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RESEARCH ARTICLE

# Nefazodone Atypical Antidepressant Associated Hepatotoxicity-Drug Induced Liver Injury (DILI) A Possible Genotoxic Effect(s)

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## Abstract

The atypical antidepressant Nefazodone used in different concentrations (2.5, 5, 10 µg/ml ) to study its genotoxic effects on HepG2 cell line by using single cell gel electrophoresis assay (Comet) and spectral karyotyping test (SKY).The results showed that Nefazodone causes a significant increment in the three important parameters (tail length, percent of DNA in tail and tail moment) of comet assay for the three used concentrations in a dose-dependent manner when compared with negative control ($p<0.01$). SKY analysis was performed at the concentration of (10 µg/ml). Both the treated and negative control cells showed abnormal chromosomal number (44-54 XY) per cell, common chromosomal rearrangements and other abnormalities in the majority of cells analyzed. As a result Nefazodone has no chromosomal aberration at 10µg/ml and the comet assay results conceder as false positive.

**Keywords:** *Nefazodone, Genotoxicity, HepG2 cell line.*

## Introduction

### Drug-induced Liver Injury

Drug-induced liver injury (DILI) is the main health matter that challenges healthcare professionals, the pharmaceutical industry and drugs regulatory agencies because it leads to failure in drugs approval, withdrawal from markets and adoption of organizational procedures [1]. The DILI can be categorized into intrinsic and idiosyncratic reactions depending on the doses of the drug and the predictability [2].

The Intrinsic hepatotoxicity can occur when a specific concentration threshold has been taken (dose dependent) [2], In contrast, Idiosyncratic DILI (I DILI) is rare, but have serious consequences. The pathogenesis of I DILI is not well known but it is unpredictable and either dependent or independent does.

However, it more frequently associated with drugs that when they are given daily in a dose over than 50 mg [3]. Several risk factors involve in DILI which can be divided into genetic factors (variations in drug-metabolizing enzymes, immunological mechanisms, damage in mitochondrial DNA) and non-genetic factors (sex, age, daily dose, drugs metabolism) [4].

### Genotoxicity

Genotoxicity referred to the destruction of genetic material (DNA) or chromosomes that caused by substances called genotoxins which is either chemical or radiations that lead to mutation and development of cancer. All the mutagens are genotoxic but not all the genotoxins are mutagens [5].

Changes of the cellular genome can generate errors in transcription and translation of DNA to proteins that are necessary for cellular signaling and function. A cell with a large amount of accumulated damaged DNA, or no longer repairs damage incurred to its DNA, can enter one of three possible states: senescence, apoptosis or unregulated cell division (cancer) [6].

### Nefazodone

Nefazodone belongs to the group of drugs called atypical antidepressant that contains other drugs such as trazodone, mirtazapine, and bupropion [7]. Nefazodone was manufactured by Bristol-Myers Squibb and marketed under the brand Serzone® as a tablet in 1994 in U.S.A and Europe For treatment of depression, panic disorder and aggressive behaviour.

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In 2004 nefazodone was withdrawn from USA after its withdrawal from Canada and Europe due to the incidence of liver injury in patients taken the drug for 1 to 8 months [8].

## Mechanism of Action

This drug acts as antagonist with relatively high affinity for postsynaptic 5-hydroxytryptamine $(5\text{-HT})_{2A}$ receptor and desensitize 5-HT1A presynaptic with chronic use, thereby inhibits reuptake of serotonin and increase its release, also inhibits norepinephrine reuptake and antagonizes alpha (1)-adrenergic receptors, and produce sedation by its action as antagonist for histamine ($H_1$) receptors [7, 9].

## Materials and Methods

### Materials

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) and Oxiselect® Comet assay kit (Biolabs / USA), Spectral karyotype (SKY) kit (ASI/USA).

### Methods

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 [10].

The cells were cultured in $25cm^2$ (T25)tissue culture vented flask with 450 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 ml of 10% Foetal bovine serum (FBS) and 0.5 mL (Penicillin 100 U/ml, Streptomycin 100 µg/ml).

The cells incubated in a humidified incubator supplemented with 5% CO2 and 95% air at 37 °C [11]. Cells were passaged to new tissue T75 flask every 4 days by washing with PBS and followed harvesting with trypsin/ EDTA and incubation at 5% CO2 and 95% air at $37^{o}C$ [12].

### Comet Assay

The comet assay used to measure the genotoxic effects at the level of a single cell for drugs, chemical and environmental contaminations. 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 is the DNA strands that containing breaks extend toward the anode [13]. Briefly the HepG2 Cells were cultured in T25 flasks one for each nefazodone concentrations (2.5, 5 and 10 µg/ml) and one without drug (vehicle control DMSO) after 24 hours of incubation, cells were detached by Trypsin-EDTA solution.

The suspension of cells was centrifuged at 1500 rpm for 2 min and washed once with ice-cold PBS and centrifuged at 1500 rpm for 2 min, then cells pellet resuspended by ice-cold PBS and the cells adjusted at1 x $10^5$ cells/ml. The cells of the sample mixed with liquefied comet agarose at 1:10 ratio (V/V) and the mixture added immediately to the comet slides by pipette and held horizontally to a dark refrigerator at 4ºC for 15 minutes. Then the slides were carried to a small container of pre-chilled lysis buffer (25ml/ slide), the slide was immersed in the buffer for 30-60min at 4ºC in a dark container.

Then the slides were immersed in pre-chilled alkaline solution (25ml /slide) for 30 min at 4ºC in the dark container. The slides were dipped for 5min in pre-chilled TBE buffer solution and carried to a horizontal electrophoresis chamber filled with a cold TBE electrophoresis solution, 1volt/cm voltage was applied to the chamber for 10-15 minutes.

Then slides were immersed for 2 minutes in per chilled distilled water (25ml/slide). The slides were carried to a container of 70% ethanol and left for 5 min then dried with air. A 100 µl of vista green stain solution was added for each well and inserted to the incubator at room temperature for 15 min [14]. The slides were observed by fluorescent microscope and the comet images were analyzed by using Comet Assay IV software. The analysis software will calculate different parameters for each comet; three parameters were estimated to indicate DNA migration

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[15], Tail length (distance from the head centre to the end of the tail). Tail moment (product of tail DNA/total DNA by the tail centre of gravity), Tail DNA%=100 X Tail DNA Intensity/Cell DNA Intensity.

### Spectral karyotyping

Spectral karyotyping (SKY) is a highly accurate, sensitive, intuitionist and novel technique comparing with another available cytogenetic tool, has been developed to visualization of all human chromosomes [16]. The SKY is a multicolor-FISH (Fluorescent in situ hybridization) system based on single hybridization for all human chromosomes and stains them with five types of fluorescent dyes, spectrum orange, spectrum green, Cy5 dye, Cy5.5 dye, Texas red and mixing each pair together. The microscope and acquisition software of the SKY system can detect the emitted signals and assign a specific color to each chromosome [17].

The SKY can detect chromosomal aberrations that can't be detected very well by G-banding technique and FISH technique [16]. This test was carried at SKY/FISH Core Facility at Roswell Park Cancer Institute Buffalo / Niagara, New York. The HepG2 Cells were cultured in T25 flasks with complete growth medium for 24 hours one for nefazodone concentration (10 µg/ml ) and one without drug (vehicle control DMSO) [14]. The metaphase and Chromosome, probe denaturation and hybridization were prepared according to a standard protocol [18, 19]. Spectral karyotyping was accomplished by using SKY computer program (Applied spectral imaging).

## Statistical Analysis

The statistical packages for social sciences (SPSS version 16) used for data analysis .The Descriptive data analysis will have expressed as mean ± standard error of mean (SEM), One-Way ANOVA test used to differentiate between more than two independent means of all studied parameters of the three groups. Followed by the Turkey test. P-value< 0.01 was considered as statistically significant.

## Results

### Comet Assay

Nefazodone HCL at concentrations (2.5, 5, 10 µg/ml) caused a significant increase in comet assays three parameters (Tail length, the percent of DNA in Tail and Tail moment) as compared to DMSO (negative control) ($p<0.01$). Also there was a significant increase ($p<0.01$) in three parameters when compering the results of each between each other as shown in Fig (1).

### SKY for Chromosomal Aberrations

To determine the chromosomal aberrations, SKY analysis was performed on 20 metaphases each for control (untreated) and HepG2 treated cell line. The treated cells were treated with 10 micrograms/ml of nefazodone for 24 hours. Both the treated and control cells have abnormal chromosomal number range from (44-54 XY) per cell. Both the treated and control cells possess a number of common chromosomal rearrangements and other abnormalities in the majority of cells analyzed as shown in Table (1) ,Fig (2) and Fig (3).

**Table 1: The chromosomal abnormities for both control and treated HepG2 cell line.**

| Type of Chromosomal rearrangement/ aberrations | Number of chromosome |
|---|---|
| Translocations | t(1:21), t(6:17), t(6:16) |
| Extra chromsomes | +1, +2, +6, +7, +14, +16, +20 |
| Missing chromosomes | -21 |
| Deleted chromosomes | (1) |

## Discussion

In the current study, the liver origin HepG2 cell line was used as cell model because it's widely used in biomedical and genotoxic studies. This cell line is qualified for detection of dietary and environmental genotoxicants because it expresses, in low capacity, a wide range of phase I enzymes such as P450 1A1, 1A2, 2B, 2C, 3A, and 2E1, cytochrome c reductase and phase II enzymes such as glutathione S-transferase (GST), uridine glucuronosyltransferase (UGT) but not express all activation enzymes compared to the conditions in vivo [20, 21].

The present study depends on maximum plasma concentration of Nefazodone to study

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its genotoxicity because the maximum plasma concentration 1-2µg/ml is reached after 200 – 400 mg daily dose. This plasma concentration may not proportion directly with increased dose [22], Moreover there are no data documented about the plasma-to-liver ratio of nefazodone [23]. Nefazodone metabolized by CYP3A4 and form conjugate with sulphate, glucuronide and eliminated thought urin (49%) and bile which represent the other major route [22, 24]. The toxicity result 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 [23, 25], where other studies showed that increase metabolism activity of CYP3A4 decrease toxicity of Nefazodone [26, 27].

### Comet Assay

The comet assay parameters showed that the single and double strand DNA breaks have been significantly increased in a Dose-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 [28]. 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 negative control [29].

The increased in light intensity and room temperature higher than 25°C can cause increase DNA damage and increase % DNA in tail [30]. 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) and cell lethality ≥ 30% can give false positive [31].Therefore SKY test is performed to ascertain that the positive result due to genotoxicity rather than cell death.

### SKY for Chromosomal Aberrations

Spectral karyotyping is a novel diagnostic tool which considered as a sensitive and highly accurate instrument for identifying chromosomal abnormalities that cannot be defined by conventional cytogenetic methods, such as translocation, deletion and duplication [32, 33].

Both the treated and control cells have abnormal chromosomal number (44-54 XY) per cell and common chromosomal rearrangements and other abnormalities in the majority of cells analyzed include translocation (t(1:21), t(6:17), t(6:16)) , Extra chromosomes (+1, +2, +6, +7, +14, +16, +20), Missing chromosomes (-21) and Deleted chromosomes (1) . This is a scribed to the use of cell line for long time in research and that lead to genomic and chromosomal instability from one passage to the next that cause changes in their phenotype, however, mutations can occur and cause characteristics and responses changes that do not exist at early passage [34].

However the cancerous cell lines actually unlike normal cells, because it possess chromosomal instability that changed from one passage to other [35].The cryopreservation conditions can also affect the karyotyping of cell lines after several passages and cryopreservation [36]. Consequently, and according to the result that obtained from the present study, it could be concluded that 10 micro/ml of Nefazodone has no chromosomal abrasion effect on HepG2 cell line and this is consistent with FDA and a study conducted by Brambilla G et al (2009) [37], where as the result of comet assay considered to be false positive.

## Conclusion

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

- Nefazodone Hcl has given false positive result in comet assay due to cell death.
- Nefazodone Hcl does not because chromosomal aberration at 10µg/ml as observed in SKY test.

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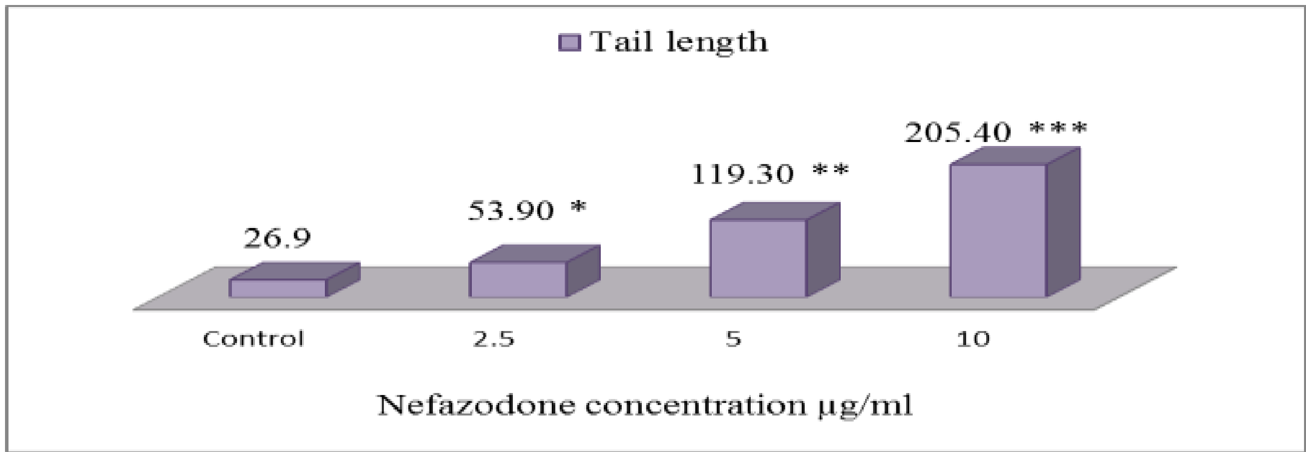
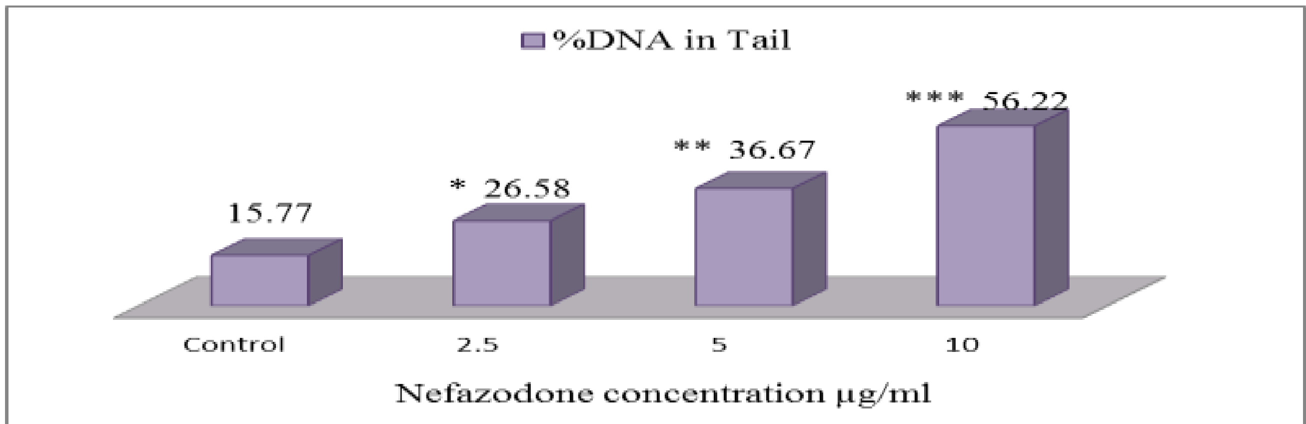


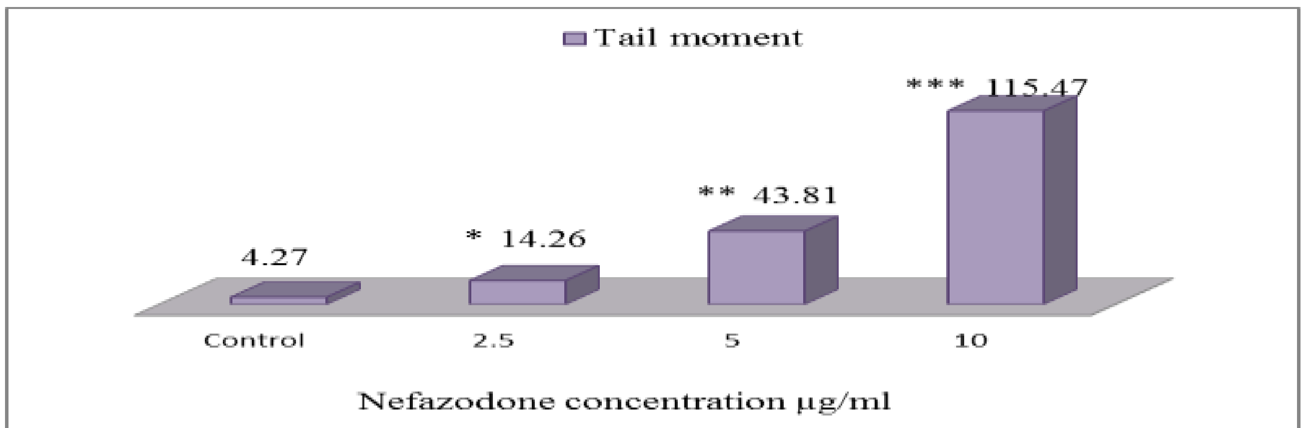
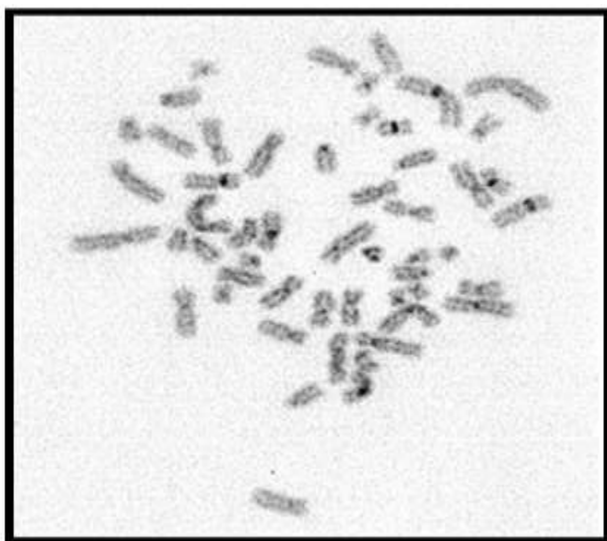
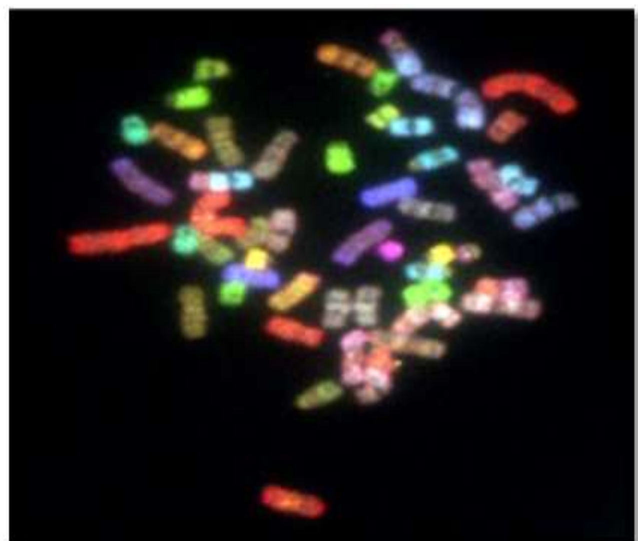




**Figure 1: the effect of Nefazodone on the comet parameters tail length, the percent of DNA in Tail and Tail moment. *, **, ***Indicates significant deference (p <0.01) in means of different concentrations**





**(A)** **(B)**

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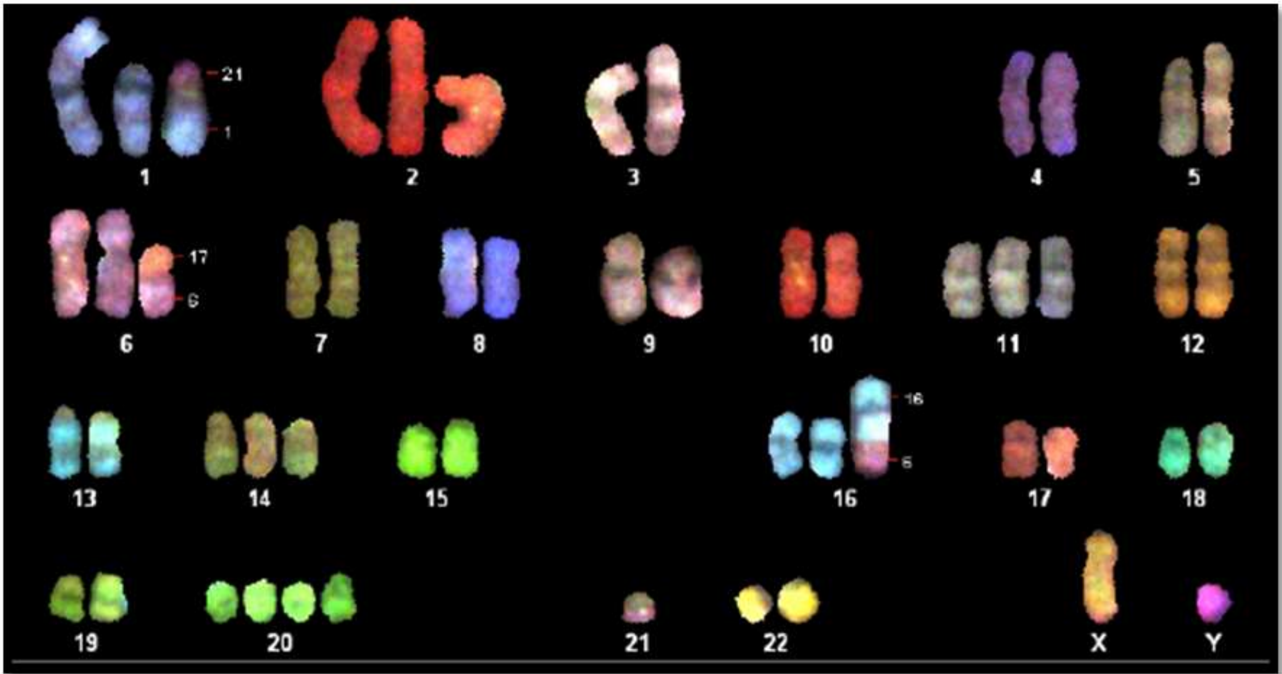
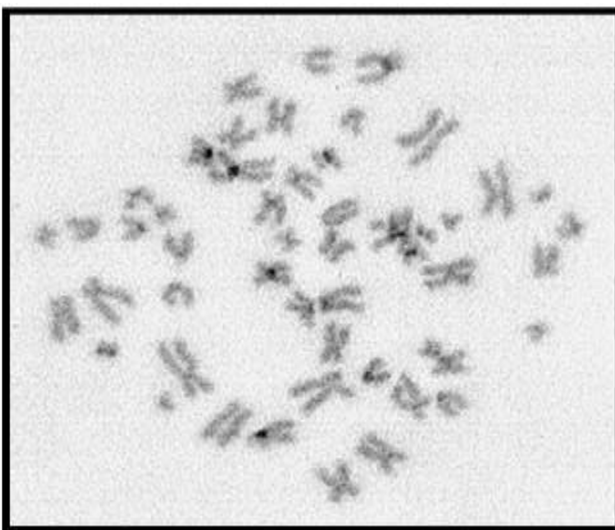


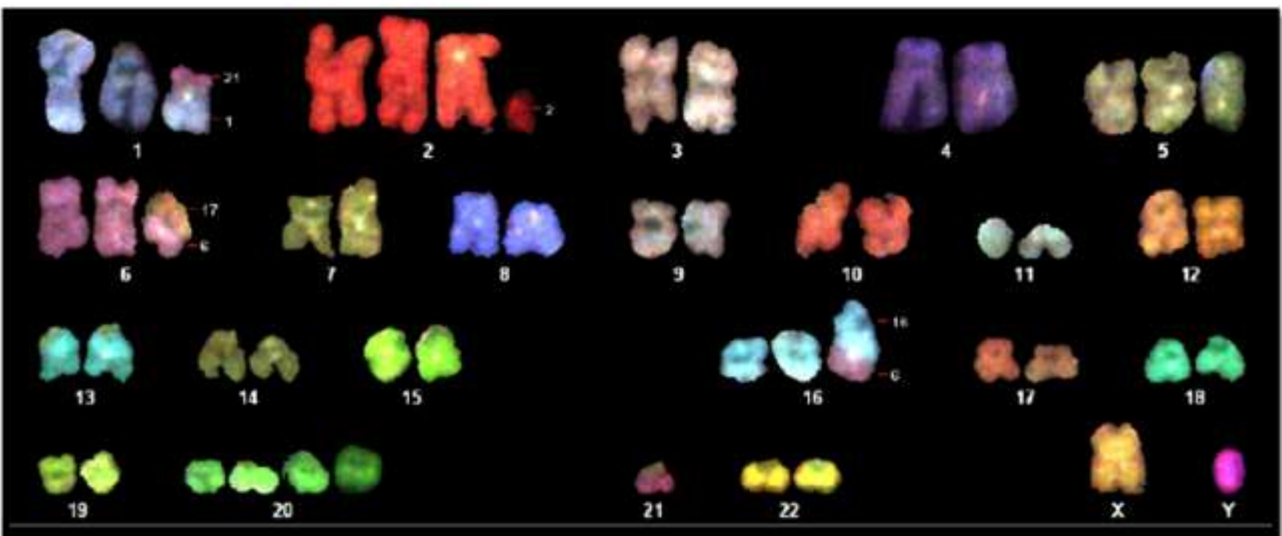

(C)

**Figure 2: SKY of metaphase HepG2 cell line after treatment with DMSO (negative control) :(A) DAPI image, (B) SKY karyotype, (C)Karyotype analysis image of SKY**



(A)

(B)




(C)

**Figure 3: SKY of metaphase HepG2 cell line after treatment with 10µg/ml Nefazodne HCL: (A) DAPI image, (B) SKY karyotype, (C) Karyotype analysis image of SKY**

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