

## Safety Assessment of a Nucleoside Analogue FNC (2'-deoxy-2'- $\beta$ -fluoro-4'-azidocytidine) in Balb/c Mice: Acute Toxicity Study

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### Abstract

**Objectives:** The present study aimed to provide an insight into the acute toxicity of a novel fluorinated nucleoside analogue (FNA), FNC (Azvudine or 2'-deoxy-2'- $\beta$ -fluoro-4'-azidocytidine). FNC showed potent anti-viral and anti-cancer activities and approved drug for high-load HIV patients, despite, its acute toxicity study being lacking. **Materials and Methods:** OECD-423 guidelines were followed during this study and the parameters were divided into four categories - behavioral parameters, physiological parameters, histopathological parameters, and supplementary tests. The behavioral parameters included feeding, body weight, belly size, organ weight and size, and mice behavior. The physiological parameters consisted of blood, liver, and kidney indicators. In histopathological parameters hematoxylin and eosin staining was performed to analyse the histological changes in the mice organs after FNC exposure. In addition, supplementary tests were conducted to assess cellular viability, DNA fragmentation and cytokine levels (IL-6 and TNF- $\alpha$ ) in response to FNC. **Results:** In the behavioral parameters FNC induced changes in the mice-to-mice interaction and activities. Mice's body weight, belly size, organ weight, and size remained unchanged. Physiological parameters of blood showed that FNC increased the level of WBC, RBC, Hb, and neutrophils and decreased the % count of lymphocytes. Liver enzymes SGOT (AST), and ALP was increased. In the renal function test (RFT) cholesterol level was significantly decreased. Histopathological analysis of the liver, kidney, brain, heart, lungs, and spleen showed no sign of tissue damage at the highest FNC dose of 25 mg/kg b.wt. Supplementary tests for cell viability showed no change in viability footprint, through our recently developed dilution cum-trypan (DCT) assay, and Annexin/PI. No DNA damage or apoptosis was observed in DAPI or AO/EtBr studies. Pro-inflammatory cytokines IL-6 and TNF- $\alpha$  increased in a dose-dependent manner. **Conclusion:** This study concluded that FNC is safe to use though higher concentration shows slight toxicity.

**Keywords:** Azvudine- antiviral agents- organization for economic co-operation and development- kidney

*Asian Pac J Cancer Prev*, 24 (6), 2157-2170

### Introduction

FNC also known as Azvudine, is a recently synthesized novel cytidine (pyrimidine) analogue (Jing et al., 2022; Smith et al., 2009). It was synthesized in 2009 for Hepatitis C virus (HCV) treatment among many other potential therapeutic molecules (Smith et al., 2009). FNC effects were more selective in HCV and it showed greater efficacy up to 125 times than the previously used treatment (Smith et al., 2009). Currently, it is a clinically approved drug for high-burden HIV patients

and showed potential activity against other viruses and cancer (NCT04303598) (Faizullina et al., 2022; Jing et al., 2022; Ren et al., 2020). The presence of 2'-fluoro substituents improves its stability in acidic media, and it mimics 2'-deoxynucleoside, thereby competing with other natural nucleoside analogues (NAs) for DNA or RNA incorporation (Faizullina et al., 2022; W. Liu et al., 2013; Yang et al., 2015). FNC strand incorporation reduces cell growth and division due to attenuated nucleotide synthesis. It is a preferred substrate for deoxycytidine kinase and phosphorylated 3-fold more efficiently than its parent,

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deoxycytidine (Faizullina et al., 2022; Klumpp et al., 2008). Viral RNA-dependent RNA polymerases (RdRp) are more sensitive toward FNC because of the presence of 2'-modification. It produces non-functional viral genomic RNA after incorporation by HCV RdRp NS5B into newly synthesized viral RNA (Smith et al., 2009). FNC is an effective inhibitor for a broad spectrum of human enterovirus (EV) pathogens. FNC got incorporated by EV RdRp 3Dpol into the positive or negative RNA strand with high specificity and efficiency (N. Xu et al., 2020). SARS-COV-2 contains a sense single-stranded RNA genome and a similar mechanism of action is suggested (Yu & Chang, 2020). 3'-OH group of FNC is not assessable to polymerases because of the masking effect of 2'-F which dramatically change the sugar conformation (Pal, Chandra, Patel, & Singh, 2022; L. Sun et al., 2020). It is potential candidate for the nucleoside reverse transcriptase inhibitor (NRTI) to inhibit RNA-dependent polymerases (L. Sun et al., 2020). It is enormously effective against HIV wild-type strain as well as NRTI-resistant and multi-resistant strains at nano-molar concentration (Wang et al., 2010). It is currently clinically approved with the name "Aztvudine" for the high-burden HIV patients (NCT04303598, (Faizullina et al., 2022; Jing et al., 2022). FNC is effective against human and duck hepatitis B viruses (HBV and DHBV, respectively) and showed activity against the HBV wild-type and lamivudine-resistant clinical isolates (Zheng et al., 2012).

Along with anti-viral activities, it showed potential anti-tumor activity. It inhibits cancer progression through cell cycle inhibition, immune system process, DNA chain termination, DNA methylation, apoptosis, etc. (Faizullina et al., 2022; Jing et al., 2022; Y. Zhang et al., 2014). FNC suppresses tumor growth through inhibition of cell surface molecules like migration, invasion, and adhesion molecules in non-small-cell lung cancer cell line H460 and two non-Hodgkin lymphoma cell lines Raji and JeKo-1 (Y. Zhang et al., 2014). FNC induces apoptosis through the mitochondrial apoptotic pathway (Jing et al., 2022), cell death receptor-mediated extrinsic pathway, or suppressing the immune system (Y. Zhang et al., 2017). It modulates the immune system to suppress the growth of tumor cells and alter the expression of genes associated with cytokines IL-2, IL-4, IL-7, IL-10, and co-stimulatory signals of T-cell activation (Y. Zhang et al., 2017). It was suggested that FNC could be a potent pleiotropic molecule for the treatment of various pathological conditions of cancer (Faizullina et al., 2022). More in-depth FNC anticancer studies are required to understand the true clinical potential of FNC. However, FNC is a potential candidate for the clinical trial in cancer and approved drug for anti-viral activities, there is no report of its acute toxicity study. As per the Organization for Economic Co-operation and Development (OECD) 423 guidelines acute toxicity study is one of the essential tests done before testing it's in vivo effects (OECD, 2002). Acute toxicity describes the adverse effect of a substance from single or multiple exposures within 24 hours, which strictly occurred within 14 days after substance administration. Some in vivo studies of FNC was performed in SCID, nude mice, rat model, and macaque, etc (Y. Liu et al., 2017; Peng et al.,

2014; L. Sun et al., 2020; Wang et al., 2011; Y. Zhang et al., 2017) and FNC showed desirable pharmacokinetics and safety profiles in the clinical trial phase-I (L. Sun et al., 2020). Thus, we performed FNC acute toxicity study in Balb/c mice, through OECD-423 (2001) guidelines. This study is divided into four sections to decipher the true acute toxicity of FNC: Behavioural parameters, physiological parameters, histopathological parameters, and supplementary tests.

## Materials and Methods

### Materials

FNC was procured from Granlen Inc., China. DAPI, AO, EtBr, and trypan were purchased from Thermo-Fisher Scientific. Annexin-V FITC and PI apoptosis kit were purchased from Invitrogen, USA. All other chemicals were purchased from Hi-media laboratory, Mumbai, India. Most of the tests were performed at the commercial diagnostic laboratory Parul Pathology, Lanka, Banaras Hindu University (BHU), Varanasi (India).

### Mice model system and Dose design

#### Animal Model

An inbred healthy population of Balb/c (H2d strain) mice model was used for the study. Mice were kept at the animal house facility of the Department of Zoology, BHU, Varanasi, India. All mice were given standard sterilized food, and water, and at strictly maintained, standard conditions of temperature (22±3°C), humidity (range 50-60 %), and light/dark (12/12 hr) cycle with proper human care. Females of age group 8-10 weeks, weight 25±2 g, were used for the experimentation. All mice experiments were approved by the Institutional animal ethical committee, BHU, Varanasi.

### Grouping and dosing of animals

Mice were divided into four groups, and each group contain 3 females, n=6 (3×2=6, repeated two times-R2), and habituated for 7 days before the dosing. Mice were fasted 4 hours before dosing, and then injected intraperitoneally (i.p.) with PBS in control and three doses of FNC viz. 1, 10, and 25 mg/kg body weight (b.wt.) on day 0. FNC dissolved in DMSO was diluted in Phosphate Buffered Saline (PBS) (pH-7.4) immediately before use, keeping DMSO concentration below 1%. Mice were visualized twice for 2 hours every day, on and after the dosing days, for any abnormal behavior and mortality up to 14 days. On day 14 mice were euthanized using light anesthesia, followed by painless cervical dislocation for organ removal for pathology, histology, and other analysis (Table 1).

### Behavioural parameters

A study of behavior before and after dosing days is very helpful to understand any external and visible side effects of the drug (Bedi & Krishan, 2020). Various behavioral parameters were taken into consideration to assess the effect of FNC on the behavior of mice. Assessment of behavior was done through daily observation of mice groups at least for 2 hours. These parameters were divided

into four groups: (a) Physical or body appearance - General physique, skin/fur color, fur appearance, skin allergy, eyes abnormality, eyes color, eyes bulge; (b) Mice to mice interaction: playing pattern, cuddling, aggressive behaviors; (c) Functional parameters - visible respiration problem, rate of respiration, body temperature, diarrhea, urination, infection of mice; (d) Mice Activity: mice movements, exploratory activity, body cleanliness, abnormal behavior, sleep pattern, sleep, normal/lethargy, drowsiness, sedation, tremors, salivation, convulsion, coma, death/live, etc.

#### *Feeding behavior*

Mice were provided with 50 g of water, and 25 g of feed daily, and after 24 hours changes in the amount of feed and water were recorded from day 0 to day 14.

#### *Body weight, belly size, and organ's weight, size, and coefficient measurement*

The body weight and belly size of the control and treated groups were measured on day 0 and then every third day and on the day of sacrifice. On the day of sacrifice organs like the Liver, kidney, lungs, brain, heart, and spleen were removed, washed with PBS dried over tissue paper, and then size and weight were measured. The organ coefficient was measured by taking the percentage of organ weight to total mice weight.

$$\text{Organ coefficient} = \frac{\text{Weight of organ (gm)}}{\text{Total body weight of mice (gm)}} \times 100$$

#### *Physiological parameter*

##### *Haematological examination*

Assessment of blood parameters helps to understand the role of drugs on the various blood constituents of the mice (Alqahtani, Ullah, & Shahat, 2022). Blood samples for the hematological analysis were collected from the tail vein. EDTA-coated vials were used for blood collection and then supplied to Parul Pathology. The blood parameters like (a) cells number - red blood cell count (RBC), white blood cell count (WBC), platelet count, and percentage of the lymphocytes, neutrophils, monocytes, eosinophils, and basophils; (b) and cellular parameter - hemoglobin (Hb) concentration, packed cell volume (PCV), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), Red cell distribution width (RDW) was recorded.

#### *Biochemical parameters*

##### *Liver function test (LFT)*

The liver and its functions were assessed through LFT. Briefly, blood was collected in a non-EDTA coated tube and stored at 4° until further analysis and supplied to Parul pathology. Serum's parameters like liver enzymes alkaline phosphatase (ALP), Aspartate transaminase or SGOT (AST), alanine transaminase or SGPT (ALT), and total protein, albumin, globulin was performed on Beckman AU480.

##### *Kidney or renal function test (RFT)*

For RFT blood collected in non-EDTA tubes was

analyzed through Beckman AU48. Different serum parameters like serum urea, blood urea nitrogen (BUN), creatinine, sodium, potassium, chloride, calcium, phosphorus, uric acid, and cholesterol, were analyzed to assess the effect of FNC on kidney functions.

#### *Histopathological tests*

##### *Histological analysis*

On day 14th all animals were sacrificed by painless cervical dislocation under light anesthesia. Mice organs liver, kidney, lungs, brain, heart, and spleen were carefully removed, and stored in 10% formalin for histological analysis. These tissues were supplied to Parul pathology for histology slide preparation through hematoxylin and eosin (H&E) and then photographed at bright field mode on a fluorescence microscope (Olympus BX63).

##### *Pathological analysis*

After sacrificing the mice detailed gross necropsy analysis was performed to check for any pathological changes. Body external and internal surface, thoracic and abdominal cavities, orifices and cranial, any abnormal growth or clot-like structure, and discoloration of organs were visualized. A detailed pathological examination of removed organs for any sign of abnormality, discoloration, or abnormal growth was done. These organs were measured through scale and weighted.

##### *Supplementary tests*

At day 14, before cervical dislocation, mice were injected with 3 ml of chilled PBS and peritoneal fluid was collected into a syringe, these cells were then washed thrice with PBS and used to perform DCT, DAPI, Ao/EtBr, and annexin/PI. Mice liver and spleen were minced to form a single cells suspension (Müller, Schreiber, Kartenbeck, & Schreiber, 1972; Tomar et al., 2018) and then used to study cell viability through DCT assay.

##### *Immunological analysis*

Inflammation due to drug-caused injury is a common occurrence during acute toxicity studies, therefore pro-inflammatory cytokines IL-6 and TNF- $\alpha$  were assessed on the Beckman colter. Briefly, blood collected in non-EDTA tubes was supplied to the Parul pathology for cytokines analysis.

##### *DCT assay for trypan positive and negative cells, i.e., cell viability*

DCT or trypan assay is based on the cell's membrane integrity to assess the viability of the cell, where the cell that had lost integrity will be stained by trypan and intact membrane integrity cells will not be stained by trypan. The extracted cells of the peritoneum, liver, and spleen were washed and then stained with our recently developed assay DCT (unpublished observation). Briefly, cells were stained with DCT standard trypan concentration for 3 minutes, washed, and counted using a hemacytometer, and the percentage of live cells was calculated using the following formula.

$$\text{Percentage of live cell} = \frac{\text{Number of live cells}}{\text{Total number of cells}} \times 100$$

Annexin PI was performed to check whether peritoneal cells are showing apoptotic characteristics. Annexin bind to apoptotic cells showing phosphatidylserine on the surface, and PI enter the cells that are undergoing apoptosis and have lost membrane integrity. For An/PI, washed cells pallet were redissolved in 200 µl of Annexin-binding buffer, stained as per manufacture instructions, and analyzed through flow cytometry (Cytotflex LX).

*DAPI staining*

DAPI (4',6-diamidino-2-phenylindole) was used to analyze the potential cytotoxic effects of FNC by assessing DNA fragmentation or chromatin condensation. For DAPI, washed cells were fixed in 4% paraformaldehyde for 10 minutes and then stained with 5 µl of DAPI (1 mg/ml) for 10 minutes.

*AO/EtBr Staining*

Live/dead cells were further characterized through differential staining AO/EtBr. AO binds to live cells and EtBr bind to the cells undergoing apoptosis or necrosis. For, AO/EtBr washed cells were stained with 3 µl of AO (100 µg/ml) and 2 µl of EtBr (100 µg/ml) for 10 minutes.

*Statistical analysis*

GraphPad Prism 5.01 software (GraphPad Software, CA) was used for statistical analysis. All experiments were performed in a duplicate and parallel manner and represent the mean ± SD unless indicated. To determine significant differences A one-way or two-way analysis of variance (ANOVA) followed by Tukey post-test and Bonferroni post-test, respectively, were used between the groups being compared. All over significance was considered at \*p<0.05.

**Results**

*Behavioral parameters*

Altered behavior due to drug effects has been reported in several studies (Meldrum, Gupta, Lowes, & Paterson, 1985; Schrand et al., 2010). Therefore, the mice's behavior

was recorded every day to check for any altered state due to the drug, compared to the control group. A total of 30 parameters were considered for the study and divided into four parts (a-d) (Table 2). (a, c) Physical and functional parameters (1-7, 11-16) - there was no significant change in the physical and functional parameters was recorded. (b) Mice-to-mice interaction (8-11) like playing patterns, cuddling, and aggressive behaviors, and (d) Mice activity (17-30) like mice movements, exploratory activity, abnormal behavior, sleep were significantly enhanced in the drug-treated group (p<0.05).

*Feeding and water behavior*

Feeding and water are important for the survival of mice, and in case of toxicity or disease, their uptake might reduce (Muia et al., 2020). To assess the effect of FNC administration, mice feeding, and water uptake were analyzed for over 14 days. It was found that FNC groups show enhanced feed (1a) and water (1b) consumption, especially the day after the drug treatment (p<0.05) (Figure 1).

*Body weight, belly size, organ weight, organ size, and organ coefficient analysis*

Loss of body weight is an important parameter indicative of drug toxicity, which also causes a reduction in belly size and organ weight (Pour, Latha, & Sasidharan, 2011). In this study, there was an overall insignificant increase/decrease in body weight (2a) or belly size (2b) in the drug-treated group compared to the control group (Figure 2). Organ weight analysis (2c, d) showed the insignificant impact of concentration on the organ weights, where randomly some organs' weight increased and others decreased, excluding the spleen. The organ coefficient (2e, f) showed an overall insignificant change in all groups, except the spleen which increase in size (p<0.05). Organs size analysis also showed that FNC didn't significantly alter the size of organs (Table 3).

*Physiological parameter*

*Haematological, RFT, and LFT examination*

Hematological and biochemical parameters were

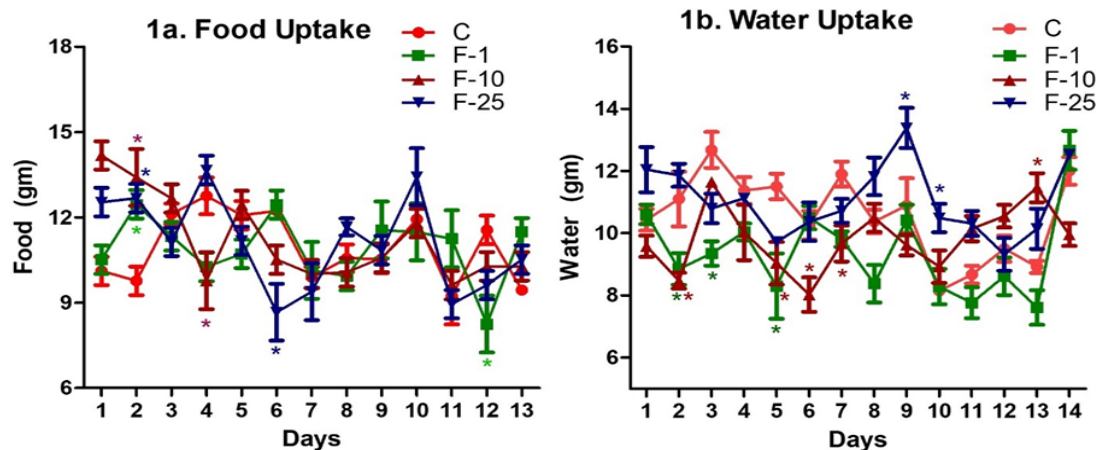


Figure 1. Feeding and Water Uptake Behavior of Mice. Mice feed and water uptake were daily analysed for 14 days and recorded consumption and significant difference is shown with a different color. The data were analyzed using two-way ANOVA followed by Bonferroni post-test. R<sup>2</sup>, Significant at p<0.05%.

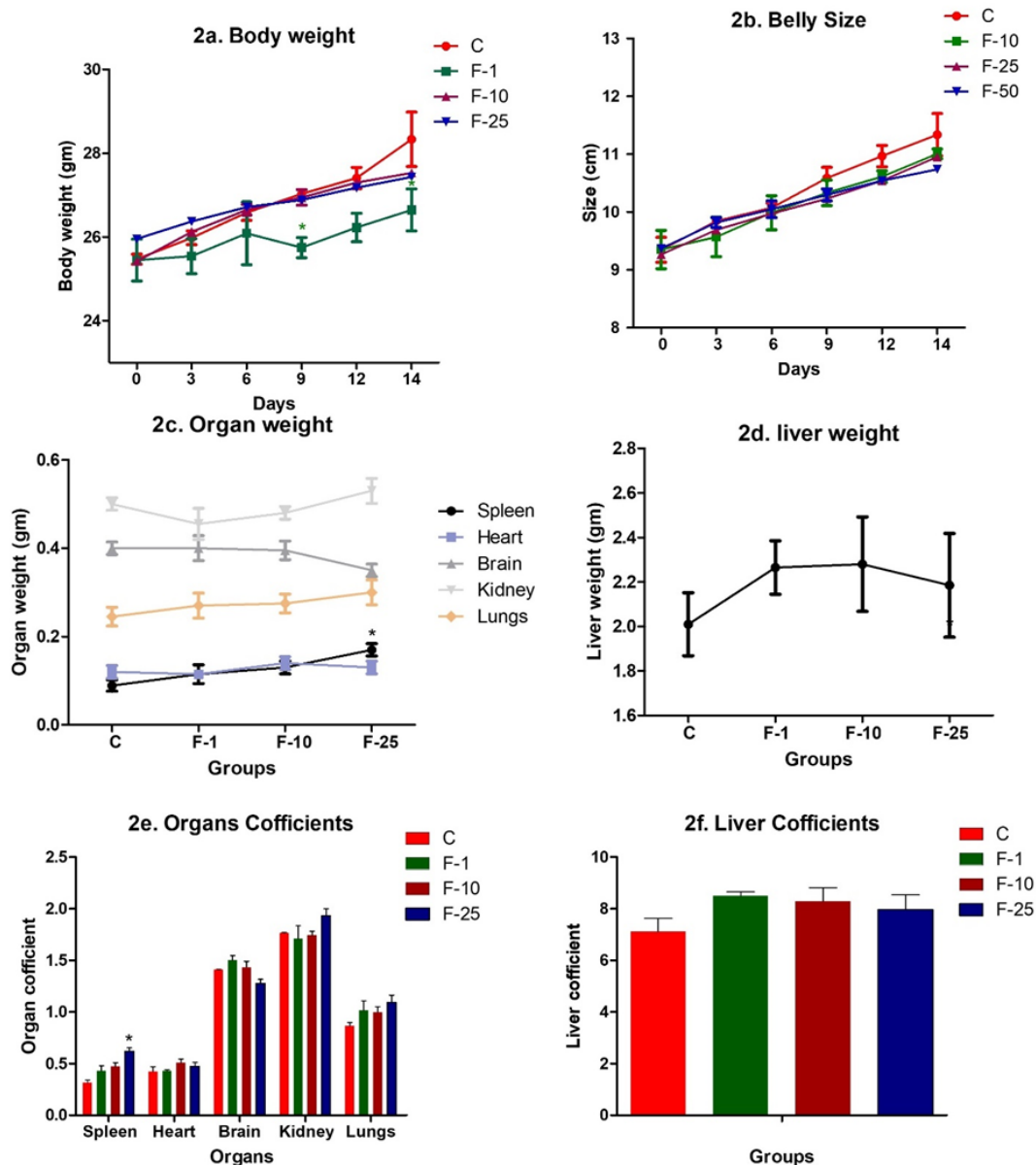


Figure 2. Analysis of Body Weight, Belly Size, Organ Weight, and Organ Coefficient. The body weight and belly size were measured at days (0, 3, 6, 9, 12, and 14). Mice organs were removed and their weight was recorded to calculate the organ coefficient. The data were analyzed using one-way or two-way ANOVA followed by post-test Tuckey and Bonferroni, respectively. R<sup>2</sup>, Significant at p<0.05%.

recorded to evaluate the effect of FNC. It was found that FNC significantly increased the number of WBC, RBC, Hb, and Neutrophil % and decreased % of lymphocytes (p<0.05) (Table 4). PCV also increased with increasing concentration of FNC. Liver enzymes ALP, and SGOT (AST) were increased (p<0.05) (Table 5). p<0.05 Renal function analysis showed that cholesterol level was significantly decreased, however, FNC didn't alter the other RFT parameters (p<0.05) (Table 5).

Table 1. Groupings of the Animals

Groups	Group name	Dose given (total 1 ml i.p.)
Group I	Control (C)	PBS only
Group II	Low dose (F-1)	1 mg/kg b.wt.
Group III	Medium dose (F-10)	10 mg/kg b.wt.
Group IV	High dose (F-25)	25 mg/kg b.wt.

### Histopathological tests

#### Histological analysis

After 14 days, mice organs were collected and then proceed for histological analysis through H&E staining. Overall, histology photographs showed that FNC didn't induce any structural changes in the mice's organs (Figure 3). The liver, kidney, lungs, brain, heart, and spleen showed a normal structure and histology comparable to the control group and showed no histopathies signs which might occur due to FNC administration.

#### Pathological analysis

A pathological examination was performed to check any visible change in the mice's bodies. It showed that FNC didn't induce changes in the body's external/internal surface, cavities, or orifices. No abnormal growth, clot, or any sign of discoloration was recorded, in the body or

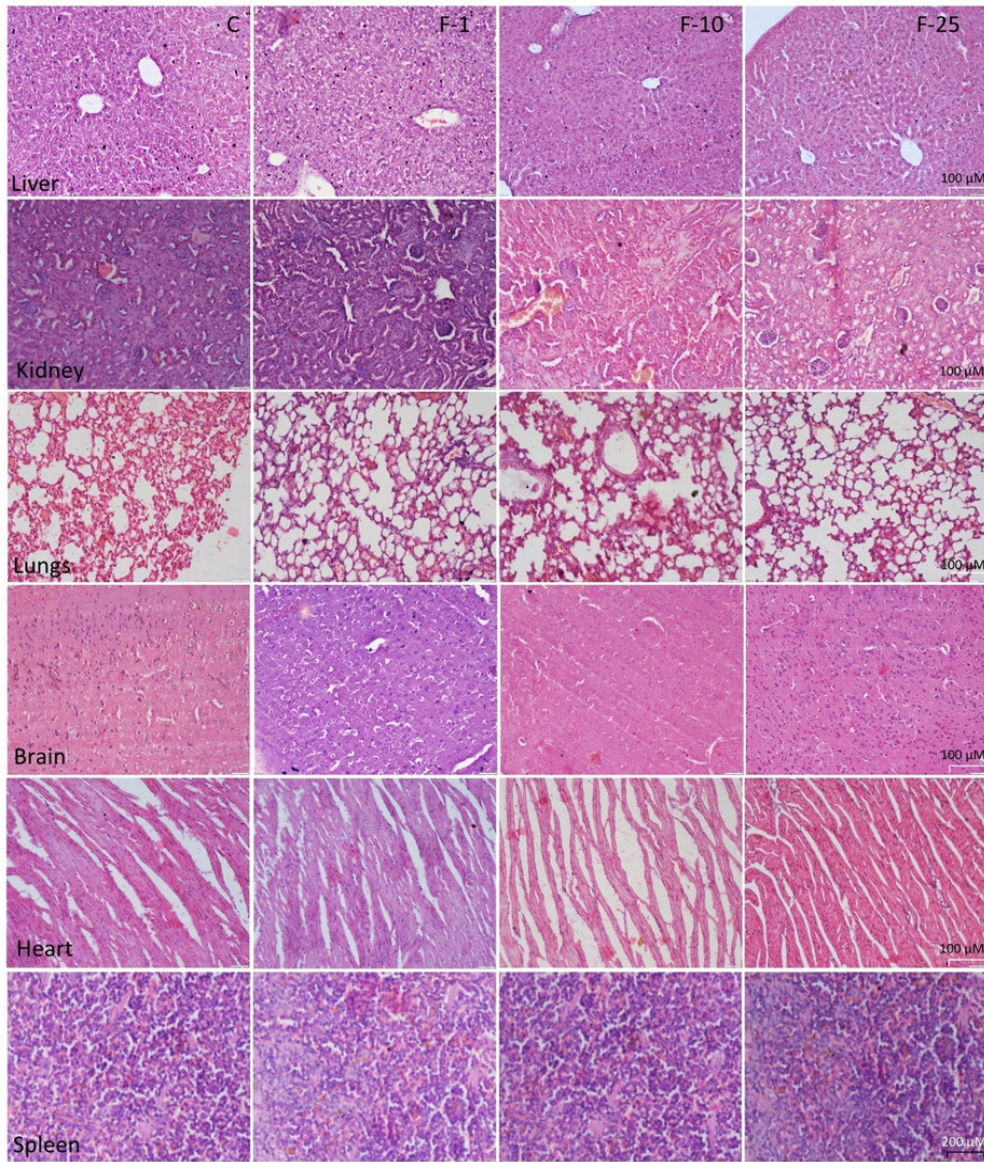


Figure 3. Histological Analysis of Mice Organs Treated with FNC. H&E staining was performed to check the impact of FNC dosing on the Liver, kidney, lungs, brain, heart, and spleen at different doses of FNC. Bar 100 μm (spleen 200 μm). R<sup>2</sup>, Significant at p<0.05%.

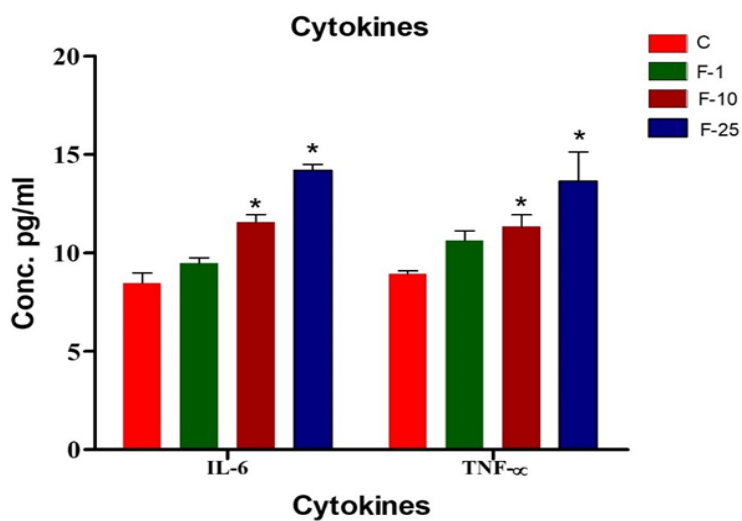


Figure 4. Effect of FNC on Immunological Parameters. The effect of FNC on the pro-inflammatory cytokines IL-6 and TNF-α was assessed on the Beckman coulter. The data were analyzed using two-way ANOVA followed by post-test Bonferroni. R<sup>2</sup>, Significant at p<0.05%.

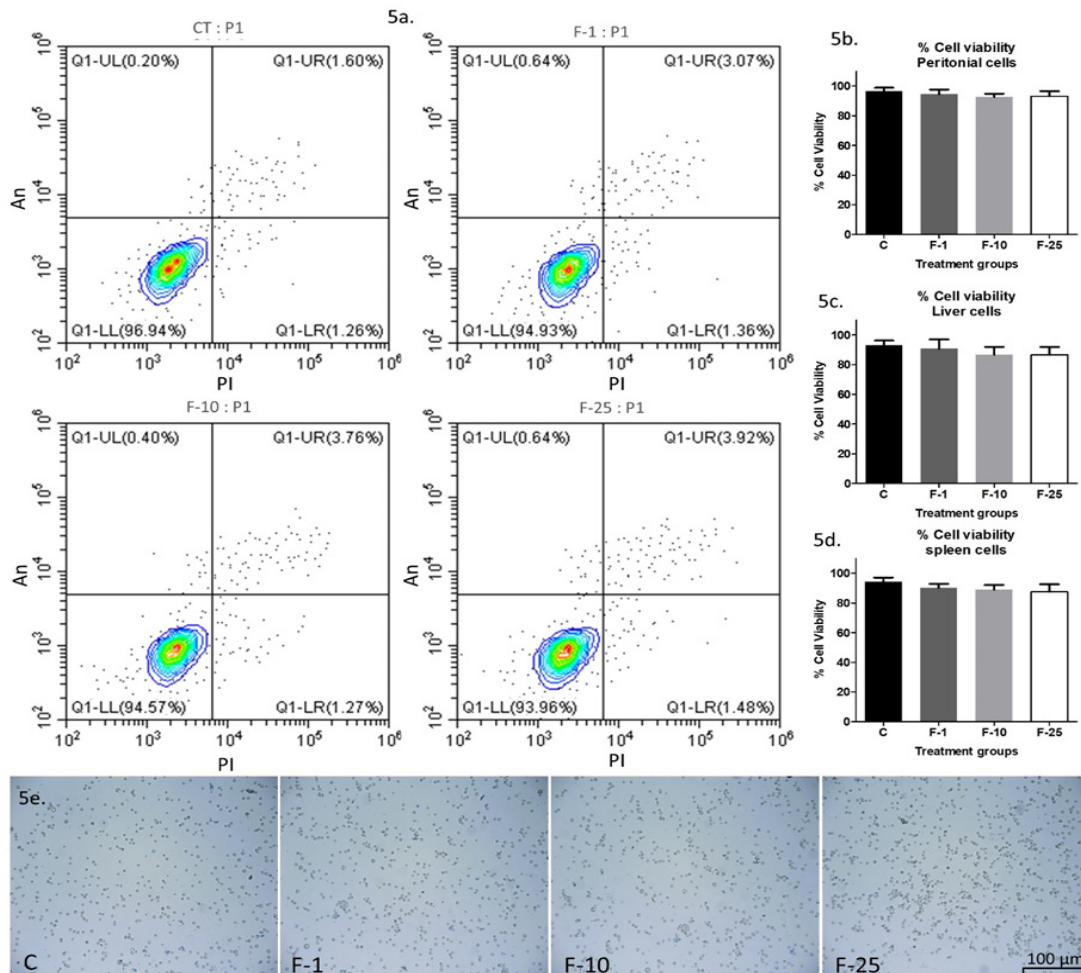


Figure 5. Live and Dead Cells Analysis through Annexin/PI and DCT. (a) Peritoneum cells stained with An/PI were analyzed through a flow cytometer. (b-d) Further, peritoneum, liver, and spleen cells proceeded for live and dead cell analysis through DCT assay. (e) Peritoneum cells DCT staining (4x) is shown at the bottom. The data were analyzed using one-way ANOVA followed by post-test Tuckey. Bar 100 μm. R2. Significant at p<0.05%.

Table 2. Impact of FNC Dosing on the Behaviour of Mice

Parameters	Groups				Parameters	C	F-1	F-10	F-25
	C	F-1	F-10	F-25					
1 General Physique	N (1)	N (2)	N (1)	N	15 Urination	N (2)	N (3)	N (3)	N
2 Skin/Fur Color	N	N	N (2)	N	16. Fecation of mice	GB (1)	GB (3)	GB (2)	GB
3 Fur appearance	A/NT/S	A/NT/S	A/NT/S	A/NT/S	17 Mice movements	N (3)	N (2)	N (4)	A (8)*
4 Skin allergy	No	No	No	No	18 Exploratory activity	N (1)	N (4)*	A (7)*	A (5)*
5 Eyes Abnormality	No	No	No	No	19. Body cleanliness	G (3)	G (4)	G (1)	G (2)
6 Eyes Color	P	P	P	P	20 Abnormal Behavior	No (1)	Y (4)*	No (1)	Y (5)*
7 (Pink/White)					21 Sleep Pattern	N (2)	N (1)	N (2)	N (3)
8 Eyes Bulge	N	N	N	N	22 Sleep	N (1)	N (2)	N (3)*	A (6)*
Playing pattern	N (1)	A (5)*	A (4)*	A (4)*	23 Lethargy	No (3)	No (3)	No (2)	No (2)
9 Cuddling	N (2)	A (8)*	A (4)*	A (5)*	24 Drowsiness	N (3)	N (4)	N (4)	N (3)
10 Aggressive behaviour	No (1)	No (1)	Y (3)*	Y (4)*	25 Sedation	No (2)	No (3)	No (2)	No (2)
11 Visible respiration problem	No	No	No	No	26 Tremors	No	No	No	No
12 Rate of respiration	N	N	N	N	27 Convulsion	No	No	No	No
13 Body temperature	N	N	N (1)	N (2)	28 Salivation	No	No	No (1)	No (1)
14 Diarrhoea	No	No	No (1)	No (2)	29 Coma	No	No	No	No
					30 Death/Live	L	L	L	L

N, Normal; A, Altered; No, Not present; Y, Yes; A/NT/S, Albino white/non-tangled/straight; G, good; GB, greenish black; P, Pink; The bracket digits indicate days of altered activity. Data of altered activities days were used to calculate significance data. R<sup>2</sup> (Repeated two times). The data were analysed using two-way ANOVA followed by Bonferroni post-test. Significant at p<0.05%.

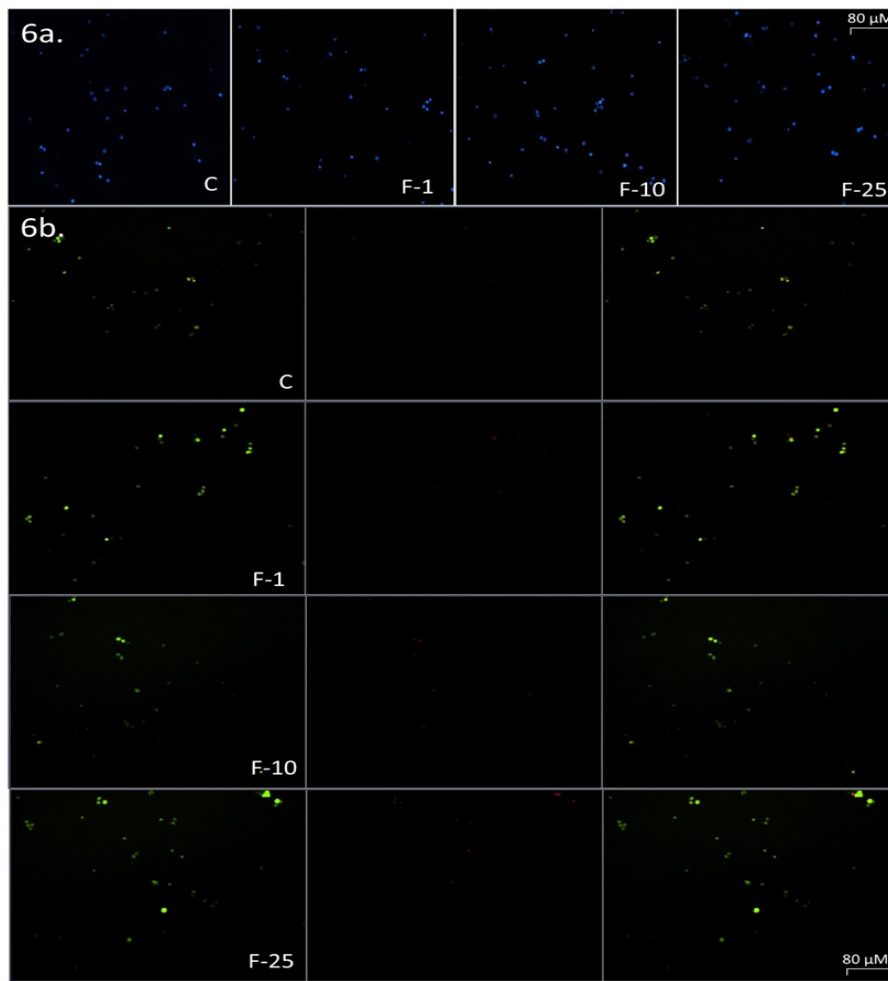


Figure 6. DAPI and AO/EtBr staining for Analysis of Cell Death or DNA Fragmentation. Bar 80 μm.

separated organs.

*Supplementary tests*

*Immunological parameters*

IL-6 and TNF-α are important cytokines that are produced during toxicity-induced inflammation or injury. It was found that FNC increases the level of IL-6 and TNF-α in a dose-dependent manner (p<0.05) (Figure 4).

*Live and Dead cells analysis through DCT and Annexin/PI assay*

Cells extracted from the peritoneum, liver, and spleen proceeded for viability analysis through DCT assay (Figure

5). It was found that more than 90 % of cells showed viable footprint. However, a certain percentage of dead cells was recorded to increase with drug concentration, but it was insignificant compared to the control group. Further, An/PI analysis was performed on the peritoneum cells showing more than 90% of cells are viable.

*DAPI and AO/EtBr staining*

DAPI and AO/EtBr were further performed to check wheatear FNC induce any DNA damage or cell death (Figure 6). In imaging, it was found that FNC didn't induce any significant DNA damage, nuclear fragmentation, or cell death. The results of all FNC groups were comparable

Table 3. Organ's Size Measurement

	Groups			
	C	F-10	F-1025	F-250
Liver	3.2±0.3 × 1.6±0.12	2.9±0.12 × 1.4±0.09	3.3±0.4 × 1.8±0.28	3.2±0.2 × 1.7±0.22
Spleen	1.5±0.21 × 0.5±0.078	1.6±0.29 × 0.57±0.010	1.6±0.12 × 0.6±0.065	1.64±0.24 × 0.5±0.026
Heart	0.85±0.15 × 0.5±0.034	0.79±0.09 × 0.5±0.086	0.90±0.25 × 0.6±0.081	0.79±0.12 × 0.6±0.014
Brain	1.10±0.18 × 0.9±0.062	0.9±0.23 × 0.8±0.071	1.15±0.22 × 1.0±0.26	1.14±0.85 × 1.0±0.093
Kidney	0.9±0.092 × 0.4±0.086	0.87±0.071 × 0.3±0.069	1.0±0.059 × 0.4±0.012	1.0±0.014 × 0.5±0.025
Lungs	1.10±0.26 × 0.8±0.041	1.12±0.18 × 0.9±0.026	1.3±0.10 × 0.9±0.21	1.20±0.10 × 0.9±0.025

Data represent organs length×breadth in cm and mean ± SD of the group. The data were analysed using two-way ANOVA followed by Bonferoni post-test. Significant at p<0.05%.



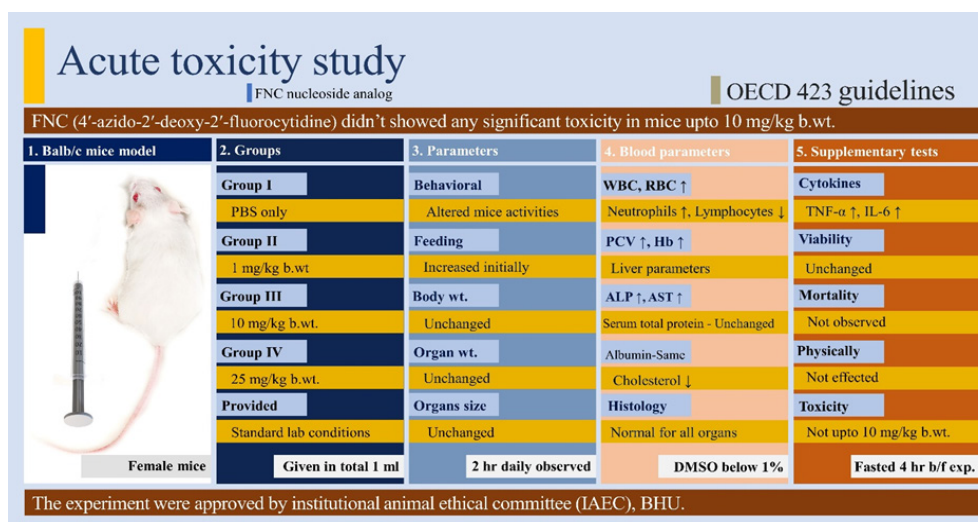


Figure 7. Overall Summary of FNC Acute Toxicity Assessment in Balb/c Mice as per OECD Guidelines 423.

to the control group.

## Discussion

Acute toxicity describes the adverse effect of a substance from single or multiple exposures within 24 hours, which strictly occurred within 14 days after substance administration. OECD-423 acute toxicity guidelines are based on stepwise evaluation of toxicity with 3 animals per step of same-sex (generally females) and depending on the output of each step (mortality or morbidity), 2-4 steps are done to judge the acute toxicity of a substance (OECD, 2002). This study describes the acute toxicity of a NA, FNC, which is a recently synthesized drug and has shown very good anticancer and antiviral efficacy.

First, we assessed the early or external signs of toxicity by studying the behavior of mice, or any external bodily changes induced by FNC. Chemical compounds above a certain limit can induce weight loss, reduce food and water

consumption, cause a behavioral change, induce organ weight loss or morbidity in mice (Akhila et al., 2007; Creton et al., 2010), can be described as an “external signs of toxicity”. NAs either may cause significant toxicity at lower doses (Jordheim et al., 2013; Moyle, 2000), or be well-tolerated in mice even at a very higher dose (Wu et al., 2014). Behaviour is the foremost parameter impacted due to any changes in the body due to drugs, and the study of behavior might indicate the early sign of toxicity. Therefore, mice behaviors were studied for 14 days and it showed that FNC administered in a single dose i.p. at 1, 10, 25 mg/kg b.wt. slightly alters the mice’s behavior at a higher dose. Mice-to-mice interactions like playing patterns, cuddling, and aggressive behaviors and mice activities like mice movements, exploratory activity, abnormal behavior, and sleep were altered in FNC-treated mice (Table 2). The drug treatment increased feed (Figure 1a) and water (Figure 1b) uptake, especially the day after the drug treatment (Figure 1). Most of these signs were

Table 4. Effect of the FNC on the Haematological Parameters of Mice

Haematological parameters				
Parameter	Control	F-1	F-10	F-25
RBC ( $\times 10^6/\mu\text{l}$ )	5.12 $\pm$ 0.38	5.55 $\pm$ 0.19	6.23 $\pm$ 0.21	6.90 $\pm$ 0.27*
WBC ( $\times 10^3/\mu\text{l}$ )	6.90 $\pm$ 0.14	6.60 $\pm$ 0.13	11.70 $\pm$ 0.685*	11.90 $\pm$ 0.69*
Platelet ( $\times 10^5/\mu\text{l}$ )	1.78 $\pm$ 0.18	2.11 $\pm$ 0.15	2.22 $\pm$ 0.21	2.98 $\pm$ 0.24
Lymphocyte %	78 $\pm$ 3.91	61 $\pm$ 3.15*	45 $\pm$ 2.15*	34 $\pm$ 1.6*
Neutrophil %	13 $\pm$ 1.65	27 $\pm$ 1.15*	46 $\pm$ 2.13*	60 $\pm$ 3.14*
Monocytes %	9 $\pm$ 0.257	12 $\pm$ 0.64	9 $\pm$ 0.15	6 $\pm$ 0.5
Hb (gm/dl)	10 $\pm$ 1.5	12.3 $\pm$ 0.915*	13.3 $\pm$ 0.365*	14 $\pm$ 0.9*
PCV %	31.4 $\pm$ 1.77	33.8 $\pm$ 1.79	32.9 $\pm$ 2.445	39 $\pm$ 1.45*
MCH (pg)	17.3 $\pm$ 0.065	17.7 $\pm$ 0.585	17.4 $\pm$ 0.67	29 $\pm$ 1.35
MCV (fl)	54.3 $\pm$ 2.115	50.9 $\pm$ 2.445	56.2 $\pm$ 2.31	59 $\pm$ 2.55
MCHC %	31.8 $\pm$ 1.39	29.1 $\pm$ 1.355	31 $\pm$ 1.85	33 $\pm$ 1.95
RDW	14.2 $\pm$ 0.51	16.1 $\pm$ 0.905	14.4 $\pm$ 0.22	14.6 $\pm$ 0.43

Data represent mean  $\pm$  SD of the group. fl, femtoliter; RBC, (Red blood cells); WBC, (White blood cells); Hb, (Haemoglobin); PCV, (Packed cell volume); MCH, (Mean corpuscular haemoglobin); MCV, (Mean corpuscular volume); MCHC, (Mean corpuscular haemoglobin concentration); RDW, (Red cell distribution width); The data were analysed using one-way ANOVA followed by Tuckeypost-test. Significant at  $p < 0.05\%$ .

Table 5. Effect of FNC on the Liver and Kidney Parameters

LFT (Liver function test)				
	C	F-1	F-10	F-25
Serum alkaline phosphatase (ALP) (IU/L)	187±9.75	161±5.05*	158±6.9*	151±8.55*
Serum total protein (gm/dl)	6.9±0.445	7.6±0.28	7.8±0.49	8±0.6
Serum albumin (gm/dl)	3.7±0.285	3.1±0.255	3.9±0.295	4.3±0.315
Serum globulin (gm/dl)	3.2±0.26	3.9±0.395	3.7±0.385	4.5±0.425
SGOT (AST) (IU/L)	99±8.26299±17.95	140±5.12*340±13.5*	198±11.09*398±15.05*	218±7.22*418±17.9 *
SGPT (ALT) (IU/L)	91±2.55	87±5.35	93±3.65	89±5.45
Bilirubin total (mg/dl)	3.4±0.57	2.9±0.245	0.9±0.145*	0.8±0.07 *
Bilirubin direct (mg/dl)	0.9±0.06	0.8±0.05	0.3±0.012	0.2±0.02
Bilirubin indirect (mg/dl)	-	-	-	0.6±0.03
Van den Bergh reaction	Positive	Positive	Negative	Negative
Anti-mitochondrial antibody (M2) U/ml	Negative	Negative	Negative	Negative
RFT (Renal function test)				
Urea (mg/dl)	38.7±1.735	37.4±1.67	42.3±2.415	43.6±2.68
Blood urea nitrogen (BUN) (mg/dl)	18.08±0.904	20.37±1.0185	21.14±1.357	23.12±1.456
Creatinine (mg/dl)	0.38±0.029	0.38±0.009	0.36±0.028	0.41±0.0405
Sodium (mEq/L)	134.6±7.73	125.2±4.26	146.7±9.335	138.7±7.935
Potassium (mEq/L)	6.8±0.54	6.2±0.41	6.9±0.245	6.3±0.615
Chloride (mEq/L)	95.3±7.765	93.6±3.68	104.2±6.21	103.7±4.185
Calcium (mg/dl)	9.1±0.255	10.3±0.415	10.3±0.315	7.3±0.165
Phosphorus (mg/dl)	4.7±0.135	5.4±0.17	6.1±0.405	4±0.4
Uric acid (mg/dl)	5.2±0.16	3.2±0.46	5.3±0.365	3.9±0.395
Cholesterol (mg/dl)	101±5.05	79±4.95*	96±3.3	92±5.6*

Data represent mean ± SD of the group. The data were analysed using one-way ANOVA followed by Tuckeypost-test. Significant at  $p < 0.05\%$ .

recorded in the early days after FNC administration and abnormality was not recorded in post days ( $p < 0.05$ ). This might be due to the fact that FNC accumulates in the mice body in early 24 hours and then excreted out in later days (Sun et al., 2020).

Another sign of toxicity that is easily observable is loss of body weight (Darnerud, 2003). FNC is well-tolerated in mice at low doses, without any weight loss (Peng et al., 2014; Wang et al., 2011; Zhang et al., 2017). FNC administered at 0.5, 1.0, and 2.0 mg/kg/day intragastric (IG), in xenograft studies on BALB/c nu/nu mice, didn't cause any significant changes in mice weight (Wang et al., 2011). In SCID mice, FNC showed no loss of body weight compared to control or toxicity up to 2 mg/kg b.wt. (Zhang et al., 2017). However, these studies are limited to low doses and/or utilize immunocompromised mice. Thus, we studied the effect of FNC on mice's body weight at high doses (1, 10, 25 mg/kg b.wt.), and found no significant changes in the body weight in all groups (Figure 2a). Consequently, changes in belly size (Figure 2b) or organ size (Table 3) were insignificant in all groups. Organ weight and coefficient analysis showed that only a significant increase occurs in the spleen at higher concentrations (Figure 2c-f). The changes in the body, organs parameters, and behavior can be understood with the insight of drug bioavailability to organs. FNC showed an absolute bioavailability of about 82.96%, and the mean time to reach  $C_{max}$  was from  $1.19 \pm 0.46$  to  $2.06 \pm 0.82$  days in the Sprague Dawley rats. This study suggested a linear pharmacokinetic characteristic (at tested

concentrations) of FNC with good oral bioavailability, rapid absorption, and reaching maximum concentration very quickly (Peng et al., 2014). However, a non-linear intestinal absorption was reported by Liu et al., (2017) at lower concentrations (30, 75, and 150  $\mu\text{g/ml}$ ). The maximum absorption was reported in the thymus, followed by the spleen and liver (Sun et al., 2020), which might explain the increased size of the spleen.

The behavioral or physical parameters showed some sign of FNC toxicity, therefore physiological sign was assessed through blood, liver, and kidney parameters. The hemopoietic system is recommended and an important target for the pathological index and profiling of toxic chemicals in both animals and humans (Abera et al., 2019; Bedi and Krishan, 2020). In toxicity studies, changes in the blood and biochemical parameters are indices of toxicities (Abera et al., 2019). Accordingly, a hematological examination of FNC-treated groups was performed and it showed that FNC alters the levels of some blood parameters (Table 4). WBC number was significantly increased from 6.90 to 11.90 ( $\times 10^3/\mu\text{l}$ ) in the FNC-25 group, however, unexpectedly lymphocyte percentage decreases from 78% to 34%. FNC is rapidly absorbed, and accumulated in the PBMCs (lymphocytes, monocytes or macrophages, etc), and shows long-term intracellular retention with an intracellular half-life of more than 100 hours (Sun et al., 2020). In addition, FNC shows a maximum distribution in the thymus with maximum concentration reaching 7-12 hrs (Sun et al., 2020). The thymus is the site for lymphocyte production,

and thymus accumulation and PBMC retention might be the reason behind the lymphocyte reduction. Although the mechanism of this lymphocyte reduction was not determined, kinetic alteration of lymphocytes might be another possibility. A similar observation of lymphocyte reduction was observed in two other FNAs, 2'-fluorouridine and 2'-fluorocytidine-HCl (Richardson et al., 1999).

Neutrophils number vastly increased from 13% to 60% (~4.5 fold) in the FNC-25 group, possibly due to inflammation or FNC activates the first line of defense, i.e., neutrophils. This might implicate that FNC may be a potent anti-bacterial and anti-fungal agent (Ermert et al., 2009). RBC numbers were also increased with FNC doses, which corroborates the dose-dependent increase in Hb from 10% (C) to 14% (F-25). The increase in RBC and Hb caused an increase in PCV (Short et al., 2012), and results showed that PCV also increased from 31 to 39%. The increased spleen weight (Figure 2) might cause increased WBC and RBC production (Xu et al., 2013). These hematological changes in hematological parameters are probably caused by FNC accumulation in organs like the thymus and spleen and retention in the PBMC (Sun et al., 2020).

The liver is the major target for the drug's toxicity because its prime function is to remove and breaks down toxic agents from the bloodstream (Singh et al., 2016). Toxicity may impact the liver cells' hepatocytes integrity leading to the release of membrane-bound enzymes (ALT and AST), or damage to the hepato-biliary system releasing essential enzymes ALP (Bernal and Wendon, 2013). These kinds of injuries impair the catabolic and biosynthetic capability of the liver. Liver size and weight analysis have shown no significant changes due to FNC (Figure 2), therefore liver function was analyzed through LFT (Table 5). LFT analysis showed a decrease in ALP from 187 to 151 (IU/L). ALP is a complex enzyme, used during multiple cellular and metabolic functions (Giannini et al., 2005). The decrease of ALP may indicate an adverse effect of FNC on the liver or hepato-biliary system, or it might be associated with pernicious anemia, biliary obstruction, and hypophosphatasia or metabolic derangement at the cellular level (Fernandez and Kidney, 2007). SGOT (AST) is another maker of enzymes linked with liver injury, necrosis, etc. AST increase indicates liver damage or conditions such as hepatotoxicity, cirrhosis, or hepatitis (Bhakuni et al., 2016). The AST increased significantly in FNC treated group from  $99 \pm 8.26$  to  $218 \pm 7.22$  (IU/L) in the F-25 group (~2.2-fold change). The significant impact of FNC on liver enzymes, ALP, and AST indicates that FNC is toxic to the liver at higher doses. A cirrhosis-specific test using an anti-mitochondrial antibody (M2) showed negative results. Overall, LFT showed possible signs of FNC toxicity, however, liver histology (Figure 3) showed no sign of abnormality in the liver. Therefore, a further study with a more specific assay of liver toxicity may provide detailed information about the toxic effect of FNC on the liver. The rest of the liver parameters were changed insignificantly. Conversely, liver toxicity remained a challenging task for the NAs, especially FNAs (Cossarizza and Moyle, 2004), therefore

FNC insignificant liver toxicity up to 10 mg/kg b.wt. might enhance its scope for the clinical trial.

Other than the liver, the kidney is the site of toxic substance excretion and assault by toxic agents might impair kidney functions (Petejova et al., 2019). The kidney function assessed through RFT is based on the renal function's markers like electrolytes, creatinine, urea, and uric acid. The increase or decrease in values of these indicates kidney dysfunction (Gowda et al., 2010). Interestingly, where LFT analysis showed significant toxicity of FNC on higher doses, RFT analysis showed no change in kidney function, except the level of cholesterol which was significantly decreased (Table 5). Studies of organ distribution analysis through LC-MS have shown that FNC is almost equally present in the liver and kidney, and mostly excreted within 72 hours of demonstration (Sun et al., 2020). There is a strong possibility that FNC-induced toxicity might have occurred within 24 hours of administration.

Liver parameters showed that FNC might be toxic at higher concentrations, and these changes might cause or caused by an alteration in the liver structure. Histological changes in the organs, especially the liver and kidney can serve as evidence of drug-induced injury or possible bioaccumulation of the drug (El Hilaly et al., 2004; Inkoom et al., 2020). Histological studies performed through H&E staining, however, showed that there were no structural changes, inflammation, or sign of injury in the liver (Figure 3). The liver showed a comparable arrangement of hepatocytes, portal area, portal, and central vein to the control group. No signs of hepatocyte necrosis, disassociation of the hepatic cord, and congestion of the central vein were reported. In addition, kidney structure was normal, without any sign of bruising, lymphocyte distortion, necrosis, infiltration, etc. Structural changes were also not observed in the SCID mice administered with 2 mg/kg b.wt. of FNC (Zhang et al., 2017). FNAs behaves unorthodoxly in case of liver toxicity, where in some studies NAs show significant toxicity to the liver at very low concentration (Sun et al., 2010), and in other no toxicity even at very high concentration (Chen et al., 2013; Inkoom et al., 2020). Organs like the spleen, heart, brain, and lungs are other sites affected by the drug, due to drug accumulation (Jiang et al., 2019; Shi et al., 2013). Like, FNAs, 2'-fluorouridine, and 2'-fluorocytidine-HCl treatment-related effects were present in the thymus and spleen (splenic extramedullary hematopoiesis) (Richardson et al., 1999). However, none of the FNC groups showed signs of toxicity in organs like the lungs, brain, heart, and spleen. In addition, the pathological examination of the body and organs showed no abnormal growth, clotting, or discoloration on the external/internal surface.

The increase in liver enzyme AST 2.2 -fold and the number of neutrophils 4.5-fold might be linked to injury or inflammatory state of the liver or any other organs. Therefore, pro-inflammatory cytokines TNF- $\alpha$  and IL-6 level was studied, which are induced during the state of injury or inflammation (Del Campo et al., 2018) (Figure 4). It was found that FNC significantly increased the level of TNF- $\alpha$  and IL-6 in a dose-dependent manner.

Contrary to this, treating SARS-CoV-2 infected rhesus macaques with FNC showed upregulated expression of anti-inflammatory interleukins (IL-4, IL-13, and IL-10), but not of IL-1 $\beta$ , INF- $\gamma$ , TNF- $\alpha$ , and IL-6 in the thymus (Zhang et al., 2021). In another study, FNC treatment significantly upregulated TNF- $\alpha$  expression in mouse xenografts (Zhang et al., 2017). This might indicate that either FNC-induced inflammation occurs in other organs or at the undetectable rate in liver tissue or possibly a site-specific action of FNC. Interestingly, inflammation and elevated level of IL-6 and TNF- $\alpha$  are linked to non-alcoholic steatohepatitis (NASH) disease of the liver, which also decrease the level of cholesterol in the blood (Contreras-Zentella and Hernández-Muñoz, 2016).

Toxicity might lead to loss of cell viability and induces DNA damage or nuclear fragmentation in cells (Hansen and Bross, 2010). Therefore, cell viability was assessed through DCT (Figure 5e), and DNA damage through fluorescent dyes DAPI, AO/EtBr (Figures 5 and 6). Live and dead cells analysis through DCT showed up to 90 % of cells were live in peritoneum, liver, and spleen extracted cells (5 b-d). An/PI on the peritoneum cells showed a similar 90% viable footprint in peritoneum cells (Figure 5a). Live and apoptotic cell characterization through DAPI (Figure 6a) and AO/EtBr (Figure 6b) staining also showed no sign of DNA fragmentation, DNA damage, apoptotic blebs, etc. The cell viability study confirmed that FNC toxicity at higher doses didn't cause the death of cells.

Overall, our study showed that FNC is not toxic up to 10 mg/kg, and it is safe to use up to this concentration and there no mortality was observed during the study (Figure 7). However, it may show some signs of toxicity up to 25 mg/kg b.wt. A further toxicological more specific liver study, at a genetic level, might help to draw a picture of true hepatic toxicity at higher concentrations.

## Author Contribution Statement

N.K., Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing-Review & Editing, Data analysis; V.S.D., Writing-Review & Editing, data analysis; A.S., Methodology, Writing-Review & Editing; R.S., Writing-Review & Editing; I.U., Writing-Review & Editing; D.F., Writing-Review & Editing; S.K., Writing-Review & Editing; A.P., Writing-Review & Editing; L.Y., Writing- Review & Editing; R.T., Writing- Review & Editing; R.P., Writing-Review & Editing; S.P.M.- Data analysis; S.K., Writing-Review & Editing; K., Writing-Review & Editing; A.A., Writing-Original Draft, Writing-Review & Editing; Conceptualization, Supervision. All authors have approved, agreed, and given consent to the submission of the manuscript, and this manuscript is part of Ph.D. thesis of Mr. Naveen Kumar.

## Acknowledgements

AA acknowledges SERB, ISLS, IoE, and DHR for their grant. We are highly thankful to the ISLS and CDC for providing the fluorescent microscopy facility.

## Funding statement

2168 *Asian Pacific Journal of Cancer Prevention, Vol 24*

The preparation of this manuscript was funded by the University grant commission (UGC), New Delhi, to NK in the form of a Senior research fellowship (UGC Ref no.- 455/(CSIR-UGC NET DEC. 2017)). Prof Acharya is highly thankful to SERB, ISLS, IoE, and DHR for their grant.

## Ethical approval

The approval for the experiment conduction was taken from the Institutional Animal Ethics Committee of the Department of Zoology, Banaras Hindu University (BHU) (BHU/DOZ/IAEC/2021-2022/003; dated 15/02/2022), Varanasi, India.

## Availability of data

This published article includes all of the data collected during this experimental study.

## Conflict of interest

The author(s) declare(s) that they have no conflicts of interest.

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