THE EFFECT OF PHENOLIC AND POLYPHENOLIC COMPOUNDS ON THE DEVELOPMENT OF DRUG RESISTANCE

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The effect of two phenolic compounds vanillin (4-hydroxy-3-methoxybenzaldehyde) and lignin on the development of drug/antibiotic resistance in *Salmonella typhimurium* was studied. Using the modified Ames test we have shown that vanillin alone has negligible effect on spontaneous mutability to ciprofloxacin and gentamicin resistance. At the tested concentrations vanillin reduces the toxicity of 4-nitroquinoline-*N*-oxide (4NQO) and reduces the ability of this compound to induce mutations leading to ciprofloxacin but not to gentamicin resistance. Lignin at higher concentrations increases mutagenicity to ciprofloxacin resistance and possess considerable inhibition effect on the spontaneous and 4NQO induced mutability to gentamicin resistance.

INTRODUCTION

The research of possibility how to reduce the frequency of both spontaneous and induced mutations in bacterial, somatic and germ cells is subject of interest for purely scientific reasons and for protection of human health. The occurrence of mutations is associated with fundamental life processes such aging, and variety of pathological conditions, including cancer and teratogenicity¹.

Mutations are also the primary cause of drug/antibiotic resistance. Bacteria have evolved diverse mechanisms of resistance to antimicrobial agents, including alteration and protection of target sites, modification and detoxification of drugs and control of uptake and efflux of drugs. Recent approaches to solving problem of resistance include identification of new target sites and antimicrobial potentiation.

A new approach consists in the prevention of the development of mutations leading to antibiotic resistance using antimutagenic agents. Coumadin and related compounds could prevent the development of antibiotic resistance². Sevag and Drable³ demonstrated that some polyamines (putrescine and spermidine) prevented development of resistance and Pillai *et al*⁴ showed that green tea catechins possess the potential to prevent or delay the development of resistance.

The purpose of this work was to examine the effect of two phenolic compounds vanillin and lignin on the mutagenesis leading to antibiotic resistance. Vanillin (4-hydroxy-3-methoxybenzaldehyde) a naturally occurring compound in vanilla beans has been used widely as a flavouring agent in confectioneries, beverages and food-stuffs. It has been demonstrated that vanillin showed strong antimutagenic effects on mutagenesis of 4NQO

and furylfuramide in *Escherichia coli*^{5,6} and suppressing effects on chromosome aberration by mitomycin C in cultured Chinese hamster cells *in vitro*⁷. The addition of vanillin to 4NQO pre-treated cells led to an increased growth of *E. coli* cells accompanied with a fall of revertant colonies being induced by this mutagen⁸. Vanillin has protective effect on radiation-induced micronuclei and chromosomal aberrations in V79 cells⁹, can protect against photosensitization-induced ssbs in the plasmid pBR322 DNA, and this effect may partly be due to its ability to scavenge ¹O₂ (ref.¹⁰). Shaughnessy *et al*¹¹ confirmed that vanillin require SOS repair genes to produce an antimutagenic effect against spontaneous mutagenesis. On the contrary, vanillin showed comutagenic effects with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) and *N*-methyl-*N*-nitroso-urea (MNU) induced mutagenesis in *E. coli* WP2s (ref.¹²). Lignin is present in the cell walls of all plants, including edible plants and due to its antioxidant effects and high adsorption activity towards mutagenic compounds has antimutagenic and anticarcinogenic properties. In the microbial system of *Salmonella typhimurium* lignin reduced mutagenicity of 2-amino-3-methylimidazo [4,5-f]quinoline (IQ) (ref.¹³), 4NQO, 2-nitrofluorene and hydrogen peroxide¹⁴. Lignin inhibited the ability of 4NQO and hydrogen peroxide to induce the SOS response¹⁴ and reduced DNA strand breaks in H₂O₂ and MNNG treated mammalian cells¹⁵.

MATERIAL AND METHODS

Bacterial strain: Salmonella typhimurium TA 98 was received from the Collection of Microorganisms, Masaryk University, Brno (Czech Republic).

Table 1. Effect of vanillin and lignin on the viability and on the spontaneous and 4NQO induced mutability to ciprofloxacin and gentamicin resistance

Concentration µg/ml		ciprofloxacin		gentamicin	
		No. of cells per culture	Frequency of mutants (RI) ^a	No. of cells per culture	Frequency of mutants (RI) ^a
Vanillin	0	8.96×10^7	1.33×10^7	7.40×10^7	2.73×10^6
	10	8.73×10^7	1.18×10^7	7.26×10^7	2.61×10^6
	50	8.46×10^7	1.44×10^7	7.04×10^7	2.51×10^6
	100	7.36×10^7	1.80×10^7	6.08×10^7	2.44×10^6
	500	7.26×10^7	1.96×10^7	5.89×10^7	1.92×10^6
Lignin	0	7.43×10^7	1.12×10^7	6.56×10^7	2.84×10^6
	10	6.99×10^7	0.94×10^7	6.03×10^7	1.50×10^6
	50	6.56×10^7	0.62×10^7	5.79×10^7	0.81×10^6
	100	4.74×10^7	1.82×10^7	4.16×10^7	0.83×10^6
	500	2.69×10^7	3.00×10^7	2.51×10^7	0.84×10^6
4NQO	0	8.42×10^7	0.98×10^7	5.08×10^7	3.12×10^6
	0.3	1.85×10^7	1.52×10^6	1.72×10^7	1.42×10^5
	0.6	0.97×10^7	2.31×10^6	0.82×10^7	1.74×10^5
	1.3	0.35×10^7	3.56×10^6	0.20×10^7	3.85×10^5
4NQO ^b and vanillin	0	1.42×10^7	2.94×10^6	1.15×10^7	1.35×10^5
	10	5.91×10^7	1.92×10^6	4.92×10^7	1.37×10^5
	50	5.61×10^7	1.56×10^6	4.19×10^7	1.21×10^5
	100	5.14×10^7	1.35×10^6	3.92×10^7	1.05×10^5
	500	4.53×10^7	1.15×10^6	3.34×10^7	1.27×10^5
4NQO ^b and lignin	0	2.05×10^7	3.11×10^6	1.85×10^7	1.28×10^5
	10	5.57×10^7	2.10×10^6	4.12×10^7	0.64×10^5
	50	4.27×10^7	0.50×10^6	3.79×10^7	0.47×10^5
	100	3.81×10^7	1.32×10^6	3.36×10^7	0.42×10^5
	500	2.48×10^7	1.51×10^6	2.19×10^7	0.37×10^5

^a The frequency of resistant mutants (resistance index RI) was expressed as the mean number of resistant colonies divided by the total number of viable cells per culture, ^b4NQO at concentration of 0.6 µg/ml. The results are mean of the at least three experiments with five parallels.

Chemicals: 4-nitroquinoline-*N*-oxide (4NQO) and vanillin (4-hydroxy-3-methoxybenzaldehyde) were purchased from Sigma-Aldrich (Germany) and the sample of lignin was obtained from Department of Chemical Technology of Wood, Pulp and Paper, Faculty of Chemical and Food Technology, Slovak Technical University, Bratislava, Slovakia.

Ames test: The test was performed using the classical plate incorporation method according to Maron and Ames¹⁶.

Estimation of mutation frequency to antibiotic resistance: 0.1 ml of overnight culture (cultivation for 16 h at 37 °C, approximate cell density $2-5 \times 10^8$ cells/ml), 0.1 ml of positive mutagen (4NQO), 0.1 ml of tested antimutagenic compound and 0.5 ml of phosphate buffer of pH 7.4 were mixed and preincubated at 37 °C for 30 min. In

tests for spontaneous mutability the positive mutagen was omitted. Then 0.8 ml of double strength nutrient broth was added and the cultures were incubated 3 h at 37 °C to allow a few cell divisions and protein expression to occur. 2 ml of melted top agar with an antibiotic were added and the mixture was poured on agar plates containing antibiotics at selective concentration ($2 \times \text{MIC}$). After 72 h incubation at 37 °C the number of resistant colonies was counted. The total number of viable cells was determined by plating an appropriate dilution of three cultures on nonselection medium. Plates were incubated aerobically for 24 h at 37 °C. The frequency of resistant mutants (resistance index RI) was expressed as the mean of resistant colonies divided by the total number of viable cells per culture.

Statistical evaluation of results: Experiments were repeated independently at least three times in five parallel estimations and results were statistically evaluated using the Student's *t*-test.

RESULTS AND DISCUSSION

The frequency of mutation for ciprofloxacin and gentamicin resistance ranged from 9.5×10^{-8} to 1.5×10^{-7} and from 2.5×10^{-6} to 3.3×10^{-6} , resp.

Vanillin alone doesn't possess a considerable effect on the mutability to ciprofloxacin or gentamicin resistance. As shown in Table 1, the resistance index of ciprofloxacin very slightly increased in the presence of vanillin and RI for gentamicin was slightly reduced (by about 30 %) at the highest used concentration of this phenolic compound.

4NQO induces the increase in the number of *his*⁺ revertants in the Ames test (data not shown) and causes a considerable increase of mutation rate to antibiotic resistance. The effective concentrations of 4NQO are comparable in both types of mutations. 4NQO at concentration of 0.6 µg/ml causes an increase in the ciprofloxacin (23 fold) and gentamicin (6 fold) resistant index for *Salmonella* strain. We used this concentration of 4NQO in the antimutagenicity studies.

Vanillin considerably reduced the toxicity of 4NQO and reduced the frequency of mutations to ciprofloxacin resistance by 35 % to 62 %, depending on the concentration. The ability of 4NQO to induce mutations to gentamicin resistance was reduced by vanillin very slightly.

Second phenolic compound tested for its possible preventive effect on the mutability for drug resistance – lignin, was more effective than vanillin.

Up to concentration of 50 µg/ml lignin was not toxic and reduced the number of spontaneous ciprofloxacin and gentamicin resistant mutants. At higher concentrations increased the mutation frequency to ciprofloxacin but not to gentamicin resistance.

The same course but with more considerable effect was noticed in the case of the influence of lignin on the 4NQO-induced mutagenicity. At the concentration of 50 µg/ml lignin reduced the RI induced by 4NQO by 83 % and at higher concentrations this reduction was less substantial.

The addition of lignin reduced the 4NQO induced mutagenesis to gentamicin resistance and a clear dose-response effect was notified. The resistance index was reduced to about 50 % still at concentration of 10 µg/ml and the use of higher concentrations of lignin resulted in a further decrease in mutation frequency (Table 1).

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