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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).

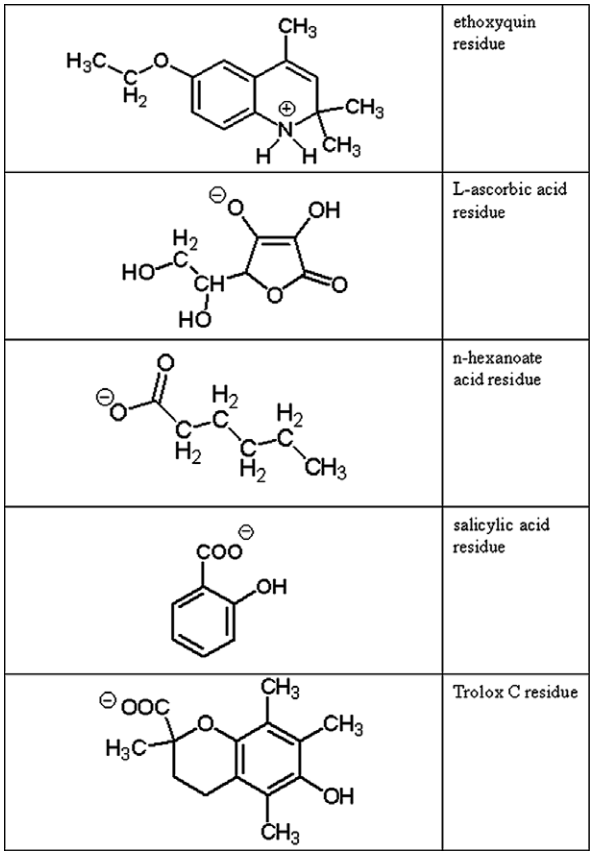

Fig. 1. Chemical structure of constituents of the studied salts.

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

(OLYMPUS BX60F5, 520 nm filter). The nuclei of apoptotic cells were dyed green, and propidium iodide treatment of the slides was used as counterstaining for all cells. The percentage of apoptotic cells was calculated for 1000 cells and the results are expressed as the mean ± SD of three independent experiments.

3. Results and discussion

The search for new antioxidant compounds, which could replace EQ showing harmful health effects in animals, resulted in the synthesis of many compounds with antioxidant activities (Błaszczyk & Skolimowski, 2005b; De Koning, 2002; Dorey et al., 2000; Skolimowska, Skolimowski, & Wedzisz, 2005; Skolimowska & Wedzisz, 2004). The first step in the studies on their properties and possible usefulness as preservatives is the cytotoxicity evaluation. The lymphocytes were incubated with the tested EQ salts and the changes in their viability were analysed using trypan blue staining. All the tested compounds affected the cell viability in the similar way (Fig. 2), so it seems that it depends only on compound concentration, but not on their structure. EQ salts used at the concentrations ranging from 25 to 100 μM did not significantly influence cell viability, except for EQ-C at 100 μM dose, which caused its statistically significant decrease (Fig. 2). Dose-effect dependences were seen after treatments with the concentrations higher than 50 μM. Low level of the cell viability was observed especially when the compounds were used at the concentration of 500 μM (Fig. 2). The trypan blue exclusion assay detects only dead cells with damaged cell membrane (necrotic cells), because trypan blue does not penetrate into living cells. During apoptosis, which is another mechanism of cell death, in contrast to necrosis the membrane integrity is not lost. Apoptotic cells show characteristic morphological and biochemical changes, among others DNA fragmentation. The TUNEL method used in this study detects free 3′-OH ends in DNA and thus apoptotic cells can be identified. The percentages of apoptotic cells determined after EQ salts treatments are shown in Fig. 3. The significant increase in the number of apoptotic cells with fragmented DNA was observed after the treatment with EQ-C, EQ-H and EQ-S when they were used at the concentration of 500 μM. At the lower concentrations studied the increase in the number of apoptotic cells was seen only after EQ-H treatments (100 and 250 μM). The results the two assays used showed that all the compounds tested caused cell death especially at the highest concentration used (500 μM) and that two mechanisms were involved: necrosis, preceded with the loss of membrane integrity, and apoptosis, in which the membrane was not disrupted. The apoptotic cells scored in the TUNEL assay could be those cells which in the trypan blue exclusion assay were identified as living ones with non-disrupted cell membrane. On the other hand, the results observed for EQ-C used at 500 μM concentration showed that the number of viable cells was lower than the number of apoptotic cells. Similar results were obtained for EQ studied earlier (Błaszczyk & Skolimowski, 2005b). Such results suggest that the TUNEL method detects not only viable cells at the early apoptosis stage but also those in late apoptosis (so-called 'secondary necrosis' or 'apoptotic necrosis') in which fragmentation of both DNA and chromatin is observed and cell membrane is altered (Kośmider, Zyner, Osiecka, & Ochocki, 2004).

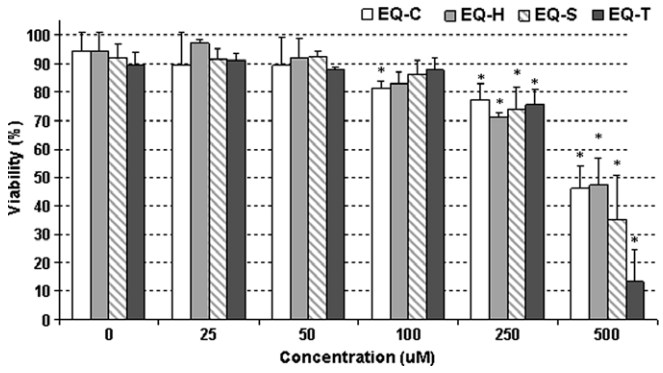

Fig. 2. The percentage of viable cells after 24-h treatment of human lymphocytes in vitro with EQ salts. * The statistically significant difference as compared with the negative control.

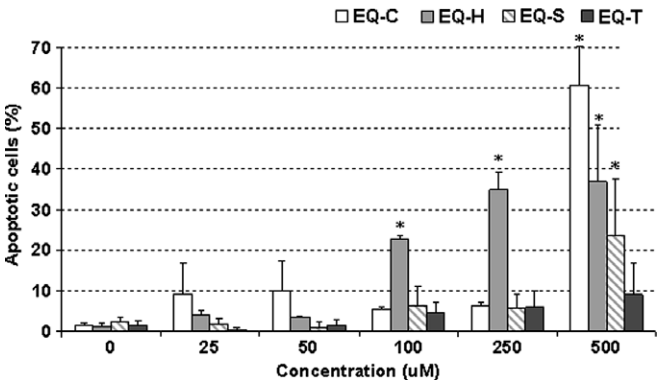

Fig. 3. The percentage of apoptotic cells after 24-h treatment of human lymphocytes in vitro with EQ salts. * The statistically significant difference as compared with the negative control.

Our results showed that all the compounds tested used at the concentration of 500 μM were strongly cytotoxic, as they caused significant decrease in cell viability as well as increase in the number of cells with apoptotic DNA fragmentation (Figs. 2 and 3). The most cytotoxic was EQ-H since it caused DNA fragmentation also at the lower concentrations: 100 and 250 μM.

The similar results obtained for the studied compounds in the trypan blue exclusion assay mean that the differences in the structure of EQ salts did not influence the cell viability, but on the other hand in the TUNEL method the higher cytotoxicity of EQ-H in comparison with EQ-C, EQ-S or EQ-T was seen. EQ-H is the only EQ salt without

ring in the structure of acid residue. It suggests that it could play role in DNA damage detected in the TUNEL method.

The goal of this study was also the comparison of the obtained results with those noted earlier for EQ which showed that this compound used at the two highest doses (250 and 500 μM) decreased cell viability to 50% and 15%, respectively (Błaszczyk & Skolimowski, 2005a). Also the numbers of the apoptotic cells were significantly increased in comparison with the control (42.5% and 83%, respectively). The comparison presented in Table 1 shows that cell viability observed after EQ and EQ salts treatments at the concentrations of 25, 50 and 100 μM was similar. The differences were noted at the concentrations of 250 and 500 μM. Cytotoxicity of EQ was usually stronger than that of EQ salts. The TUNEL method also showed that EQ was stronger inducer of DNA fragmentation than its salts. The comparison of the results obtained for EQ and EQ salts suggests that converting EQ into salt influence its cytotoxicity; it was especially seen when the higher doses of the compounds were used. It is known that EQ in the presence of oxygen can be oxidized a little, and as a result a yellow oil changes to brown oil. The products of EQ oxidation have also antioxidant activities and it is one of the reason of its high antioxidant efficiency. EQ oxidation products are numerous, and only some were identified (De Koning, 1998; Thorisson, Gunstone, & Hardy, 1992). In fish meal or oxidized fish oil two compounds were reported as a major oxidation products: 2,6-dihydro-2,2,4-trimethyl-6-quinolone and 1,8′-di[1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline] (dimmer of EQ; Thorisson et al., 1992).

Table 1
Comparison of the cytotoxic activities of EQ and EQ salts. EQ data were taken from the earlier published paper (Błaszczyk & Skolimowski, 2005b)

Concentration (μM)	EQ/EQ-C	EQ/EQ-H	EQ/EQ-S	EQ/EQ-T
Cell viability[a]				
0	0.99	1.01	1.02	1.03
25	1.01	0.93	0.98	0.99
50	1.01	0.99	0.98	1.03
100	1.07	1.04	1.01	0.99
250	0.66*	0.71*	0.67*	0.67*
500	0.36*	0.35*	0.47*	1.23
Apoptotic cells[b]				
0	0.98	1.08	1.00	1.04
25	0.30	0.72	1.71	5.70
50	0.36	0.99	3.66	2.15
100	1.48	0.36*	1.29	1.79
250	6.95*	1.21	7.53*	7.19*
500	1.37	2.24*	3.50*	9.22*

*The statistically significant difference between EQ and EQ salt treatments ($P < 0.05$).

The comparison is presented as the ratio of the values obtained for EQ and those obtained for EQ salts.

[a] The percentages of viable cells observed after 24-h treatment cell treatment with the tested compounds were compared.

[b] The percentages of apoptotic cells with fragmented DNA observed after 24-h treatment cell treatment with the tested compounds were compared.

Processes of oxidation in EQ molecule occur among others at N atom and as a result aminyl radical can arise (Brannegan, 2000); it can be the cause of cytoxic or genotoxic effects. Converting EQ in the form of salt eliminates that possibility to some degree and in our opinion it can be possible reason of lower cytotoxic activity of EQ salts in comparison with that of EQ.

In conclusion, the new EQ salts studied were less cytotoxic than EQ which is a positive feature for potential antioxidants. Further detailed studies on their antioxidant properties are needed to estimate their usefulness as preservatives.

Acknowledgements

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