ORIGINAL ARTICLE

Structure-efficiency relationship in derivatives of stilbene. Comparison of resveratrol, pinosylvin and pterostilbene

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Abstract

OBJECTIVES: Oxidative stress is related to a number of autoimmune diseases, e.g. rheumatoid arthritis, cancer, etc. The main source of pathologically working reactive oxygen species (ROS) are activated polymorphonuclear leukocytes (PMNL).
OBJECTIVE: There are some papers comparing structure – pharmacological efficiency relationship of vegetal substances from the stilbenoid group. We compared the effect of trans-resveratrol, which is well-known by its antioxidative activity, with the effect of pinosylvin and pterostilbene.
METHODS: Luminol-enhanced chemiluminescence (CL) was used to study the antioxidative action. The effect was observed in whole blood and in isolated PMNL. The concentrations of substances tested were 0.01–100 μM. Due to the different abilities of luminol and isoluminol to pass through the cell membrane, we studied the effect of the substances tested on intracellular and extracellular ROS. To stimulate the production of ROS we used phorbol-myristate-acetate (PMA), which activates PMNL *via* protein kinase C.
RESULTS: Resveratrol, pinosylvin and pterostilbene inhibited significantly the CL of whole blood and extra- and intracellular CL of isolated PMNL in a dose-dependent manner. Depending on different functional groups of the stilbene molecule, resveratrol inhibited CL of whole blood and isolated PMNL, whereas pinosylvin influenced mainly intracellular CL and pterostilbene extracellular CL.
CONCLUSION: The presence of different functional groups in the molecules of stilbenoids influence their antioxidative effect. Modification of these functional groups may result in derivatives with required antioxidative properties, targeting mainly extracellular ROS which are responsible for tissue damage during chronic inflammation.

Abbreviations

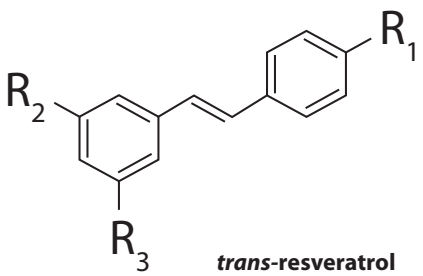
CL - chemiluminescence
ROS - reactive oxygen species
PMA - phorbol-myristate-acetate
PMNL - polymorphonuclear leukocytes

INTRODUCTION

Resveratrol was first isolated from *Veratrum grandiflorum O. Loes* and it is wide-spread in many plant species. In plants it works as phytoalexin, i.e. it protects the plant against infections. Aggarwal *et al.* (2004) mentioned the suppressive effect of resveratrol on inflammation, adhesion and angiogenesis, on the other side it induces apoptosis. Its effect on tumour cells makes this compound an eligible anticancer stuff. It can also suppress transcription factors, e.g. NF-κB, AP-1, etc. This molecule possesses strong antioxidative properties. Resveratrol is well-known because of the „French paradox" – lower incidence of cardiovascular diseases in red wine drinkers (Aggarwal *et al*., 2004; Šmidrkal *et al*., 2001; Lastra and Villegas 2005).

Structural analogues of resveratrol may possess some of these effects and potentially even more benefits. Pinosylvin and pterostilbene are chemically related to resveratrol (Figure 1). They are also wide-spread in variety of plants. Pterostilbene is studied because of anticancerous, antiinflammatory and antioxidative properties. *Pterocarpus marsupium*, which contains pterostilbene is used in traditional medicine for treatment of diabetes. Glover *et al*., demonstrated the ability of the extract from *P. marsupium* and pterostilbene alone to decrease blood glucose (Remsberg *et al*., 2008). Pinosylvin has been studied because of its anticancer, antifungal and antioxidative properties (Roupe *et al*., 2006).

In our work we synchronised the antioxidative effects of these three substances, focusing on their activity in extracellular and intracellular space of isolated human PMNL and in whole human blood. During inflammation, professional phagocytes, e.g. PMNL, are recruited from the circulation to tissues where they produce ROS using the membrane-asociated enzyme NADPH-oxidase. The activity of NADPH-oxidase in dormant



	R_1	R_2	R_3
***trans*-resveratrol**	-OH	-OH	-OH
pterostilbene	-OH	$-OCH_3$	$-OCH_3$
pinosylvin	-H	-OH	-OH

Figure 1. Structure of stilbene derivatives

PMNL is rapidly activated by a variety of mediators in the process called oxidative burst (Jang *et al*., 1999; Lastra and Villegas 2005). ROS are formed to eliminate intruding agents. However, ROS are generated both intracelullarly and extracellularly. Intracellular ROS are produced to phagosome, whereas extracellular ROS are produced outside the cell and thus may damage surrounding tissue. The latter is involved in chronic inflammation injury.

Finally, the cytotoxicity of the substances tested was measured using ATP-release test.

MATERIAL AND METHODS

trans-resveratrol, pinosylvin and pterostilbene were synthetised in the Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic. Luminol and isoluminol, D-luciferin and luciferase, phorbol-myristate-acetate, horseradish peroxidase, superoxide dismutase and catalase were purchased from Sigma, Germany.

Isolation of PMNL

Blood was collected in 2×9 ml citrate tubes, purified by 3% dextrane centrifugation and separated on Lymphoprep (Fresenius, Norway) (Jančinová *et al*., 2006). The erythrocytes were removed with hypotonic, cold haemolysis. Cells were washed with phosphate-buffered saline before counting on Counter (Beckman Coulter). Concentration of cells in suspension was 10×10^6 cells/ml. The sample contained 500 000 PMNL/250 µl.

Measurment of chemiluminescence

CL of whole human blood and isolated PMNL was measured in 96-well microplate on Luminometer Immunotech LM-01T at 37 °C (Nosáľ *et al*., 2006) after adding phorbol-myristate-acetate (PMA, F.C. = 0.05 µM). Total volume of the sample was 250 µl. To ensure sufficient concentration of extracellular peroxidase in stimulated cells, we added horseradish peroxidase (HRP, F.C. = 8 U/ml). To examine extracellular CL we used isoluminol. In examination of intracellular CL we added catalase (2 000 U/ml) and superoxide dismutase (100 U/ml) to luminol because of elimination of extracellular ROS. In case of whole blood the samples were tested for 1 hour, in both extracellular and intracellular measurment it was 30 minutes. We examined the area under the curve.

Dose-response toxic effect of substances tested

To investigate the cytotoxic effect of concentrations of the substances tested we used ATP-release test. Per sample 30 000 PMNL were incubated with 1, 10 and 100 µM substances tested for 15 minutes in dark (37 °C). Then 10 µl of luciferin-luciferase solution was added and CL was immediatly measured for 60 seconds. The amount of released ATP was calculated from the calibration curve.

Table 1. Effect of resveratrol (RES), pterostilbene (PTE) and pinosylvin (PIN) on the CL of whole human blood shown as percentage of inhibition.

	Inhibition of whole human blood chemiluminescence (%), Mean ± SEM, n = 6.				
(μM)	**0.01**	**0.1**	**1**	**10**	**100**
RES	3.33±3.79	–1.65±3.63	17.39±2.99	67.83±2.13	94.34±0.50
PTE	–5.18±5.21	7.92±3.99	28.59±2.82	92.05±0.73	96.09±0.46
PIN	4.65±1.72	7.04±3.97	–0.71±8.63	17.73±11.74	70.33±2.01

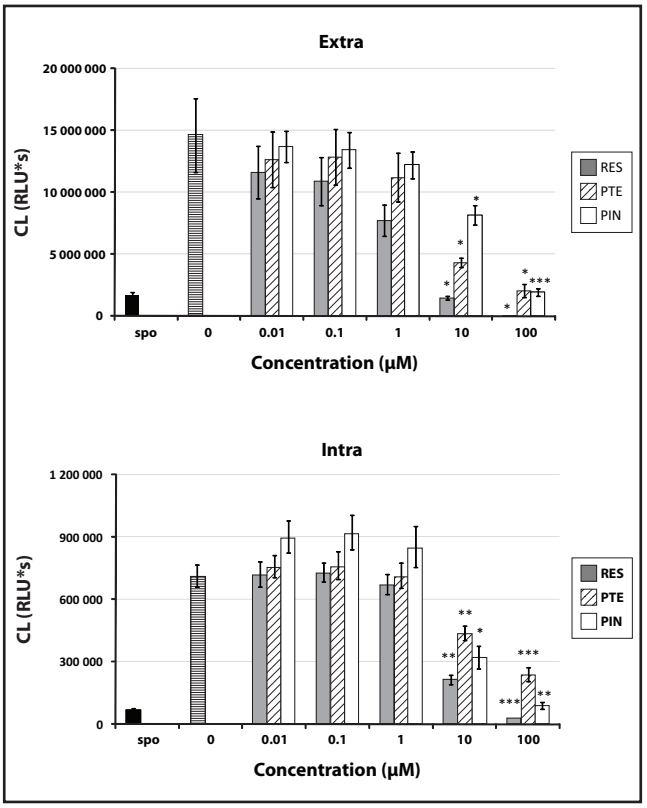


Figure 2. Inhibition of extracellular and intracellular CL of isolated human PMNL stimulated with PMA (0.05 μM). Mean ± SEM, n=6. RES - resveratrol, PIN - pinosylvin and PTE - pterostilbene. *p<0.05; **p<0.01; ***p<0.001

Statistics

Data were examined using the Student t-test and p-values below 0.05 were considered statistically significant. Standard Error of the Mean (SEM) was calculated as standard deviation divided by square root of number of sample size.

RESULTS

All three substances tested inhibited stimulated (PMA, 0.05 μM) whole human blood CL in dose-dependent manner (see Table 1). Significant inhibition of whole human blood CL was reached at concentration 1 μM for resveratrol and pterostilbene (p<0.05) and at 100 μM for pinosylvin (p<0.001), in comparison with control. According to the dose-response course in whole human blood, pterostilben was found to be the most effective, followed by resveratrol and the least efficient was pinosylvin.

Figure 2 shows the effects of the substances tested on extra- and intra-cellular CL of isolated human PMNL stimulated with PMA (0.05 μM). Resveratrol and pterostilbene were more effective than pinosylvin in extracellular space of human PMNL in the 0.01–10 μM concentration scale. However, the most effective concentration tested was 100 μM in all three substances, reaching 99% inhibition for resveratrol, and 87% for both pinosylvin and pterostilbene. The effective rank of the substances tested in extracellular space of PMNL is resveratrol > pterostilbene > pinosylvin.

In the intracellular milieu of human PMNL, no effects of the substances tested were observed at concentrations 0.01–1 μM (Figure 2). However, the effects differed at concentrations 10 and 100 μM, i.e. the effect of resveratrol and pinosylvin increased rapidly, whereas pterostilben was less operative. The effective rank was: resveratrol ≥ pinosylvin > pterostilbene. This may be due the different ability of the substances tested to pass through the cell membrane. There was considerable evidence that the most effective substance on isolated neutrophils was resveratrol. The effect of pterostilbene approaches that of resveratrol in extracellular space, while in intracellular space of PMNL, the effect of pinosylvin was comparable to that of resveratrol. This difference is discussed below.

None of the substances tested at concentrations 1, 10 and 100 μM did affect cell viability measured by ATP-release test (data not shown).

DISCUSSION

The structure – effect relationship is well known. Stilbenes are naturally occurring more in Z form, which is also more effective compared to the E form (Aggarwal *et al*., 2004; Šmidrkal *et al*., 2001). The molecule of stilbene is conjugated. Depending on the character of the substituent, phenols in stilbene could be either saturated or drawn off with the electrons. This may influence electron donor/acceptor properties of stilbene derivatives and thus the antioxidative activity.

Taking into account the structure of ***trans*-resveratrol**, which is **3,5,4´- trihydroxystilbene**, we could discuss the effect of substitution. Resveratrol is actually well known because of its anticancerous, antiinflammatory and antioxidative properties and may inhibit activated immune cells, mainly PMNL (Lastra and Villegas 2005; Roupe *et al*., 2006). In our study we investigated the antioxidative effect of resveratrol both in extra- and intra-cellular space of PMNL (Figure 2). Partition coefficient (logP) of resveratrol is 3.1 (Haneke 2002) and

thus it can enter the cell. As shown below, the number and position of hydroxyl and methoxyl groups may play a role in ROS inhibition, which is consistent with Jang *et al*. (1999).

Removal of the hydroxyl group from resveratrol in position 4´ results in **pinosylvin**. By this change, pinosylvin is more lipophilic than resveratrol, with logP 3.8. At the concentration of 10 μM, it was the least effective of the substances tested in whole blood (Table 1), but it is still antioxidatively active. In our experiment, pinosylvin at the concentration 10 and 100 μM was as effective as resveratrol at the same concentrations in intracellular space of PMNL (Figure 2). We could hypothesise that the removal of 4´-OH does not affect intracellular antioxidative activity. However, the 4´-OH group is necessary for a strong whole blood and extracellular antioxidative effect, which is in agreement with other studies (Roupe *et al*., 2006). Stojanovič *et al*. (2001) discussed this finding for *trans*-resveratrol, indicating that its *para*-hydroxyl group dominated in the radical-scavenging efficiency whereas its *meta*-hydroxyl groups showed only minor reactivity. Our study indicates that the *para*-hydroxyl group is not required in intracellular space activity.

Changing resveratrol in a different way, i.e. methoxylation in 3,5 – position, leads to **pterostilbene** (logP 4.1). Pterostilbene is known to scavange DPPH radical (Remsberg *et al*., 2008). In our study, pterostilbene at the concentration 10 μM was the most effective of the substances tested in inhibiting stimulated whole blood CL. But its activity was lower than that of resveratrol in extracellular space of isolated PMNL (Figure 2). Despite the highest lipophilicity among the substances tested, pterostilbene was the least effective against intracellular CL of isolated PMNL. This may be due the requirement of free 3,5-OH groups in intracellular activity. The results are indicating that 3,5-*meta*-methoxyl groups increase whole blood antioxidative activity but decrease the extracellular and especially intracellular activity compared to resveratrol. Thus logP is not the only condition operative in intracellular activity.

CONCLUSION

Numerous previous studies have demonstrated the relationship between the molecular structure of some compound and its effect. Changing the substituents of a molecule is the basic algorithm in synthesis of new effective molecules with desired properties. On doing this we could design molecules with specific actions *in vitro.* In our study we showed the effect of different molecular structure on antioxidative actions of resveratrol, pinosylvin and pterostilbene in various cell microspaces. This may be used to advantage in target cell approach.

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REFERENCES

1 Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y (2004). Role of Resveratrol in Prevention and Therapy of Cancer: Preclinical and Clinical Studies. Anticancer Res. **24**: 3–60.
2 Haneke KE (2002). *trans*-Resveratrol [501-36-0] Review of Toxicological Literature. Integrated Laboratory Systems.
3 Jančinová V, Drábiková K, Nosáľ R, Holomáňová D (2006). Extra- and intracellular formation of reactive oxygen species by human neutrophils in the presence of pheniramine, chlorpheniramine and brompheniramine. Neuroendocrinol Lett. **27**(2): 141–143.
4 Jang DS, Kang BS, Ryu SY, Chang IM, Min KR, Kim Y (1999). Inhibitory Effects of Resveratrol Analogs on Unopsonized Zymosan-Induced Oxygen Radical Production. Biochem Pharmacol. **57**: 705–712.
5 Lastra CA, Villegas I (2005). Resveratrol as an anti-inflammatory and anti-aging agent: Mechanisms and clinical implications. Mol Nutr Food Res. **49**: 405–430.
6 Nosáľ R, Drábiková K, Jančinová V, Mačičková T, Pečivová J, Holomáňová D (2006). On the pharmacology and toxicology of neutrophils. Neuroendocrinol Lett. **27**(2): 148–151.
7 Remsberg CM, Yáñez JA, Ohgami Y, Vega-Villa KR, Rimando AM, Davies NM (2008). Pharmacometrics of Pterostilbene: Preclinical Pharmacokinetics and Metabolism, Anticancer, Antiinflammatory, Antioxidant and Analgesic Activity Phytother Res. **22**: 169–179.
8 Roupe KA, Remsberg CM, Yáñez JA, Davies NM (2006). Pharmacometrics of Stilbenes: Seguing Towards the Clinic. Curr Clin Pharmacol. **1**(1): 81–101.
9 Šmidrkal J, Filip V, Melzoch K, Hanzlokova I, Buckiova D, Krisa B (2001). Resveratrol. Chem Listy. **95**: 602–609.