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The antiproliferative effect of pinostrobin on human umbilical vein endothelial cells (HUVEC)

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Abstract. – BACKGROUND: Atherosclerosis and stent re-stenosis are problems that are accompanied with high morbidity and mortality. Endothelial cell proliferation plays a role in both diseases, so the quest for potent inhibitors is still ongoing.

AIM: The flavonoid pinostrobin previously showed cytotoxic effects on different cell lines. In this investigation, we tested the antiproliferative effect of pinostrobin on human umbilical vein endothelial cells (HUVEC).

MATERIALS AND METHODS: The effect of pinostrobin on human umbilical vein endothelial cells after 1 hour and after 48 hours of treatment was tested. A dose- and time-dependent antiproliferative effect of pinostrobin was observed.

RESULTS: After 1 hour of treatment, no significant differences between the control group and the cells treated with pinostrobin could be detected. After 48 h of pinostrobin treatment, the number of cells decreased significantly. Higher doses had stronger inhibitory effects on the proliferation. Furthermore, we tested the change of membrane potential on cells that were treated with different concentrations of pinostrobin. We could show that the change of membrane potential was also time- as well as dose-dependent.

CONCLUSIONS: Our hypothesis is that pinostrobin leads to depolarisation of the cell potential of endothelial cells. Since the membrane potential remains less negative, this could lead to instability of the membrane, resulting in cell death.

Key Words:
Pinostrobin, HUVEC, Endothelial, Proliferation, Membrane potential.

Introduction

Atherosclerosis is one of the main causes of vascular obstruction, leading to hypoxia of the following tissues. Furthermore atherosclerosis leads to myocardial infarction, if persistent in the coronary artery system[1]. The pathogenesis of atherosclerotic plaque formation includes imbalance of the lipid metabolism, maladaptive immune response, chronic inflammation at the vessel wall and endothelial dysfunction[2,3]. Treatment of atherosclerosis by now includes life style changes, medical treatment and stent implantation in cause of symptomatic obstruction, especially in the heart. Proliferation of endothelial cells leads to stent re-obstruction[4]. Although there are several drugs to prevent and treat stent re-obstruction and atherosclerosis, the quest for new therapies is an ongoing process.

The flavonoid pinostrobin (Sigma Aldrich Corporation, St. Louis, MO, USA) is a (S)-2,3-dihydro-5-hydroxy-7-methoxy-2-phenyl-4H-1-benzopyran-4-one (Figure 1). The Molecular Weight amounts to 270.28[5]. In the past, the flavonoid pinostrobin has been isolated from different plants. Previously, it has been extracted from *Artemisia campestris*[6], *Boesenbergia pandurata*[7], *Boesenbergia rotunda*[8], *Sarcandra glabra*[9], dried leafs of *Polygonum ferrugineum*[10], *Cajanus cajan*[11] and *Polygonum lapathifolium*[12]. Even from honey, the isolation of pinostrobin succeeded[13].

Previous experiments showed that pinostrobin has a cytotoxic effect on different cell lines. The apoptotic response has been tested in leukemic cells. The study showed that the effect of pinostrobin is dose-dependent. With rising concentration of this flavonoid, the number of apoptotic cells increased[12]. Furthermore, pinostrobin showed an antitumor activity in cell culture of human mammary carcinoma. DNA topoisomerase I activity could be inhibited by pinostrobin[14].

Another study showed the effect of pinostrobin on herpes simplex virus-1. Herpes simplex virus-1 could be inhibited by the tested flavonoid

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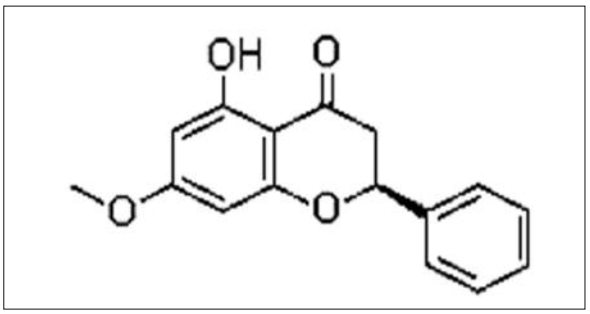

Figure 1. Molecular structure of pinostrobin.

pinostrobin[15]. Moreover, another investigation lead to the conclusion that voltage-gated sodium channels of mammalian brain could be inhibited by pinostrobin[16].

Furthermore, one report showed the ability of pinostrobin to reduce the viability of amastigote stages of Leishmania amazonensis[17]. Researches showed that the flavonoid pinostrobin induces phase 2 detoxication enzymes in mammals[13].

All these previous researches show that the flavonoid pinostrobin has various effects in different cells. It has also been proven that pinostrobin has a strong antiproliferative and cytotoxic effect in several cell types. Pinostrobin might as well act antiproliferative on HUVEC, which would lead to the conclusion that it could become an important part in atherosclerosis treatment.

Materials and Methods

Isolation and Cultivation of Human Umbilical Vein Endothelial Cells (HUVEC)

Endothelial cells were isolated from human umbilical cord veins by a collagenase digestion procedure. After isolation the endothelial cells were cultivated on 12 well plastic plates in Endothelial Cell Basal Medium (Promocell, C-22210, Heidelberg, Germany) added to Endothelial Cell Growth Medium (Promocell, C-39210) and Gentamycin (Promocell, C-42060). After 24 hours, the medium was replaced by serum-free medium, which contains Endothelial Cell Basal Medium (Promocell, C-22210), Gentamycin (Promocell, C-42060) and hydrocortisone (HC 500, Promocell C-31061).

Proliferation Experiments

Another 24 hours later, cells were stimulated with different concentrations of pinostrobin [solved in dimethyl sulfoxide (DMSO)] in Medium. To determine the effect of pinostrobin on HUVEC cells after 48 hours, we used pinostrobin concentrations of 1 µmol/l, 10 µmol/l, 50 µmol/l, 100 µmol/l, 500 µmol/l and 1 mmol/l. In addition, we conducted experiments to determine the effect of two pinostrobin concentrations on the cells, depending on the time of exposure. Therefore, we added pinostrobin at concentrations of 100 µmol/l and 500 µmol/l to the cells and analysed the number of cells after 1 hour and after 48 hours. At the end of the experiments, cells were washed twice in Hank's BSS (balanced salt solution) (PAA, H15-009), trypsinized and counted.

Membrane Potential Measurements

The membrane potential of a huge conglomeration of cells was determined using the plate reader TECAN INFINITE M200 (Tecan, Grodig, Austria).

Statistical Analysis

A post-hoc Tuckey's test was conducted to determine the significance of the results. $p < 0.05$ was considered statistically significant

Results

Inhibition of Proliferation by Pinostrobin is Dose-Dependent

The proliferation of HUVEC cells was reduced after 48 hours of pinostrobin treatment. Higher doses had stronger effects on the proliferation. Compared to control, 1 µmol/l pinostrobin decreased the number of cells by 11%, which was not significant. The following concentration induced significant decreases in cell number. 10 µmol/l lead to a decrease of 37%, 50 µmol/l reduced the cells by 50%, 100 µmol/l by 65% and 500 µmol/l by 87%. The highest reduction was seen by a concentration of 1 mmol/l pinostrobin. Here, the cells were reduced by 99% (Figure 2).

Inhibition of Proliferation by Pinostrobin is Time-Dependent

The proliferation of HUVEC was significantly reduced by pinostrobin in concentrations of 100 µmol/l and 500 µmol/l after 48 hours, whereas the effect after 1 hour of treatment did not show any significant differences (Figure 3).

Changes in Membrane Potential

The reaction of cells treated with pinostrobin concentrations of 1 µmol/l, 10 µmol/l and 25

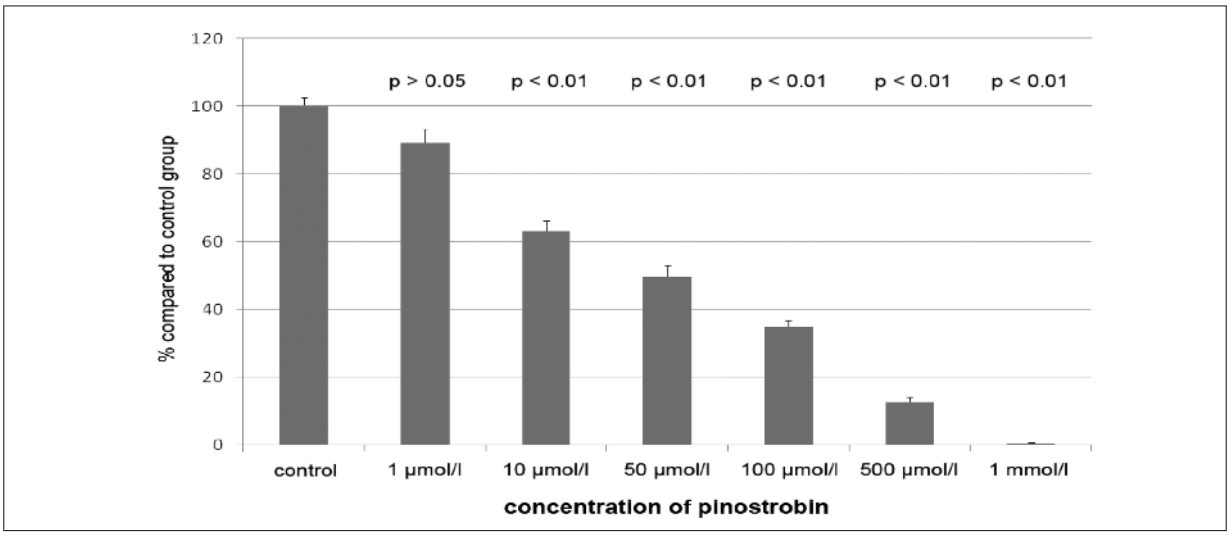

Figure 2. Relative number of cells after 48 hours of treatment with different concentrations of pinostrobin compared to untreated control group; $p > 0.05$: not significant; $p < 0.01$: highly significant.

µmol/l did not show significant differences, compared to the control group. The values for 50 µmol/l and 100 µmol/l start on higher levels than the control values. After 2 minutes, the curves converge at about 11% resp. 30% higher than control values. 300 µmol/l and 500 µmol/l reach their highest values after 10 minutes. The curve of 300 µmol/l starts 85% higher than control curve. Maximum value is 128% higher than control. The values converge at 106%. 500 µmol/l started at 111% of control values, maximum value is 197% of control value. The values converge at 154%. The highest concentrations of 700 µmol/l, 900 µmol/l and 1000 µmol/l displayed

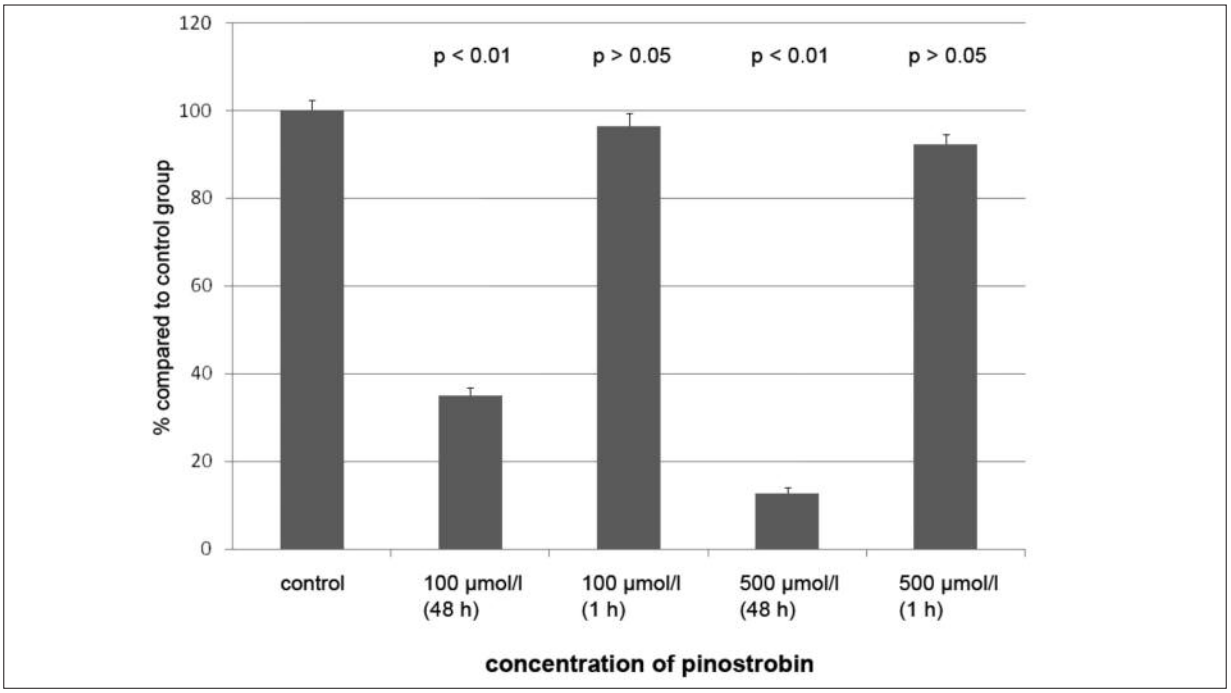

Figure 3. Relative number of cells after 1 hour resp. 48 hours of treatment with different concentrations of pinostrobin compared to untreated control group; $p > 0.05$: not significant; $p < 0.01$: highly significant.

similar membrane potential changes, although the intensity of those varied widely. At 700 µmol/l first value was 198% of control, maximum after 5 min at 271% and converges at about 161%. At 900 µmol/l first value was 236%, maximum after 5 min was 333 and converges at 193%. At 1000 µmol/l first value was 229%, maximum after 6 min was 377% and converges at 202% (Figure 4). These changes represent potential changes of the conglomerate of cells in the measuring chamber. It is not possible to deduce from these changes to the amount of the potential change of a single cell.

Discussion

To our knowledge, this is the first work to describe the effect of pinostrobin on endothelial cells. Due to this reason; the study was conducted without having any data about lipidic parameters or cell viability. Previous experiments revealed an antiproliferative effect of pinostrobin on leukaemia cells[12] and mamma carcinoma cells[14]. This effect was mainly conducted by inhibition of the DNA topoisomerase A. As carcinoma cells are abnormal cells with high proliferation rates, it is interesting whether pinostrobin is also able to affect normal cells. Additionally, recent discoveries concerning pinostrobin revealed that it can also prevent cells from cytotoxicity[11]. In that case, neurotoxicity of β-amyloid in PC12-cells was reduced.

Therefore, and as endothelial cell proliferation is important in the development of atherosclerosis, it is of utmost interest to test the effect of pinostrobin on endothelial cells. We utilized a HUVEC cell line. Treatment with rising concentrations of the flavonoid lead to decreasing proliferation rates of the cells, which seems to be due to its antiproliferative effects. High concentrations reduced the cell numbers significantly, indicating not only antiproliferative but also cytotoxic effects of pinostrobin, as has already been described in other cells[12,14].

The following experiments aimed to clarify whether the antiproliferative and cytotoxic effects of pinostrobin were solely dose or also time dependent. The time dependency of the effects of the flavonoid in HUVEC has not been investigated so far. Our findings indicate that the effect of Pinostrobin is higher after 48 hours compared to the values after 1 hour.

Additional experiments revealed that the membrane potential of HUVEC is strongly influenced by pinostrobin. The change of the membrane potential was correlated to the concentration of the flavonoid. The greatest change was achieved by adding high concentrations of pinostrobin. The membrane potential remained less negative throughout the whole investigation, especially under high concentrations of pinostrobin.

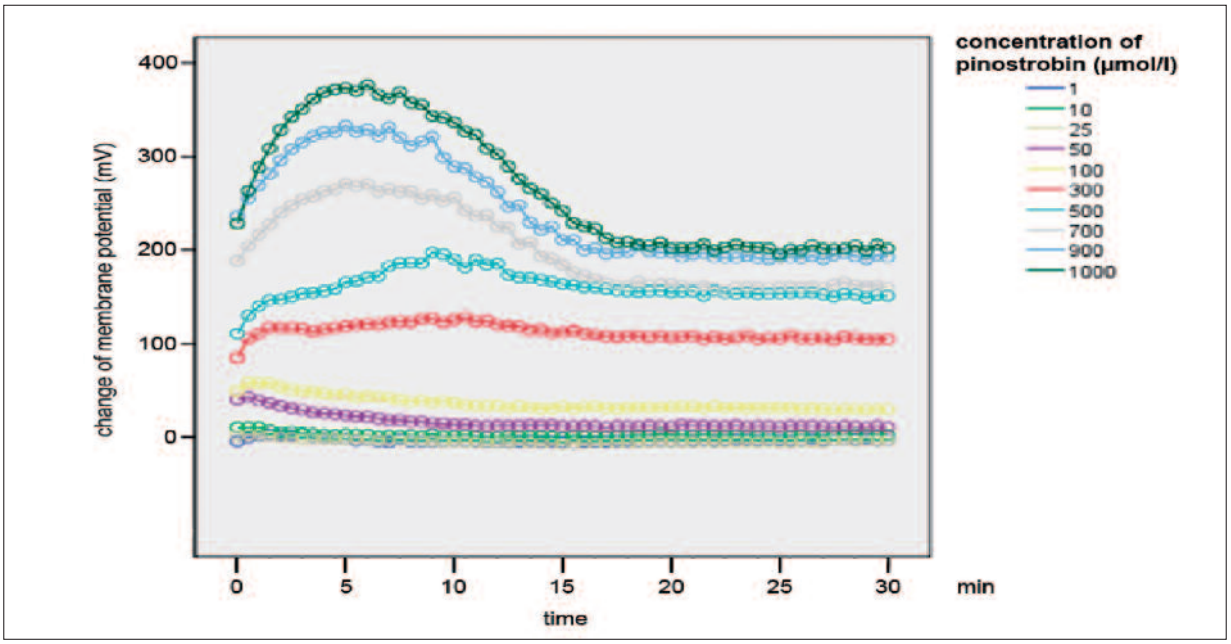

Figure 4. Change of fluorescence measured with TECAN INFINITE M200 over 30 minutes.

Conclusions

Pinostrobin leads to depolarisation of the membrane of endothelial cells. As the membrane potential remains less negative, this could lead to instability of the membrane, resulting in cell death. This hypothesis is supported through the facts that the effect of pinostrobin is time dependent as well as dose dependent.

This work gives basis for more experiments in order to identify the potential of pinostrobin in the treatment and prevention of atherosclerosis. Further experiments regarding the effect of pinostrobin *in vivo* will follow.

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