

## Cytotoxic Effect of Nefazodone on HepG2 Cell Line and the Reliability of Comet Assay to Determine Genotoxicity.

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### Abstract:

Nefazodone is atypical antidepressant which was manufactured by Bristol-Myers Squibb in 1994 to avoid the adverse effects associated with other antidepressants, including nausea, sedation, insomnia, cardiovascular toxicity, weight gain and dysfunction.

In 2004, nefazodone was withdrawn from USA after its withdrawal from Canada and Europe due to the reports of liver injury in patients treated with this drug. The current study was performed to investigate the cytotoxic and genotoxic effect of nefazodone on HepG2 cell line at different concentrations by using MTT assay and comet assay, respectively. The results showed that nefazodone causes a reduction in cells viability of HepG2 cell line with an IC50 4.682 µg/ml. Comet assay showed a significant increment in the three parameters (tail length, percent of DNA in tail and tail moment) in a concentration-dependent manner, when compared with negative control ( $p < 0.01$ ), but these results considered as false positive due to cells death.

**Key words:** Nefazodone, Cytotoxicity, Genotoxicity, Comet assay, HepG2 cell line.

## التأثير السمي لعقار النيفازودون على خط خلايا الكبد HepG2 السرطانية وموثوقية اختبار المذنب لتحديد سميته الجينية

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### الخلاصة:

النيفازودون هو مضاد للاكتئاب غير نمطي تم تصنيعه بواسطة شركة بريستول مايرز سكويب في عام 1994 لتجنب الآثار الجانبية المرتبطة بمضادات الاكتئاب الأخرى بما في ذلك الغثيان، الخمول، الأرق، تسمم القلب والأوعية الدموية، زيادة الوزن والعجز الجنسي. في عام 2004 تم سحب دواء النيفازودون من الولايات المتحدة الأمريكية بعد سحبه من كندا وأوروبا بسبب تقارير عن تسمم الكبد عند المرضى الذين عولجوا بهذا الدواء. تم إجراء الدراسة الحالية للتحقق من التأثير السمي للخلايا والجينات في خط خلايا الكبد (HepG2) السرطانية باستخدام تراكيز مختلفة من دواء النيفازودون بواسطة اختبار MTT و اختبار المذنب على التوالي. أظهرت النتائج أن النيفازودون تسبب في الحد من بقاء خلايا HepG2 حية بنسبة خمسين بالمائة IC50 4.682 ميكروغرام/مل. كما أظهرت نتائج اختبار المذنب زيادة في المؤشرات الثلاث (طول الذنب، النسبة النووية للحمض النووي في الذنب وزخم الذنب) بطريقة تعتمد على الجرعة عند مقارنتها بمجموعة السيطرة ( $p < 0.01$ ), لكن هذه النتائج اعتبرت إيجابية خاطئة بسبب موت الخلايا.

**الكلمات المفتاحية:** نيفازودون، السمية الخلوية، السمية الجينية، اختبار المذنب، خط خلايا الكبد (HepG2) السرطانية.

## Introduction:

Drug-induced liver injury (DILI) is the health problem that leads to failure in drugs approval and withdrawal from markets [1]. The DILI can be classified into intrinsic (dose dependent) and idiosyncratic, which is a rare, unpredictable and either dependent or independent doses [2].

Antidepressants are a group of drugs that prescribed to manage psychiatric illnesses, like depression and anxiety. At therapeutic dose and the absence of risk factors such as cirrhosis and hepatitis, antidepressant may cause liver injury. The occurrence of hepatotoxicity varies from 5 days to 3 years after starting the treatment. Citalopram, paroxetine, fluoxetine, mirtazapine causes reversible liver injury; while sertraline, nefazodone, and duloxetine can cause death to the patient use these drugs [3].

Nefazodone is atypical antidepressant which was manufactured by Bristol-Myers Squibb and marketed under the brand name Serzone® in 1994 for treatment of depression, panic disorder and aggressive behavior, and to avoid the adverse effects associated with other antidepressants including nausea, sedation, insomnia, cardiovascular toxicity, weight gain and sexual dysfunction. In 2004 nefazodone was withdrawn from USA after its withdrawal from Canada and Europe due to reports of liver injury in patients treated with this drug for 1 to 8 months [4].

Nefazodone acts as antagonist to 5-hydroxytryptamine (5-HT)<sub>2A</sub> receptors, alpha (1)-adrenergic receptors and desensitize 5-HT<sub>1A</sub> receptor with chronic use, thereby inhibits reuptake of serotonin, norepinephrine and increase their release. Nefazodone cause sedation by antagonizing the histamine (H<sub>1</sub>) receptors [5, 6].

Nefazodone is available as tablets which is showing rapid and complete absorption after oral administration with peak plasma level observed within 2 hours and the steady-state are attained within 4 days.

Nefazodone metabolized by and inhibit CYP3A4 and act as a weak inhibitor for CYP2D6, therefore only a little amount of parent drug reaches the systemic circulation [7].

Nefazodone the only atypical antidepressant that carries the warning black box of FDA concerning to its hepatotoxicity and appeared to cause severe idiosyncratic liver injury [8].

Nefazodone induce hepatotoxicity through different suggested mechanisms includes Cholestasis hepatocellular injury by inhibition of bile salt export pump, mitochondrial toxicity by inhibition mitochondrial respiratory chain complex I and to less extent complex IV [9], endoplasmic reticulum stress and reduction in ATP synthesis [10].

The aim of study is to determine the cytotoxic effect of nefazodone on HepG2 cell line and the reliability of Comet Assay To determine genotoxicity.

## Materials and Methods

In this study, the following materials was used:

Nefazodone HCL powder (Sigma-Aldrich / USA), Dulbecco's modified Eagle's medium (DMEM) media (Santa Cruz / USA), fetal bovine serum (Sigma-Aldrich/ Germany), dimethyl sulfoxide (DMSO) (Sigma Aldrich /USA ), HepG2 Hepatoma G2 cell line obtained from the ATCC /USA, phosphate buffer saline (PBS) (Sigma Aldrich/USA), MTT [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide] (Intron Biotech/Korea) and Oxiselect® Comet assay kit (Biolabs / USA).

The HepG2 cell line in this study was obtained from ATCC company and cultured in tissue culture laboratory of Biotechnology research center/Al-Nahrain University.

The cells culturing process including aseptic techniques, sterilization of glassware, culturing, sub-culturing, cryopreservation, thawing, and maintenance of cells culture was

accomplished according to the standard techniques of cells culture procedures <sup>[11]</sup>

### MTT Assay

The cytotoxicity assay (MTT) is a simple method to evaluate cells proliferation or death. The MTT is reduced by the mitochondria of living cells to formazan and give the purple color which related to the number of viable cells. The absorbance can be measured by spectrophotometer at a wavelength between 550-600nm <sup>[12]</sup>.

The cytotoxic effect of nefazodone HCl was performed at 11 different concentrations by using MTT kit, as described in standred protocol <sup>[13]</sup>.

### Comet Assay

The comet assay is used to measure the genotoxic effects at the level of a single cell. The cells are lysed by detergent after embedded in agarose to form a super coiled DNA connected to nuclear matrix. Electrophoresis under alkaline solution results in structures likes comets can be observed under fluorescent microscope. The principle involves the DNA strands that containing breaks extend toward the anode <sup>[14]</sup>. The comet images were analyzed by using Comet Assay IV software. The analysis software can calculate different parameters for each comet. Three parameters were estimated to indicate DNA migration, Tail length (distance from the head centre to the end of the tail), and Tail moment (product of tail DNA/total DNA by the tail centre of gravity), Where tail DNA%=100 X Tail DNA Intensity/Cell DNA Intensity. The genotoxic effect of nefazodone HCl was performed by using Oxiselect® Comet assay kit, as described in standred protocol <sup>[15]</sup>.

### Statistical Analysis

The statistical packages for social sciences (SPSS version 16) was used for data analysis. The descriptive data analysis was expressed as mean  $\pm$  standard error of mean (SEM), One-Way ANOVA test was

used to differentiate between more than two independent means of all studied parameters of the three groups. Followed by the Tukey test. P-value < 0.01 was considered as statistically significant. The IC<sub>50</sub> of nefazodone was determined by graphpad prism 7 software.

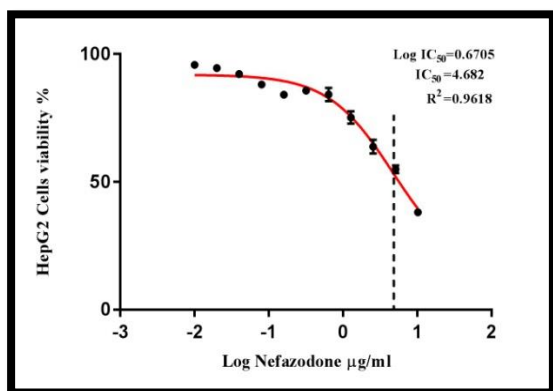
## Results

### Cytotoxic Assay

The MTT assay was used to determine the cytotoxic effect and cells viability after treatment of HepG2 cell line with a range of nefazodone concentrations for 24 hours of incubation at 37°C. The results in table (1) indicated that nefazodone HCl causes a reduction in cells viability of HepG2 cell line in concentration dependent with an IC<sub>50</sub> 4.682  $\mu$ g/ml, as shown in figure (1).

**Table (1): Cells viability of HepG2 cell line after 24hs treatment with nefazodone HCl.**

Concentration s ( $\mu$ g/ml)	% Mean of cells viability $\pm$ SEM
0.01	95.76 $\pm$ 0.85
0.02	94.56 $\pm$ 1.12
0.04	92.21 $\pm$ 0.40
0.08	88.16 $\pm$ 1.18
0.16	84.14 $\pm$ 0.37
0.32	85.74 $\pm$ 1.17
0.64	84.20 $\pm$ 2.58
1.28	75.24 $\pm$ 2.44
2.56	63.81 $\pm$ 2.71
5.12	54.95 $\pm$ 1.47
10.24	38.14 $\pm$ 0.79



**Figure (1): Cytotoxic effect of nefazodone HCl on HepG2 cells after 24hs incubation at 37°C.**

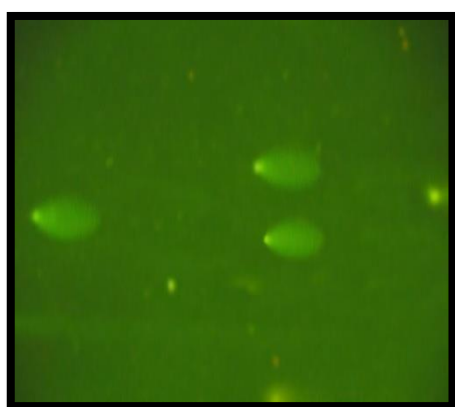
**Table (2): Measurement of Tail Length, Percent of DNA in Tail and Tail Moment in HepG2 Cell line Cells Treated with Different Concentration of Nefazodone HCl.**

Nefazodone Conc. (µg/ml)	Tail Length of HepG2 cells	Percent of DNA in Tail of HepG2 cells	Tail Moment of HepG2 cells
control	26.9±1.07	15.77±0.81	4.27±0.32
2.5	53.90±1.76 <sup>a</sup>	26.58±0.96 <sup>a</sup>	14.26±0.52 <sup>a</sup>
5	119.30±1.27 <sup>b</sup>	36.67±0.62 <sup>b</sup>	43.81±0.99 <sup>b</sup>
10	205.40±1.22 <sup>c</sup>	56.22±0.62 <sup>c</sup>	115.47±1.30 <sup>c</sup>

Each value is expressed as mean ±SEM.

Different lower-case letters indicate significant difference (p <0.01) among different mean concentrations.

One –way ANOVA followed by post hoc Tukey.



**Figure (2) :Comet shape appears after treatment with 10 µg/ml of nefazodone HCl**

**Discussion**

In the current study, the HepG2 cell line was used as cell model because its widely used in biomedical and genotoxic studies

**Comet Assay**

Nefazodone HCl at concentrations (2.5, 5, 10 µg/ml) caused a significant increase in comet assays parameters (Tail length, the percent of DNA in Tail and Tail moment) as compared to control (DMSO) (p<0.01) figure (2). Also, there was a significant increase (p<0.01) in three parameters when comparing the results of each among each other, as shown in table (2).

[16]. The present study depends on maximum plasma concentration of Nefazodone to study its genotoxicity because the maximum plasma concentration is 1-2µg/ml reached after 200 – 400 mg daily dose. This plasma concentration may not proportion directly with increased dose [17], moreover there were no data documented about the plasma-to-liver ratio of nefazodone [18]. Nefazodone metabolized by CYP3A4 and form conjugate with sulphate, glucuronide and eliminated through urine (49%) and bile, which represents the other major route [17]. The toxicity results from saturation of conjugation and inhibition of CYP3A4 and BSEP that lead to accumulation of the nefazodone as parent drug in liver and inhibit its elimination [18], where previous study showed that increase

metabolism activity of CYP3A4 decrease toxicity of Nefazodone <sup>[19]</sup>.

### The Cytotoxic Effect of Nefazodone

According to the obtained results, the IC<sub>50</sub> of nefazodone was 4.682 µg/ml, as shown in figure (1), which is considered as cytotoxic according to the American National Cancer Institute (NCI), a compound is considered cytotoxic on cancer cell lines when the IC<sub>50</sub> lower than 50 µg/ml <sup>[20]</sup>. The cytotoxicity of nefazodone can be explained by inhibition of the mitochondrial respiratory chain (MRC) complexes I and to less extent complex IV, the inhibition of MRC lead to inhibition of ATP synthesis and increase production of reactive oxygen species (ROS) and increased susceptibility to cell death <sup>[21,22]</sup>.

Nefazodone cause collapse in mitochondrial membrane potential ( $\Delta\psi_m$ ) which is generated by Complexes I, III and IV and it is essential for ATP synthesis <sup>[23]</sup>. The collapse of  $\Delta\psi_m$  lead to elevation of ROS and reduction in levels of glutathione, all the previously mentioned events lead to mitochondrial dysfunction, depilation of ATP synthesis and activation of cell death pathways <sup>[24]</sup>. The cytotoxicity of nefazodone also may occur through the endoplasmic reticulum stress and activation of mitogen-activated protein kinase (MAPK) <sup>[25]</sup>. Endoplasmic reticulum stress mean disrupting the function of endoplasmic reticulum which is intracellular calcium homeostasis, protein folding and trafficking <sup>[26]</sup>. Meanwhile, the activation of MAPK leads to activation of its three arms the c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK1/2) and p38. Generally, p38 and JNK activated by cell damage and lead to cell death while ERK1/2 associated with cell survival, but some studies showed that ERK1/2 may promote cell death <sup>[27,28]</sup> and inhibition of ERK1/2 reduce endoplasmic reticulum stress caused by nefazodone <sup>[25]</sup>.

### Comet Assay

The comet assay parameters showed that the single and double strand DNA breaks have been significantly increased in a concentration-dependent manner. In general, the comet assay could show false positive results because of its sensitivity to instrumental factors such as light source intensity, and microscope quality and/or cleanliness, camera sensitivity and image analysis system <sup>[29]</sup>. Whoever, the DNA damage may occur by fluorescent light that is used during the experiment and that could explain the slight increase in three parameters measured for the control <sup>[30]</sup>. The increased in light intensity and room temperature higher than 25°C can cause increase DNA damage and increase % DNA in tail <sup>[31]</sup>.

The cells death by cytotoxic effect of drugs can cause DNA fragmentation and apoptotic or necrotic cells can result in comet images with small head and large diffuse tails (hedgehog) (figure 2) and cell lethality  $\geq 30\%$  can give false positive <sup>[32]</sup>.

### Conclusion

In the current study, the cytotoxic and genotoxic effect of Nefazodone has been studied on HepG2 cell line by using MTT test and comet assay respectively. Depending on the results obtained, it could be concluded that:

Nefazodone HCl has a cytotoxic effect on HepG2 cell line in concentration dependent manner. Nefazodone HCl give false positive result in comet assay due to cell death, therefore using comet assay alone to study the genotoxicity of nefazodone is not reliable due to the high level of dead cells.

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