

Evaluation of the possible proapoptotic effect on Tetrodotoxin on Ehrlich Ascites Carcinoma Cells (EAC) using P53 & Caspase 3 immunohistochemistry in mice's liver

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ABSTRACT

It is becoming obvious that all cancers have a defective p53 pathway, either through *TP53* mutation or deregulation of the tumor suppressor function of the wild type TP53. In this study we examined the expression of P53 and Caspase 3 in transperitoneally injected Ehrlich Ascites carcinoma cells (EAC) treated with Tetrodotoxin in the liver of adult mice in order to evaluate the possible pro apoptotic effect of Tetrodotoxin. Results: Early in the treatment, numerous EAC detected in the large blood vessels & central veins and expressed both of P53 & Caspase 3 in contrast to the late absence of P53 expressing EAC at the 12th day of Tetrodotoxin treatment. In the same context, predominantly the perivascular hepatocytes expressed Caspase 3 in contrast to the more diffuse expression pattern late with Tetrodotoxin treatment. Non of the hepatocytes ever expressed P53 neither with early nor late Tetrodotoxin treatment. Conclusion: Tetrodotoxin therapy has a proapoptotic effect on Ehrlich Ascites carcinoma Cells (EAC). This may be through enhancing the tumor suppressor function of the wild type TP53 with subsequent Caspase 3 activation.

KEYWORDS: Proapoptotic, Tetrodotoxin, P53, Caspase 3 and liver.

INTRODUCTION

P53 is a tumor suppressor gene critically involved in cell cycle regulation, DNA repair, and programmed cell death [1]. *P53* mutations are very common in cancer and generally been associated with poor prognosis and poor response to treatment in several types of cancers. However, *P53* mutation is not the only rout for carcinogenesis. Demolishing of tumor suppression functions of wild-type P53 is considered to also have a role in carcinogenesis [2]. On the other hand, P53 can promote apoptosis through *trans*-activation and down-regulation of specific pro- and anti- apoptotic genes [3]. Several studies implied that the pro-apoptotic activity of P53 is independent of its function as a transcription factor [1]. Activation of P53 death signals lead to Caspases activation and thus can induce apoptosis through release of mitochondrial Cytochrome *c* into the cytoplasm which then facilitates activation of Caspase 9, leading to activation of Caspase 3 and other effector caspases. This represents the independent intrinsic apoptotic pathway [1].

Activation of the caspase-3 pathway is a hallmark of apoptosis [1]. Caspase 3 is an “effector” marker; associated with initiation of apoptotic signaling pathway. Caspase-3 is activated by the upstream caspase-8 and

caspase-9, and accordingly it serves as a convergence point for different signaling pathways [1]. Multiple pathways play a role in triggering the caspase-3 activation.

Tetrodotoxin (TTX) is a marine toxin. Although this is not a formal anti-tumor agent [4], it is in fact in Phase III trials as an agent (Tectin[®]) used against the neuropathic pain resulting from chemotherapy-induced peripheral neuropathy [5,6] and [7]. Ehrlich Ascites Carcinoma Cells (EAC) used a model in anti-cancer research in many studies [8]. The action mechanism of Tetrodotoxin on EAC is probably not different from that of other cells in the nervous system through blocking Na channels thus preventing Na influx into the cells [9].

In this study we studied the possible mechanism of anticancer action of Tetrodotoxin on Ehrlich Ascites Carcinoma cells (EAC) through elaboration of possible pro-apoptotic effect of P53. Accordingly we examined the expression of P53 and Caspase 3 in the livers of mice having transperitoneally injected Ehrlich Ascites carcinoma cells and treated with Tetrodotoxin.

MATERIAL AND METHODS

Experimental animals:

Animals (female mice about 20-30g in body weight) were obtained from the Egyptian Organization for Vaccine and Biological Product. Animals were housed in groups (i.e. 10 mice in each cage) and exposed to 12 hours of regular light/dark period. The animals were fed daily with vegetable, milk, commercial pellet and tap water. All animals were cared in accordance with the institutional and national guide for the care and use of laboratory animals

Preparation of Tetrodotoxin extract:

Tetrodotoxin (TTX) was extracted from the gonads of Puffer fish *Lagocephalus lunaris* obtained from the Red Sea. The gonads of five females Puffer fish was homogenized and soaked in 10% acetic acid in methanol as described by Fouda [10]. The homogenate was boiled in water bath for 10 min and filtrated using a cotton funnel. The supernatant containing crude Tetrodotoxin was concentrated by evaporating the excess of methanol [11]. The crude extracts obtained were then stored at -4°C till the time of experiment.

A mouse bioassay expressed in terms of mouse units (MU) was used according to the methods described by Kawabata [12]. Several concentrations of Tetrodotoxin were injected inter-peritoneally into male mice (about 20 g) and time of death was recorded. The amount of Tetrodotoxin that killed the mice within 30 min after injection is considered as one mouse unit (MU) [7] and [13].

Cancer cell line: The initial inoculation of EAC cells was kindly provided by the National Cancer Institute (Cairo University, Egypt). EAC cells were thereafter propagating in our laboratory by weekly interaperitoneal injection of 2.5×10^6 cells per mouse, which was carried out according to the method recommended by the Egyptian National Cancer Institute, Cairo University. Cells were counted before injection using the bright line hemocytometer and dilutions were made by physiological saline, and the desired number of cells was injected in a volume of 0.5 ml per mouse.

Experimental animals:

Animals were divided into four different groups (15 mice in each) that received inter-peritoneal injection as follows:

1. The control group (A): mice were injected with 0.5 ml of saline solution (KCl 0.9%) on the first day of the experiment.
2. The control group (Group B) with Tetrodotoxin treatment only (no EAC cells) : extracted from female-fish gonads of *L. lunaris*: mice were injected with Tetrodotoxin (at a dose of 1/20MU representing 0.051mg of the toxin).
3. Animals in group (C) were inoculated on the zero day of the experiment with 0.5 ml of EAC diluted in saline solution (containing about 1 million cells). EAC-bearing mice were anaesthetized and sacrificed on the days 1, 6, and 12 after tumor cell inoculation.
4. Animals in group (D) were injected with 1×10^6 of EAC cells on the zero day and treated after 24 h. with Tetrodotoxin (at a dose of 1/20MU representing 0.051mg of the toxin).

Five animals from each group were dissected on each day of the days 1, 6, 12. Animals of group D received a total of six doses within 12 days. An interval of 48 hours was left between the injections of each dose of toxin.

Pathological analysis with immuno-histochemical stains:

Immunohistochemical staining performed on 4- μ m, formalin-fixed, paraffin-embedded sections using P53 & Caspase 3 antibodies at 1:50 dilution (DAKO, Carpinteria, CA). Antigen retrieval performed in all cases by steam heating the slides in a 1-mmol/L solution of EDTA (pH 8.0) for 30 minutes. After blocking of endogenous biotin, staining performed using an automated immunostainer (DAKO) followed by detection by using a streptavidin-biotin detection system (DAKO). Positive and negative control sections used for each assay.

The mean number of the P53 expressing EACs per ten high power fields (10 HPFs x400) calculated for each case. Caspase 3 expression in hepatocytes evaluated according to [14].

Statistical Analysis:

All results were expressed as mean \pm S.E. of the mean. Statistical Package for the Social Sciences program (SPSS), version 11.0 was used to compare significance between each two groups. Difference was considered significant when $P \leq 0.5$.

Results:

Tetrodotoxin treatment (day 1) Fig (1):

A lot of Ehrlich carcinoma cells expressed both p53 & Caspase 3 and present predominantly in large blood vessels central veins and less in sinusoids and periportal areas. Also apoptotic hepatocytes expressing Caspase 3 present mainly in the pericentral veins areas. None of the hepatocytes expressed p53 as an indication that there is no neoplastic transformation.

Tetrodotoxin treatment (day 6) (Chart 1):

Fewer Ehrlich cells expressing p53 detected ($p < 0.5$). Also the apoptotic hepatocytes expressing Caspase 3 increased significantly in a proportionate fashion ($p < 0.5$) (Table 1 & Fig 2). None of the hepatocytes expressed p53 as an indication that there is no neoplastic transformation.

On the 12th day of Tetrodotoxin treatment (Fig 3):

There was complete absent of Ehrlich cells expressing P53. In contrast, the increase of apoptotic hepatocytes expressing Caspase 3 was significant ($p < 0.5$). None of the hepatocytes expressed p53 as an indication that there is no neoplastic transformation.

Table 1: Average number of P53 expressing EAC cells /10 HPFs and % of Caspase 3 expressing hepatocytes

	Average number of P53 expressing EAC cells /10 HPFs	% of Caspase 3 expressing hepatocytes
Group C (1 X10 ⁶ of EAC cells only) control no Tetrodotoxin treatment	10	5
Group D (day 1 Tetrodotoxin treatment)	9	7
Group D (day 6 Tetrodotoxin treatment)	2	19
Group D (day 12 Tetrodotoxin treatment)	0	30

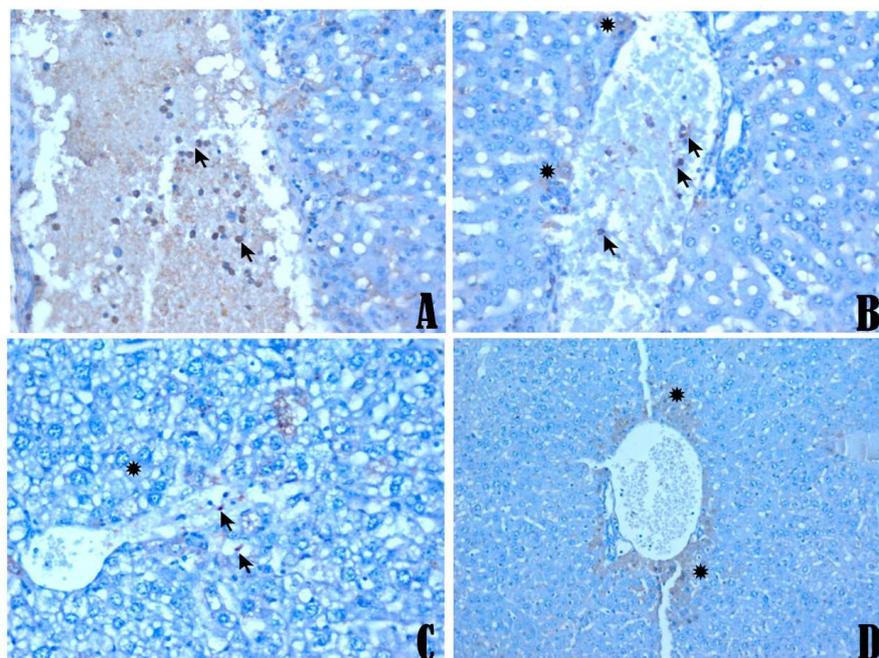


Fig. 1: Day 1 Tetrodotoxin treatment: A- A lot of Ehrlich carcinoma cells expressing nuclear P53 (arrows) in large blood vessels Immunohistochemistry x400. B- Ehrlich carcinoma cells expressing nuclear Caspase 3 (arrows) in large blood vessels. Also few perivascular hepatocytes expressing cytoplasmic Caspase 3 (stars). Immunohistochemistry x400. C- Ehrlich carcinoma cells expressing nuclear P53 (arrows) in large blood vessels however negative hepatocytes for P53 . Immunohistochemistry x400. D- Few perivascular hepatocytes expressing weak cytoplasmic Caspase 3 (stars). Immunohistochemistry x100.

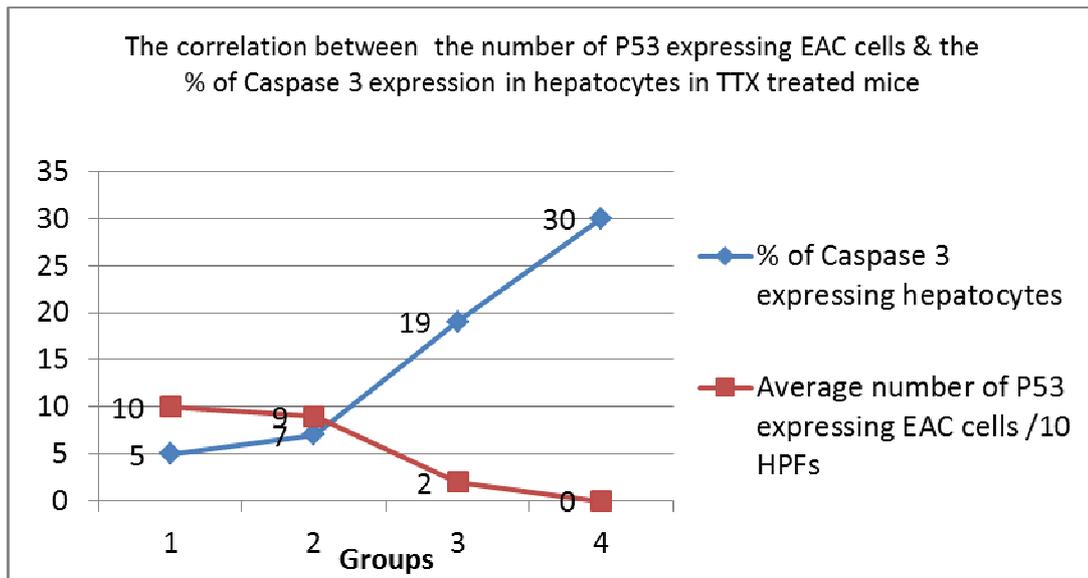


Fig. 2: The correlation between the number of P53 expressing EAC cells & the % of Caspase 3 expression in hepatocytes in Tetrodotoxin treated mice.

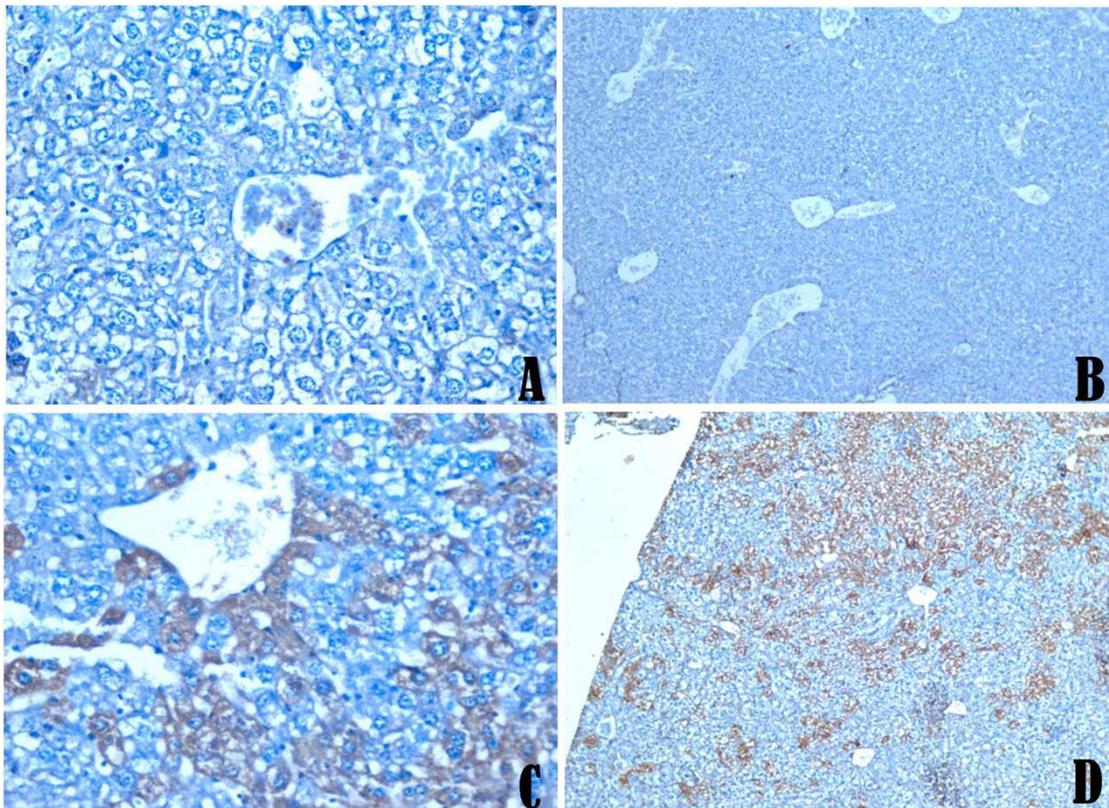


Fig. 3: Day 12 Tetrodotoxin treatment : A- No Ehrlich carcinoma cells expressing nuclear P53 in large blood vessels. Immunohistochemistry x400. B- Negative hepatocytes for P53. Immunohistochemistry x100. C- No Ehrlich carcinoma cells expressing Caspase 3 in large blood vessels, however the hepatocytes show wide diffuse moderate cytoplasmic Caspase 3 expression. Immunohistochemistry x400. D- Hepatocytes show wide diffuse moderate cytoplasmic Caspase 3 expression. Immunohistochemistry x100.

Discussion:

In this study we evaluate the effect of Tetrodotoxin on p53 expression in Ehrlich carcinoma cells. The study showed that Tetrodotoxin has an inhibitor effect on EAC in liver, which came in agreement with several previous results of Fouda *et al.* [8], Abd El-Motelp *et al.* [9] and Abd El-Dayem *et al.* [15]. P53 is a tumor

suppressor protein, encoded by the *TP53* gene (OMIM 191170), to control cell proliferation [2]. The process of carcinogenesis involves accumulation of genetic alterations causing loss of tumor suppressor genes and over activation of oncogenes [2]. Goldstein *et al.* [2] suggested that P53 activation occurs through two different pathways in which the subsequent functions are different. One pathway is through accumulation of non mutated wild type TP53 in the nucleus, where it regulates the transcription of numerous target genes using specific DNA response elements with subsequent several biological responses occur such as apoptosis, cell cycle arrest, DNA repair, differentiation or senescence, regulation of mitochondrial functions [2]. The other pathway is *TP53* mutation through missense or frame shift mutations, leading to the production of a different protein expressed at higher level than wild-type p53 leading to loss of the tumor suppression function with subsequent tumor progression and distant metastases; which is the major event in human cancer [2]. Studies showed that p53 is a druggable target by several ways one of them through restoring the p53 wild-type pro apoptotic function. However, these compounds still need to be assessed for their functional and biological effects [2].

Several studies showed that wild type p53 is required for the apoptotic cell death particularly if induced by some anticancer drugs through several pathways [1,2] and [3]. Recently, some therapeutics work through activation of wild-type p53 protein in tumors described. Our study showed that the EAC disappeared completely on the 12th day post intra peritoneal injection. We suggest that cancer cell death is due to genotoxic stress in addition to the activation of the tumor suppressor function of the P53 through restoring wild type (not the mutant type) p53 activity leading to apoptosis. This comes in context with Goldstein *et al.* [2] that stated that genotoxic stress may induce a senescent state, which has a role in tumor suppression and is regulated also by p53. Thus Tetrodotoxin helped to restrain the proliferation of EAC cancer cells which came along with Xiao [16]. On the other hand, our study showed EACs exhibit both cytoplasmic & nuclear Caspase 3 staining, which is similar to findings of Eckle *et al* [17]. Moreover, our study showed cytoplasmic expression of Caspase 3 in both early and late Tetrodotoxin treatment. These findings came along with Eckle *et al* [17] in which stated that Caspase-positive apoptotic hepatocytes could be reliably identified and quantified both in normal and neoplastically transformed liver tissue [17]. However our study showed no nuclear expression of P53 by the hepatocytes whether with early or late Tetrodotoxin, indicating absence of neoplastic transformation in hepatocytes. Furthermore we showed that the expression of Caspase 3 expression by hepatocytes directly proportionate with the duration of Tetrodotoxin treatment (Fig 2); where Active caspase-3 is confined primarily to the cytosol [18] and according to Eckle *et al.* [17], Caspase-stained figures were either immuno-positive apoptotic bodies or pre-apoptotic hepatocytes showing cytoplasmic and/or nuclear caspase-staining with otherwise normal cellular appearance. This proapoptotic activity of P53 is independent of its function as a transcription factor. This is through the release of Cytochrome *c* from mitochondria is a central event in the death receptor-independent, “intrinsic,” apoptotic pathway which facilitates activation by Caspase 9 of the effector Caspases [1], which subsequently activates Caspase 3, and ultimately leads to the apoptotic cell death.

Conclusion:

In conclusion, the present study suggests that Tetrodotoxin administration had a powerful inhibitory effect on growth of Ehrlich Ascites Carcinoma cells, mediated through restoring wild type p53 activity and activation of caspase-3 leading to apoptosis. It can be extended further to develop therapeutic protocols for treatment of cancer.

List of abbreviations:

EAC: Ehrlich Ascites Carcinoma Cells; TTX: Tetrodotoxin; HPFs: High Power Fields

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Conflicts of Interest(s):

Authors declare that there is no conflict of interest regarding the publication of this paper.

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Authorship:

The authors fulfilled the following criteria:

- Substantial contributions to research design, or the acquisition, analysis or interpretation of data.
- Drafting the manuscript or revising it critically.

- Approval of the submitted and final versions.

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