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Apoptosis and cytotoxicity caused by ethoxyquin salts in human lymphocytes in vitro

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Abstract

Cytotoxicity of four salts of ethoxyquin (EQ; 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline), an antioxidant used in various food products (mainly in animal feeds), was studied: ethoxyquin ascorbate, ethoxyquin hexanoate, ethoxyquin salicylate and ethoxyquin salt of Trolox C (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid). This study is part of the research aimed at searching for new potential preservatives, which was undertaken due to many unfavourable side-effects observed in animals fed with EQ containing feeds and in people working with it. In this study the trypan blue exclusion assay was used to study the viability of human cultured lymphocytes after 24-h treatment with the tested compounds, and the TUNEL method was applied to detect apoptotic DNA fragmentation. All the compounds tested decreased cell viability when they were used at the two highest concentrations: 250 and 500 μM. Significantly increased numbers of apoptotic cells were observed after treatments with ethoxyquin ascorbate, ethoxyquin hexanoate and ethoxyquin salicylate.

Keywords: Ethoxyquin; Preservatives; Cytotoxicity; Apoptosis; Human lymphocytes

1. Introduction

Ethoxyquin (EQ, 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) is an antioxidant used in various food products, mainly as a preservative in animal feeds, to effectively extend their shelf life. It is also applied to control scald in the apple and pear and to preserve colour of powdered paprika and chilli. However, in the late 1980' many unfavourable side-effects were observed in animals fed with EQ containing feeds and in people working with it (Alanko, Jolanki, Estlander, & Kanerva, 1998; Dzanis, 1991; Little, 1990; Rubel & Freeman, 1989). In 1997, after reviewing a study done voluntarily by Monsanto, a major manufacturer of EQ, FDA asked manufacturers to lower voluntarily the amount of EQ in animal feeds to 75 ppm (150 ppm was the permitted dose of EQ in the feed). These regulations triggered a search for new efficient antioxidants which might replace EQ (Błaszczyk & Skolimowski, 2005a; De Koning, 2002; Dorey, Lockhart, Lestage, & Casara, 2000). Two of the factors that might affect animal health are EQ's cytotoxicity and genotoxicity. It was earlier shown that EQ used at the concentrations of 250 μM and 500 μM decreased the viability of cultured human lymphocytes significantly; moreover, the concentrations of 100, 250, and 500 μM markedly increased the number of apoptotic cells compared with the control (Błaszczyk & Skolimowski, 2005a, 2005b). In the comet assay performed on human lymphocytes, strong DNA fragmentation was observed (Błaszczyk, 2006; Błaszczyk & Skolimowski, 2006). Abnormal chromosomes were also found in cell cultures treated with EQ (Błaszczyk, Osiecka, & Skolimowski, 2003). Recently performed experiments showed that converting EQ into a salt changed its biological properties. EQ hydrochloride and EQ phosphate were significantly less cytotoxic (Błaszczyk & Skolimowski, 2005b) and genotoxic

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than EQ, but unfortunately their antioxidant activities were also lower (unpublished results).

In this paper, which is part of the research aimed at searching for new potential preservatives and comparing them with EQ activities, the cytotoxic properties of other EQ salts synthesized in our laboratory are presented. The trypan blue exclusion assay was used to determine the viability of cultured lymphocytes and the TUNEL method to detect DNA fragmentation in apoptotic cells. The results will be helpful in selecting compounds for further detailed studies, which are necessary to estimate their usefulness as preservatives.

2. Materials and methods

2.1. Chemicals

The following ethoxyquin salts were synthesized in the Department of Organic Chemistry, University of Łódź (Poland): ethoxyquin L-ascorbate (EQ-C, purity >96%), ethoxyquin *n*-hexanoate (EQ-H, purity >98%), ethoxyquin salicylate (EQ-S, purity >98%) and ethoxyquin salt of Trolox C (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (EQ-T, purity >97%) (Fig. 1). The synthesis was performed with the use of earlier synthesized ethoxyquin (EQ; $C_{14}H_{19}NO$; 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline; CAS 91-53-2; purity >97%) (Błaszczyk & Skolimowski, 2005b) and with the use of L-ascorbic acid (Vitamin C, $C_6H_8O_6$; CAS 50-81-7; Sigma), *n*-hexanoic acid ($C_6H_{12}O_2$; CAS 142-62-1; POCH, Poland), salicylic acid ($C_7H_6O_3$; CAS 69-72-7, POCH, Poland) and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox C, $C_{14}H_{18}O_4$; CAS 53188-07-1, Fluka). The compounds were synthesized in general according to the method described elsewhere for other ethoxyquin salts (Błaszczyk & Skolimowski, 2005a, 2005b).

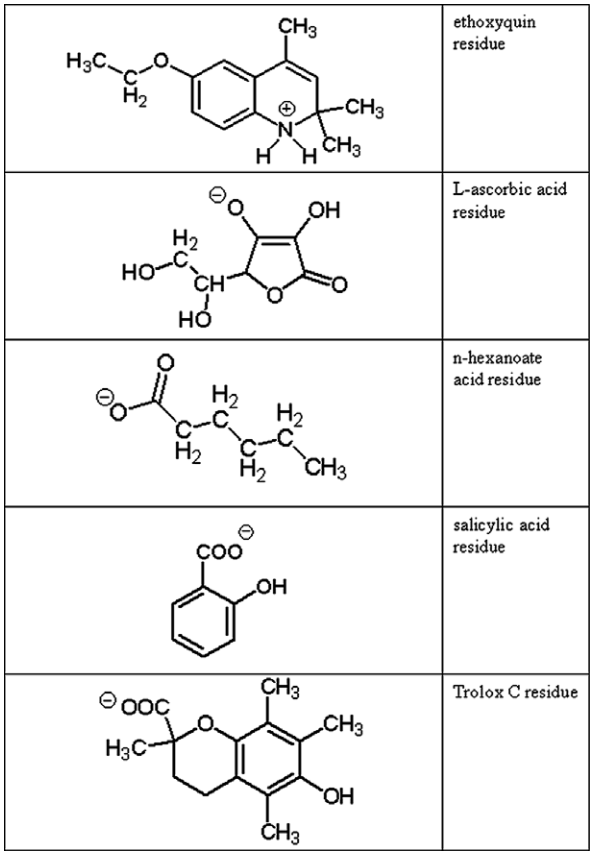

Fig. 1. Chemical structure of constituents of the studied salts.

RPMI 1640 medium, foetal calf serum, antibiotics (penicillin and streptomycin), trypan blue and propidium iodide were purchased from Sigma Chemical Co. (USA). Phytohaemagglutinin (PHA) was obtained from Gibco BRL (UK), and paraformaldehyde from Polysciences (USA). The DeadEnd™ Fluorometric TUNEL System detecting the fragmented DNA labelled at its 3′-OH ends with fluorescein in apoptotic cells was obtained from Promega (USA).

2.2. Lymphocyte isolations and cell cultures

Lymphocytes were isolated from peripheral blood of healthy non-smoking donors by centrifugation in a density gradient of Histopaque 1077 (15 min, 280*g*). The cells were washed with RPMI 1640 medium and the viability of the isolated lymphocytes measured by trypan blue exclusion assay was found to be about 99%. The cells were added to the RPMI 1640 medium supplemented with 15% foetal calf serum and 1% penicillin/streptomycin solution. The cells were stimulated with 1% phytohemagglutinin M. The final concentration of the lymphocytes in the medium was about 0.8×10^6 per ml.

2.3. Cell treatment

After 24-h incubation of the cells at 37 °C, EQ salts were added to the cultures at five concentrations: 25, 50, 100, 250, and 500 μM, followed by another 24-h incubation period. All EQ salts were dissolved in ethanol (EtOH) and diluted with medium to a final concentration of alcohol of 0.05%.

2.4. Cytotoxicity evaluation

The viability of the treated cells was determined by the trypan blue exclusion assay. The cells were centrifuged (7 min, 200*g*), washed with RMPI 1640 medium, and mixed with 0.4% trypan blue reagent. The percentage of viable cells was determined in a bright-field microscope (Olympus BX60F5). In each experiment 200 cells were analysed. The results are presented as the mean ± SD of three independent experiments.

To detect the fragmented DNA of apoptotic cells, the lymphocytes were fixed on the slides with 4% paraformaldehyde (Polysciences) and then incubated (1 h, 37 °C) with a mixture of TdT and fluorescein-labelled nucleotides. The analysis was performed using the fluorescence microscope