

Received 26 July 2004  
Accepted 19 October 2004

Short Communication

## **APOPTOSIS AND CYTOTOXICITY CAUSED BY ETHOXYQUIN AND TWO OF ITS SALTS**

ALINA BŁASZCZYK<sup>1\*</sup> and JANUSZ SKOLIMOWSKI<sup>2</sup>

<sup>1</sup>Department of Cytogenetics and Plant Molecular Biology, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland, <sup>2</sup>Department of Organic Chemistry, University of Łódź, Narutowicza 68, 90-136 Łódź, Poland

**Abstract:** In our study, we analyzed the cytotoxicity of ethoxyquin (EQ) and its two salts, ethoxyquin hydrochloride (EQ-HCL) and ethoxyquin phosphate (EQ-P). It was shown that EQ was the most cytotoxic compound ( $IC_{50} = 0.09$  mM), while the lowest cytotoxic effect was observed for EQ-P ( $IC_{50} = 0.8$  mM). The properties of ethoxyquin and its salts were also analyzed with the TUNEL method, which evaluates their ability to induce apoptosis. It was shown that EQ induced apoptosis in cultured human lymphocytes, especially at concentrations of 0.25 and 0.5 mM.

**Key Words:** Ethoxyquin, MTT Assay, TUNEL Method, Apoptosis, Cytotoxicity

### **INTRODUCTION**

Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) is used in various food products and in animal feeds because of its powerful antioxidant activity. Recently, many unfavourable side-effects have been observed in animals fed with EQ-containing feeds [1]. Adverse effects were also observed in people who are exposed to this compound at work [1, 2]. EQ was nominated for carcinogenicity testing by the U.S. Food and Drug Administration (FDA) [1]. The nomination was based on the increased use and high levels of EQ in animal

---

\*Corresponding author; e-mail: [ablasz@biol.uni.lodz.pl](mailto:ablasz@biol.uni.lodz.pl)

Abbreviations used: EQ – ethoxyquin; EQ-HCL – ethoxyquin hydrochloride; EQ-P – ethoxyquin phosphate;  $IC_{50}$  – the compound concentration that effectively inhibits 50% of cell growth after a 72-h exposure of cells to the tested compound; MTT – 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; SDS – sodium dodecyl sulphate; DMF – dimethylformamide; TUNEL – TdT-mediated dUTP Nick-End Labelling; TdT – terminal deoxynucleotidyl transferase; ET – ethanol; MI – the mitotic index.

feed; there was also no certain data concerning the potential toxicity of EQ. In a report prepared by Dewhurst [2], studies of genotoxicity in mammalian systems were recommended since they would provide information useful for evaluating the compound. An *in vitro* chromosome aberration test revealed that this compound induced chromosome aberrations; the following were observed: dicentrics, atypical translocated chromosomes and chromatid exchanges [3, 4]. On the other hand, EQ was observed to have antimutagenic and anticarcinogenic properties [5-7]. However, due to the harmful effects observed in animals fed with EQ-containing feeds and due to its toxicity, proved in many studies, other compounds, including analogues of EQ, are being researched and prepared to replace EQ. The properties and antioxidant efficacy of these new agents are being intensively studied [8, 9].

In our laboratory, we attempted to study the *in vitro* cytotoxicity and genotoxicity of EQ, and we compared EQ properties with the properties of some new antioxidants. The cytotoxicity of EQ was observed earlier during the chromosome aberration test [4]. In this study, we investigated the cytotoxicity and ability to induce apoptosis of two EQ salts (EQ-HCL and EQ-P) and we compared this with the properties of EQ. There is no published data on the *in vitro* cytotoxicity of EQ-HCL and EQ-P. These compounds have similar protective properties to EQ, and they have been proposed for use in animal feeds [10, 11].

## MATERIAL AND METHODS

### Chemicals

The chemicals were synthesized in the Department of Organic Chemistry, University of Łódź. The synthesis of ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, CAS number: 91-53-2, purity > 97%, Fig. 1) was described elsewhere [4]. The two EQ salts, ethoxyquin hydrochloride (EQ-HCL, 1:1, purity > 96%) and ethoxyquin phosphate monobasic (EQ-P, 1:1, purity > 98%), were synthesized according to the method presented in our earlier paper [12]. Briefly, these compounds were obtained in the reaction of EQ water-methanol solution with concentrated hydrochloric or concentrated phosphoric acid, respectively. The obtained products, EQ-HCL (light-beige) and EQ-P (light-grey), slowly darkened over 7-10 days at room temperature, and they were soluble in water.

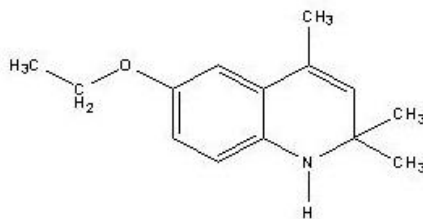


Fig. 1. The chemical structure of EQ.

**Lymphocyte isolation and culture**

Lymphocytes were isolated from peripheral blood obtained from healthy non-smoking donors by density-gradient centrifugation (15 min., 280 g) with Histopaque-1077 (Sigma). The concentration of the lymphocytes in culture was  $10^5$  per mL (MTT assay) or  $0.8 \times 10^6$  per mL (TUNEL assay). The lymphocytes were cultured in RPMI 1640 medium (85%) supplemented with antibiotics (100 IU/mL penicillin and 0.1 mg/mL streptomycin, Sigma), PHA (1% v/v, Gibco) and fetal calf serum (15%, Sigma).

**The MTT assay**

The MTT assay is based on the cleavage of the yellow dye MTT to purple formazan crystals by mitochondrial dehydrogenases; this conversion only occurs in viable cells. Lymphocytes were seeded on a 96-well microplate 24 hours before drug treatment. The cells were incubated with different concentrations of the tested compounds for 72 hours. Then, 15  $\mu$ l of MTT solution (5 mg/mL) was added to each well, and after 2 hours, formazan crystals were dissolved in 20% SDS/50% DMF. After a further 24 hours, the absorbance was measured at 580 nm using an Awareness microplate reader. Three independent experiments were performed for each compound tested. The  $IC_{50}$  values were estimated on the basis of survival curves.

**The TUNEL assay**

The DeadEnd<sup>TM</sup> Fluorometric TUNEL System (Promega) was used to detect the fragmented DNA of apoptotic cells labelled at their 3'-OH ends with fluorescein. After 24 hours in culture, lymphocytes were treated with the tested compounds. The lymphocytes were fixed on slides with 4% paraformaldehyde (Polysciences) and then incubated (1 hour, 37°C) with a mixture of TdT and fluorescein-labelled nucleotides. The analysis was performed using a fluorescence microscope (OLYMPUS, 520 nm filter). The nuclei of apoptotic cells were dyed green, and propidium iodide treatment of the slides was used as a counterstaining for all cells. The percentage of apoptotic cells was calculated for 1000 cells in three independent experiments. The statistical analysis was performed using the exact Fisher test.

**RESULTS****The MTT assay**

In the MTT assay, lymphocytes were incubated with the tested compounds for 72 hours. The test showed that EQ was the most cytotoxic compound (Fig. 2); the concentration causing 50% growth inhibition was 0.09 mM. For EQ-HCL, the  $IC_{50}$  value was 0.13 mM, while EQ-P was the least cytotoxic with an  $IC_{50}$  of 0.8 mM.

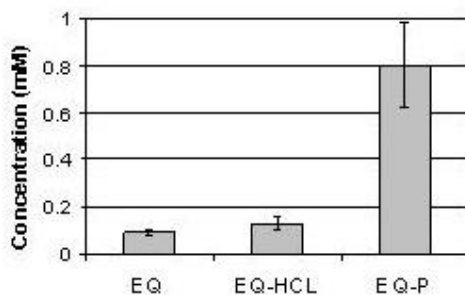


Fig. 2. The IC<sub>50</sub> values for EQ, EQ-HCL and EQ-P determined after a 72-hour treatment of cells.

### TUNEL method

Fig. 3 shows the relationship between the time of treatment and the number of apoptotic cells observed. In earlier studies, we showed that if EQ was present in the lymphocyte cultures for 24 hours, this effectively induced apoptosis [12]. Here, we compare these results with the results obtained for 48-hour and 72-hour incubations.

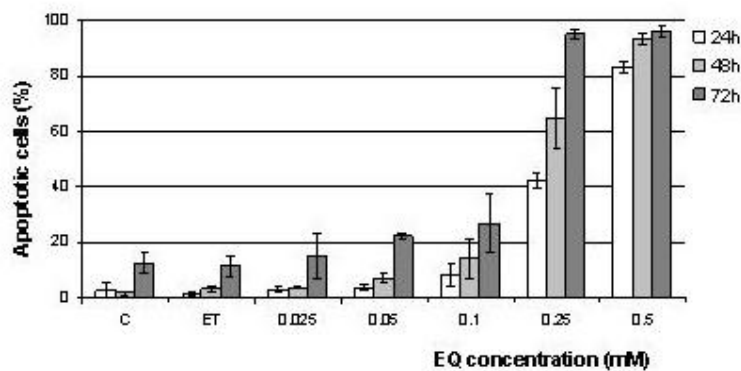


Fig. 3. The percentage of apoptotic cells following 24-, 48- or 72-hour EQ treatment of lymphocytes; C – the control without any compound added; ET – the control with ethanol (the EQ solvent) added. The apparent differences between ET and the treatments of 0.05 mM EQ concentration and higher were statistically significant.

It was observed that cytotoxicity of EQ depended on the concentration used and the time of treatment. Especially high numbers of apoptotic cells were observed when the applied concentrations were higher than the IC<sub>50</sub> value (0.25 and 0.5 mM) and after 72 hours' treatment. With lower doses of EQ, an increase in the number of apoptotic cells in comparison with the control was also seen (Fig. 3).

Fig. 4 shows the comparison of the ability of the tested compounds to induce apoptosis after a 24-hour treatment. The number of apoptotic cells was the highest after EQ application. A dose-effect relationship was observed. A statistically significant increase in the number of apoptotic cells after EQ-HCL application was only observed with the highest dose used (0.5 mM). In lymphocyte cultures treated with EQ-P, no increase in the number of apoptotic cells in comparison with the control was noticed.

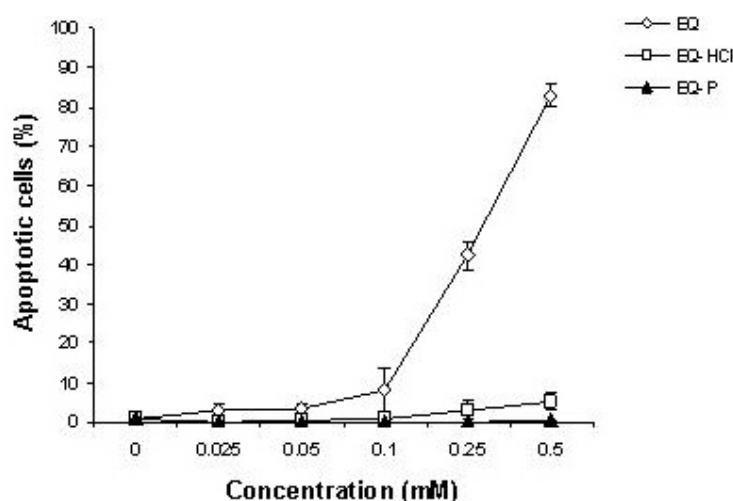


Fig. 4. A comparison of the ability of EQ and its salts to induce apoptosis in cultured human lymphocytes (exposure time – 24 hours). Statistically significant differences between the treatments and the respective controls were observed for EQ (0.05-0.5 mM) and for EQ-HCL (0.5 mM). The numbers of apoptotic cells observed after EQ-HCL and EQ-P lymphocyte treatments were statistically lower than those after EQ treatments.

## DISCUSSION

Because of the adverse health effects observed in animals given EQ-containing feeds and in people working with it [1, 2], there is a great deal of research aimed at finding new compounds, including EQ analogues, with similar antioxidant properties to replace EQ [8, 9]. In this paper, we report on the results of studies on the cytotoxicity and ability to induce apoptosis of EQ and its two salts, which show similar antioxidant activities as EQ.

EQ proved to be most cytotoxic. The  $IC_{50}$  value determined after a 72-hour lymphocyte treatment was 0.09 mM. We showed that high EQ concentrations (especially 0.25 and 0.5 mM) induced apoptosis; thus, the cytotoxicity observed in the MTT assay probably resulted from this process. An induction of apoptosis by EQ was indicated in PHA-activated lymphocytes. There are no published results concerning apoptosis induction by this compound in non-activated cells; such lymphocytes are more resistant to this process. In the comet assay, we

observed that EQ also induced apoptosis in non-activated cells when EQ was used at concentrations of 0.1 mM and higher for 1 hour [unpublished data].

These results concur with our previous observations [4]. In the chromosome aberration test, we noted a statistically significant decrease (> 50%) in the mitotic index in lymphocyte cultures (24-hour treatment; 0.25 mM) as compared to the negative control. The analysis of MI also showed that EQ was less cytotoxic for human lymphocytes after the application of metabolic activation with an S9 fraction obtained from the rat liver [4]. The results of the HPLC assay showed that this compound was effectively metabolized *in vitro* by enzymes of the liver microsomal fraction [4]; EQ at doses of 0.01-0.05 mM was entirely metabolized (2-hour treatment), but in cultures with higher doses of EQ, the unchanged compound was also found. In animals, EQ is also metabolized [13] and the *in vivo* cytotoxic effect of EQ may not only depend on the dose of the compound but on the efficiency of the metabolic system as well.

In this study, the cytotoxic effect of EQ was especially observed at the higher doses used: this was clearly seen with doses higher than  $IC_{50}$ . In the chromosome aberration test performed earlier, the highest number of aberrations and a decrease in MI were also found after using EQ at concentrations over 0.1 mM [4]. Many antioxidants used at high concentrations can have a pro-oxidant effect, and it is possible that it was this EQ property that caused growth inhibition and apoptosis induction.

The two EQ salts studied, EQ-HCL and EQ-P, were less cytotoxic and also less able to induce apoptosis than EQ; the modifications of the EQ structure changed the cytotoxicity of its salts in comparison with EQ. The compound that did not induce apoptosis was EQ-P ( $IC_{50} = 0.8$  mM in comparison to 0.09 mM for EQ). EQ-P was not tested with regard to its influence on living organisms, but there is some information available concerning EQ-HCL: it was proved to induce some adverse side effects in animals, similarly to EQ [10]. In the MTT assay, the  $IC_{50}$  value for EQ-HCL was only a little higher than that for EQ (0.13 mM in comparison with 0.09 mM), but it only slightly increased the number of apoptotic cells in comparison to the negative control (contrary to ethoxyquin). These results indicate that EQ and EQ-HCL can affect cells in slightly different ways. It should be noted that the TUNEL method allows the detection of DNA fragmentation in cells which could still be classified as living cells during staining with fluorochromes (cells in early apoptosis). The other stages of apoptosis are not detected with this method and we also cannot observe necrotic cells. Thus, further research is necessary to elucidate on the cytotoxic effects of EQ and EQ salts.

**Acknowledgements.** This study was supported by grant No. 3 PO4C 021 24 from the State Committee for Scientific Research (KBN).

## REFERENCES

1. Little, A.D. Chemical Committee Draft Report, Ethoxyquin, CAS Number 91-53-2, Submitted to National Toxicology Program, Executive Summary of Safety and Toxicity Information (1990) [http://ntp-server.niehs.nih.gov/htdocs/Chem\\_Background/execSumm.Ethoxyquin.html](http://ntp-server.niehs.nih.gov/htdocs/Chem_Background/execSumm.Ethoxyquin.html)
2. Drewhurst, I. Ethoxyquin. JMPR Evaluations (1998) <http://www.inchem.org/documents/jmpr/jmpmono/v098pr09.htm>
3. Gille, J.J.P., Pasman, P., vanBerkel, C.G.M. and Joenje, H. Effect of antioxidants on hyperoxia-induced chromosomal breakage in Chinese hamster ovary cells: protection by carnosine. **Mutagenesis** 6 (1991) 313-318
4. Błaszczuk, A., Osiecka, R. and Skolimowski, J. Induction of chromosome aberrations in cultured human lymphocytes treated with ethoxyquin. **Mutat. Res.** 542 (2003) 117-128.
5. Renner, H.W. Antimutagenic effect of an antioxidant in mammals. **Mutat. Res.** 135 (1984) 125-129.
6. Manson, M.M., Green, J.A. and Driver, H.E. Ethoxyquin alone induces preneoplastic changes in rat kidney whilst preventing induction of such lesions in liver by aflatoxin B<sub>1</sub>. **Carcinogenesis** 8 (1987) 723-728.
7. Bammler, T.K., Slone, D.H. and Eaton, D.C. Effects of dietary oltipraz and ethoxyquin on aflatoxin B<sub>1</sub> biotransformation in non-human primates. **Toxicol. Sci.** 54 (2000) 30-41.
8. Dorey, G., Lockhart, B., Lestage, P. and Casara, P. New quinolinic derivatives as centrally active antioxidants. **Bioorg. Med. Chem. Lett.** 10 (2000) 935-939.
9. De Koning, A.J. The antioxidant ethoxyquin and its analogues: a review. **Int. J. Food Properties** 5 (2002) 451-461.
10. Kim, H.L. Preparation and dietary effect of ethoxyquin hydrochloride. **J. Toxicol. Environ. Health** 15 (1985) 663-671.
11. Novus International, Inc.; Filing of food additive petition (animal use) – ethoxyquin phosphate, The Food and Drug Administration, **Federal Register** 66 (2001) 36791.
12. Błaszczuk, A. and Skolimowski, J. Synthesis and studies on antioxidants: ethoxyquin (EQ) and its derivatives. **Acta Pol. Pharm.** 62 (2005), in press.
13. Burka, L.T., Sanders, J.M. and Matthews, H.B. Comparative metabolism and disposition of ethoxyquin in rat and mouse. II. Metabolism. **Xenobiotica** 26 (1996) 597-611.