Chapter 4
Study the pro-oxidant action of 5-F fluorouracil and 5-F fluorouracil-Cu (II) combination in presence of white light
**Oxidative DNA damage study**

Copper may be responsible for the cytotoxicity of many anticancer drugs and is likely to be involved in the reaction generating ROS leading to oxidative damage to nuclear DNA. The oxidative DNA damaging properties of 5-FU in white light was supposed to be through generation of ROS which is enhanced in presence of Cu (II). It is very well established that Cu (II) is converted to Cu (I) when it participates in Fenton reaction as well as Haber-Weiss reaction and produce hydroxyl radicals (Ali and Naseem, 2002). From fig. 32 we can say that the 5-FU in presence of Cu (II) drastically increased the oxidative degradation of DNA which was indicated by increased in the formation of acid soluble nucleotides. In case of 5-FU alone there was increase in the formation of acid soluble nucleotides at lower concentration while at higher concentration the formations of acid soluble nucleotides were dropped as expected. To further emphasize on our work, we performed plasmid nicking assay using 5-FU alone which has shown that on exposure to white light the plasmid was converted to open circular form at 50 µM concentration. Fig. 33a also show that in presence of white light, 5-FU increased the super twist around the helical axis of the plasmid thus converting it into more compact form as was depicted by new band which was moving faster and was at lower position when compared to supercoiled and nicking was completely inhibited. In case of combination (5-FU-Cu (II)) the plasmid was degraded to open circular and linear form and this conversion was dependent on the concentration of 5-FU (fig. 33b). The presence of H$_2$O$_2$ scavenger (catalase) and 'OH radical scavenger (thiourea) did not effect much when added along with 5-FU in the reaction lane 3 and 4 respectively. However, the presence of superoxide dismutase (O$_2^-$) has completely inhibited the reaction lane 2 fig. 34a. The pattern of inhibition with 5-FU-Cu (II) combination was different from when 5-FU alone was used as shown in fig. 34b. In this case maximum inhibition was observed with thiourea indicating that 'OH was the major ROS participating in the reaction.
Fig. 32. DNA (calf thymus) damage

The formation of acid soluble nucleotides when incubated in white light for 2 hours where (■) denotes 5-FU with increasing concentration, (□) 5-FU-Cu (II) combination with Cu (II) at concentration of 50 μM.
Fig. 33a. Effect on Plasmid pUC19 DNA with photoilluminated 5-FU

5-FU alone where Lanes representing

Control plasmid   C,
5-FU (50 µM)   1,
5-FU (100 µM)   2,
5-FU (200 µM)   3,
5-FU (300 µM)   4,
5-FU (400 µM)   5

Reaction mixture was incubated with white light for 2 hours.
Fig. 33b. Treatment of plasmid pUC19 DNA with photo illuminated 5-FU in presence of Cu (II)

5-FU-Cu (II) combination, Cu (II) (50 µM)

Control plasmid C, 5-FU-Cu (II) (50 µM) 1, 5-FU-Cu (II) (100 µM) 2, 5-FU-Cu (II) (200 µM) 3, 5-FU-Cu (II) (300 µM) 4, 5-FU-Cu (II) (400 µM) 5

Reaction mixture was incubated with white light for 2 hours.
**Fig. 34a. Effect of reactive oxygen scavengers on treatment of plasmid pUC19 DNA with photoilluminated 5-FU**

Control plasmid C,

5-FU (50 μM) (1),

5-FU + SOD 20 μg/ml (2),

5-FU + Catalase 20 μg/ml (3),

5-FU + TU 0.1 mM (4)

Reaction mixture was incubated with white light for 2 hours.
Fig. 34b. Effect of reactive oxygen scavengers on treatment of plasmid pUC19 DNA with photoilluminated 5-FU-Cu (II) combination

5-FU-Cu (II) both at 50 µM  (1),

5-FU-Cu (II) + SOD 20 µg/ml  (2),

5-FU-Cu (II) + Catalase 20 µg/ml  (3),

5-FU-Cu (II) + TU 0.1 mM  (4)

Reaction mixture was incubated with white light for 2 hours.
Using a cellular system of peripheral lymphocytes isolated from human blood, alkaline single cell gel electrophoresis (Comet assay), we have tried to confirm that copper may inhibit the formation of N1–C5-linked hydrate dimer of 5-FU in presence of white light. This may lead to enhancement in an oxidative degradation of macromolecules in cells as compared to 5-FU alone following Fenton/Haber-Wiess reaction. It may be noted that when 5-FU was used alone nuclear DNA breakage progressively decreased with increasing concentrations of drug. The breakage was more pronounced when 5-FU was used in combination with Cu (II) indicated by the increase in the tail length of the comet (fig. 37a). In fig. (37b) the data is plotted as % DNA in tail of comet, as the percentage of tail DNA content or tail length is an index of DNA breakage in comet assay (Renschler, 2004). In order to rule out the possibility that 5-FU alone can mobilize chromatin bound copper in the reaction the neucuproine (a membrane permeable copper chellator) was added and the results were similar as in the absence of neucuproine fig. 36a, fig. 36b as well as from table 2. It is well established that the nuclear pore complex is permeable to small molecules (Nakagawa et al., 2007). The nuclear DNA breakage observed in these experiments exhibited radial movement that probably is due to larger fragment generation as shown in fig. 35a, fig. 35b. Tail formation in comet is because of smaller fragments as shown in fig. 37a, fig 37b.
Fig. 35a. Effect of photoilluminated 5-FU on lymphocyte DNA breakage

Single cell gel electrophoresis of human peripheral lymphocytes (control), lymphocyte nuclei showing comets after treatment with photoilluminated 5-FU with increasing concentration. The incubation period was 2 hours under white light.
Fig. 35b. Plot of tail length from fig 35a data
Fig. 36a. Effect of photoilluminated 5-FU-Neu on lymphocyte DNA breakage

Comparison of DNA breakage in lymphocytes as compared to control using photoilluminated 5-FU-Neu, 5-FU ↑ concentration (50 µM, 100 µM, 200 µM, 300 µM, 400 µM) and Neu at concentration of 50 µM. The incubation period was 2 hours in white light.
Fig. 36b. Plot of tail length from fig 36a data
Fig. 37a. Effect of 5-FU-Cu (II) combination on lymphocyte DNA breakage

Comparison of DNA breakage in lymphocytes as compared to control using photoilluminated 5-FU-Cu (II) combination, 5-FU ↑ concentration (50 µM, 100 µM, 200 µM, 300 µM, 400 µM) and Cu (II) at concentration of 50 µM. The incubation period was 2 hours in white light.
Fig. 37b. Plot of tail length from fig 37a data
Table 2. The amount of DNA breakage in lymphocyte on treatment with 5-FU, 5-FU Neu and 5-FU-Cu (II) combination, when incubated in presence of white light for 2 hours

Increase in tail length is an indicator of DNA breakage. Column 1 shows the reaction mixture containing lymphocyte alone as a control (c) with increasing 5-FU concentration. Column 2, shows the reaction mixture containing lymphocyte alone as a control (C) and with 5-FU-Neu with Neu at concentration of 50 µM. Column 3, shows the reaction mixture containing lymphocyte alone as a control (c) and with 5-FU-Cu (II) combination with Cu (II) at concentration of 50 µM. The incubation period was 2 hours under white light. Values reported are ± SEM of three independent experiments.

*P < 0.05 as compared to control +
We have shown the effect of ROS scavengers (Superoxide dismutase, catalase, thiourea) on 5-FU alone as well as 5-FU-Cu (II) combination induced DNA breakage in lymphocyte nuclei using Comet assay fig. 38a, fig. 38b, fig. 39a, fig 39b. ROS scavengers caused inhibition of DNA breakage indicated by decrease in the tail length of the comet. It may be mentioned that due to the site-specific nature of the reaction of hydroxyl radicals with DNA it is difficult for any trapping molecules to intercept them completely (Shamim et al., 2008). The maximum inhibition was observed with superoxide dismutase, an O$_2^-$ anion scavenger, in case of 5-FU alone while in case of combination (5-FU-Cu (II)) the maximum inhibition was observed with thiourea which is a $^\cdot$OH radical scavenger. Hence, we suggest that superoxide anion and H$_2$O$_2$ are essential components in the pathway that leads to the formation of hydroxyl radical following Fenton/Haber-Wiess reaction when divalent metal like Cu (II) was present with 5-FU as depicted by table 3.
Fig. 38a. Effect of reactive oxygen scavengers on 5-FU induced DNA breakage in lymphocyte

The reaction mixture contained lymphocyte (a), 5-FU (50 µM) (b), and active oxygen scavengers, 5-FU + SOD 20 µg/ml (c), 5-FU + Catalase 20 µg/ml (d) and 5-FU + Thiourea 0.1 mM (e). The incubation period was 2 hours under white light.
Fig. 38b. Plot of tail length from fig 38a data
Fig. 39a. Effect of reactive oxygen scavengers on photoilluminated 5-FU-Cu (II) combination induced DNA breakage in lymphocyte

The reaction mixture contained lymphocyte alone (a), with 5-FU-Cu (II) (b), 5-FU-Cu (II) + SOD 20 µg/ml (c), 5-FU-Cu (II) + Catalase 20 µg/ml (d) and 5-FU-Cu (II) + Thiourea 0.1 mM (e). The incubation period was 2 hours under white light.
Fig. 39b. Plot of tail length from fig 39a data
Table 3. The effect of reactive oxygen scavengers on the amount of DNA breakage in lymphocytes on treatment with 5-FU and 5FU-Cu (II) combination, when incubated in presence of white light for 2 hours

Increased in tail length as indicator of DNA breakage. Column 1 shows the reaction mixture containing lymphocyte alone as a control (1), with 5-FU (50 µM) (2), 5-FU + SOD 20 µg/ml (3), 5-FU + Catalase 20 µg/ml (4) and 5-FU + Thiourea 0.1 mM (5). Column 2, shows the reaction mixture containing lymphocyte as control (1), 5-FU-Cu (II) combination both at concentration of 50 µM (2), 5-FU-Cu (II) + SOD 20 µg/ml (3), 5-FU-Cu (II) + Catalase 20 µg/ml (4) and 5-FU-Cu (II) + Thiourea 0.1 mM (5). The incubation period was 2 hours at 37 ºC. Values reported are ± SEM of three independent experiments.

*P < 0.05 when compared to control
**P <0.03 when compared to absence of scavengers
**TBARS estimation**

Primary products of lipid peroxidation, which are highly reactive and relatively short-lived, undergo further reactions to form secondary products of lipid peroxidation that include a variety of aldehydes such as malondialdehyde, the 4-hydroxyalkenals, and acrolein. The aldehydes are more stable than the primary products and can diffuse throughout the cell where they damage cellular components and interfere with cellular functions. Because of their electrophilic character, the aldehydes can bind to nucleophilic groups of amino acids, such as cysteine, lysine, histidine, serine, and tyrosine, which are critical components of enzyme active sites or are necessary for maintaining the tertiary structure of proteins. Thus, oxygen radical mediated damage to DNA is considered to give rise to TBA reactive material (Ramanathan et al., 1994). We therefore determined the formation of TBA reactive substance (TBARS) as a measure of oxidative stress in lymphocyte nuclei. TBARS were determined using 5-FU alone as well as in combination with Cu (II) in presence of white light as shown in fig. 40a, fig 40b. Effect of active oxygen scavengers like superoxide dismutase, catalase and thiourea was also studied. Inhibition to oxidative DNA damage was observed in all cases when ROS scavengers were added in the reaction. However, inhibition was greater in case of SOD when 5-FU was used alone and it was thiourea when Cu (II) was present with 5-FU. The binding of aldehydes to proteins may result in enzyme inhibition and alteration of the structure of cellular receptors and may subsequently account for the cell cycle arrest (Conklin, 2004). Thus, 5-FU–Cu (II) combination in presence of white light could possibly generate oxidative stress on specific cell receptors which may lead to cell cycle arrest.
Fig. 40a. Amount of TBARS as a measure of oxidative stress in Nuclei by 5-FU and in presence of reactive oxygen scavengers

Effect of pre-incubation of lymphocyte nuclei with active oxygen scavengers SOD 20 µg/ml (3), Catalase 20 µg/ml (4) and Thiourea 0.1 mM (5) on TBARS generated by 5-FU (2) at concentration of 50 µM as compared to control (1). The incubation period was 2 hours at 37 ºC; Values reported are ± SEM of three independent experiments.

P value <0.05 when compared to control in absence of scavengers

In presence of scavengers, P value <0.03 with respect to absence of scavengers
Fig. 40b. Amount of TBARS as a measure of oxidative stress in Nuclei by 5-FU-Cu (II) combination and in presence of reactive oxygen scavengers

TBARS formation in case of 5-FU-Cu (II) combination (2), both at concentration of 50 µM and in presence of active oxygen scavengers like SOD 20 µg/ml (3), Catalase 20 µg/ml (4) and Thiourea 0.1 mM (5). The incubation period was 2 hours under white light. Values reported are ± SEM of three independent experiments.

P value <0.05 when compared to control in absence of scavengers.

In presence of scavengers P value <0.03 when compared to absence of scavengers.
Treatment of red blood cells

Erythrocytes are the most abundant cells in the human body, have potential carrier capabilities for the delivery of drugs. Erythrocytes are biocompatible, biodegradable, possess long circulation half life and can be loaded with a variety of biologically active compounds using various chemical and physical methods (Herlinde et al., 2003). Oral or parenteral administrations of chemotherapeutic agents are transported to the tissues by the blood in different fractions: plasma water, plasma proteins or cells. Erythrocytes may also be used as carriers for antimetabolites drugs (Schrijvers, 2003). We have previously used human RBCs as the targets of riboflavin in combination with Cu (II) (Ali et al., 2000) as well as riboflavin with aminophyiline, the antiasthemetic drug (Ali and Naseem, 2002) and 5-FU is another popular antimetabolite anticancer drug. We examined the effect of 5-FU alone and with Cu (II) on human RBCs. Echinocytes are formed when treated with 5-FU (fig. 41b) which became more pronounced with the presence of Cu (II) (fig.41c) in the reaction.

Finally, we can conclude with this study that the chemotherapeutic concentration of 5-FU used are high enough for the drug to dimerize, especially when used with photodynamic therapy. Hence, we hypothesize that the endogenous copper in cancer cells helps the cytotoxic action of the drug in two ways one by preventing dimerization and secondly by enhancing the production of ROS. Thus, it would essentially be an alternative, non-enzymatic and copper-dependent pathway for the cytotoxic action of 5-FU.
Fig. 41. Scanning electron microscopy of RBCs treated with photoilluminated 5-FU and photoilluminated 5-FU-Cu (II) combination

Images of RBCs (a) control, RBCs treated with 5-FU (50 µM) (b), RBCs treated with 5-FU-Cu (II) combination (c) for 2 hours under white light.