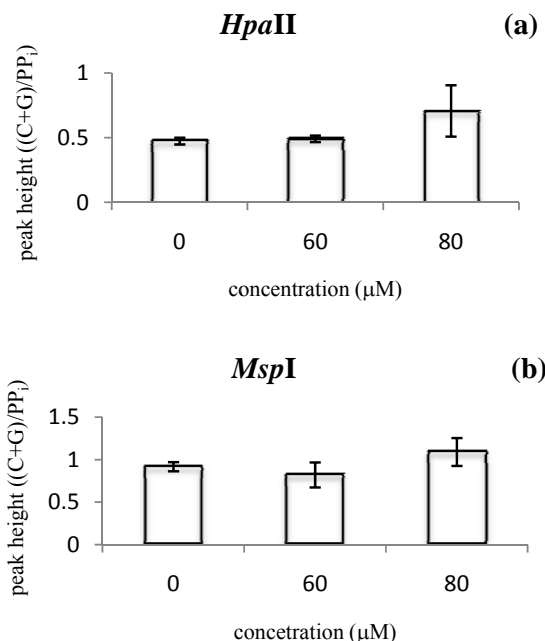


Fig. 2 DNA methylation level after 24 h of lawsone exposure analyzed by LUMA. DNA was digested with *HpaII* (a), *MspI* (b). A higher level refers to a lower level of DNA methylation

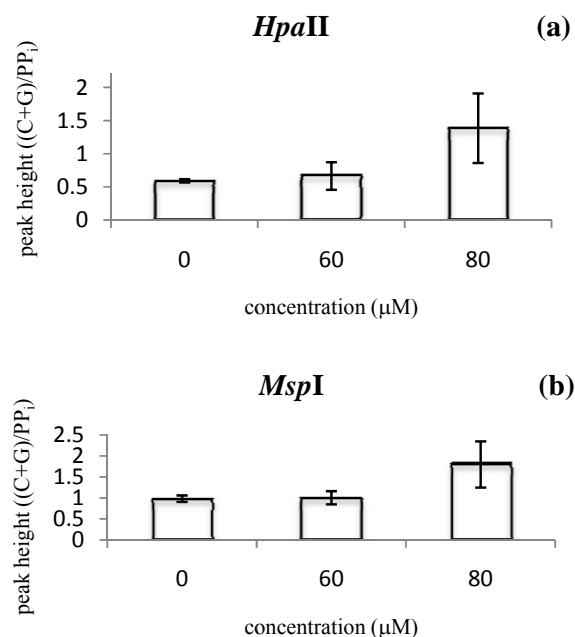


Fig. 3 DNA methylation level after 48 h of lawsone exposure analyzed by LUMA. DNA was digested with *HpaII* (a), *MspI* (b). A higher level refers to a lower level of DNA methylation

The level of lipid peroxidation after lawsone treatment was detected to proof its ability to create ROS that can react with DNA and form DNA adducts. The final product of lipid peroxidation, malondialdehyde (MDA), was determined spectrophotometrically at 532 nm and 600 nm. Our results

showed an increase in MDA production, with the highest amount at 100 μ M concentration (Fig. 4). These results could correlate with the cell growth as well as with the changes of DNA methylation level.

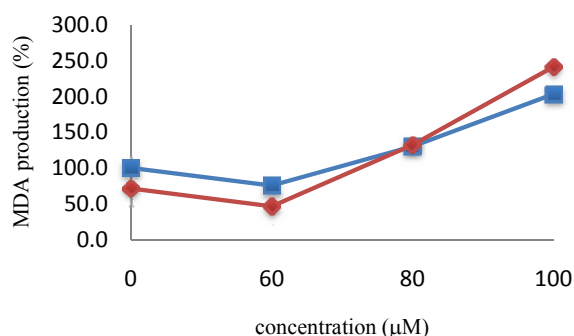


Fig. 4 Effect of lawsone on the malondialdehyde production. BY-2 cells treated for 24 h (\square); BY-2 cells treated for 48 h (\diamond). Each point represents the mean of three independent experiments

IV. DISCUSSION

Lawsone, a secondary metabolite derived from 1, 4-naphthoquinone, has been used as a dye for hair and skin since 1400 BC [4]. Nevertheless, it has been described as a cytotoxic agent, similarly as other naphthoquinones. Cytotoxic activity of metallic complexes with lawsone [13] and its amino derivatives [14] were reported.

In this study, the influence of lawsone on the cell suspension culture BY-2 was determined in two different time treatments. Our results demonstrate that lawsone can effectively affect tobacco BY-2 cell growth in the concentration-dependent manner. The inhibiting effect of naphthoquinones on the cell viability has already been reported [3]. Furthermore, it is known that naphthoquinones can interact with electron transport and create reactive oxygen species (ROS) that subsequently cause damage of biomolecules, such as DNA, lipids, and proteins and lead to programmed cell death [2]. Therefore, a product of lipid peroxidation was evaluated by spectrophotometric method. Increased free radical creation was observed in both time intervals after treatment with lawsone at 80 μ M and 100 μ M concentrations as indicated the malondialdehyde (MDA) production. The amount of MDA correlated with the lawsone-induced growth depression.

DNA methylation was studied after lawsone treatment for 24 h and 48 h. Changes in the level of DNA methylation were analysed by LUMA method [11]. Methylation changes were detected in the CCGG sequence. DNA sequence was recognized and cut by restriction enzymes according to the methylation status of cytosine residues. We found out that lawsone exposure for both 24h and 48 h causes DNA hypomethylation at higher evaluated concentration.

Epigenetic modifications, including DNA methylation, histone modifications, and chromatin remodelling, can be affected by different environmental factors. All these

epigenetic traits can cause an alteration of gene expression without modification to the underlying genetic sequence [15]. DNA methylation is nowadays intensively studied epigenetic mark. The correlation between DNA methylation and gene inactivity has been reported [16]. In mammals and plants, transcriptionally inactive heterochromatin is associated with the hypermethylation of DNA at CG sites. In contrast to this, DNA hypomethylation is characteristic for active euchromatin [17]. One of the stress factors that can change DNA methylation are ROS. Therefore we presume that changes in DNA methylation level observed after lawsone exposure may be caused by overproduction of ROS.

REFERENCES

- [1] P. Babula, R. Mikelova, V. Adam, D. Potesil, J. Zehnalek, R. Kizek, L. Havel, and Z. Sladky, "Chromatographic analysis of naphthoquinones in plants," *Chem. Listy*, vol. 100, pp. 271-276, Jan. 2006.
- [2] P. Babula, V. Adam, L. Havel, and R. Kizek, "Noteworthy secondary metabolites naphthoquinones – their occurrence, pharmacological properties and analysis," *Curr. Pharm. Anal.*, vol. 5, no. 1, pp. 47-68, Jan. 2009.
- [3] P. Babula, V. Adam, R. Kizek, Z. Sladky, and L. Havel, "Naphthoquinones as allelochemical triggers of programmed cell death," *Environ. Exp. Bot.*, vol. 65, pp. 330-337, March 2009.
- [4] S. M. G. Saeed, S. A. Sayeed, S. Ashraf, S. Naz, R. Siddiqi, R. Ali, and M. A. Mesaik, "A new method for the isolation and purification of lawsone from *Lawsonia inermis* and its ROS inhibitory activity," *Pak. J. Bot.*, vol. 45, no. 4, pp. 1431-1436, Aug. 2013.
- [5] R. Sauriasari, D. H. Wang, Y. Takemura, K. Tsutsui, N. Masuoka, K. Sano, M. Horita, B. L. Wang, and K. Oqino, "Cytotoxicity of lawsone and cytoprotective activity of antioxidants in catalase mutant *Escherichia coli*," *Toxicology*, vol. 235, no. 1-2, pp. 103-111, Jun. 2007.
- [6] H. Sano, "Inheritance of acquired traits in plants. Reinstatement of Lamarck," *Plant Signal. Behav.*, vol. 5, no. 4, pp. 346-348, Apr. 2010.
- [7] H. H. Ng, and A. Bird, "DNA methylation and chromatin modification," *Curr. Opin. Genet. Dev.*, vol. 9, no. 2, pp. 158-163, Apr. 1999.
- [8] R. A. Martienssen, and V. Colot, "DNA methylation and epigenetic inheritance in plants and filamentous fungi," *Science*, vol. 293, no. 5532, pp. 1070-1074, Aug. 2001.
- [9] T. Murashige, and F. Skoog, "A revised medium for rapid growth and bio assays with tobacco tissue cultures," *Phys. Plant Pathol.*, vol. 15, no. 3, pp. 473-497, July 1962.
- [10] T. Nagata, Y. Nemoto, and S. Hasegawa, "Tobacco BY-2 cell-line as the HeLa-cell in the cell biology of higher plants," *Int. Rev. Cytol.*, vol. 132, pp. 1-30, 1992.
- [11] M. Karimi, S. Johansson, D. Stach, M. Corcoran, D. Grandér, M. Schalling, G. Bakalkin, F. Lyko, C. Larsson, and T. J. Ekström, "LUMA (Luminometric Methylation Assay)-A high throughput method to the analysis of genomic DNA methylation," *Exp. Cell Res.*, vol. 312, no. 11, pp. 1989-1995, July 2006.
- [12] F. Q. Zhang, Y. S. Wang, Z. P. Lou, and J. D. Dong, "Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorrhiza*)," *Chemosphere*, vol. 67, no. 1, pp. 44-50, Feb. 2007.
- [13] S. Oramas-Royo, C. Torrejón, I. Cuadrado, R. Hernández-Molina, S. Hortelano, A. Estévez-Braun, and B. de las Heras, "Synthesis and cytotoxic activity of metallic complexes of lawsone," *Bioorg. Med. Chem.*, vol. 21, no. 9, pp. 2471-2477, May 2013.
- [14] A. Esteves-Souza, D. V. Figueiredo, A. Esteves, C. A. Camara, M. D. Vargas, A. C. Pinto, and A. Echevarria, "Cytotoxic and DNA-topoisomerase effects of lapachol amine derivatives and interactions with DNA," *Braz. J. Med. Biol. Res.*, vol. 40, no. 10, pp. 1399-1402, Oct. 2007.
- [15] S. A. Tammen, S. Friso, and S. W. Choi, "Epigenetics: The link between nature and nurture," *Mol. Aspects Med.*, vol. 34, no. 4, pp. 753-764, Jul.-Aug. 2013.
- [16] A. Razin, and H. Cedar, "DNA methylation and gene expression," *Microbiol. Mol. Biol. Rev.*, vol. 55, no. 3, pp. 451-458, Sep. 1991.

- [17] M. Tariq, and J. Paszkowski, "DNA and histone methylation in plants,"
Trends Genet., vol. 20, no. 6, pp. 244-251, Jun. 2004.