# A STUDY ON THE CYTOTOXIC EFFECTS OF MITOMYCIN C AND 5-FLUOROURACIL IN CULTURED RAT FIBROBLASTS

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**ABSTRACT:** *To investigate the cytotoxicity and genotoxicity of the DNA alkylating agnet, mitomycin C and the antimetabolite, 5-Fluorouracil (5-FU) in cultured rat fibroblasts, the colorimetric assay of neutral red (NR) for cytotoxicity and for genotoxicity, sister chromatid exchange (SCE) assay and the measurement of the rate of DNA synthesis were performed in cells cultured in media containing various concentrations of mitomycin C and 5-FU. The uptake ability of neutral red decreased dose-dependently. $NR_{90}$ and $NR_{50}$ values of mitomycin C were 1.49 $\mu M$ and 6.87 $\mu M$ and 5-FU were 38.4 $\mu M$ and 284.4 $\mu M$ respectively. Mitomycin C and 5-FU induced dose-dependent increase of SCE but dose-dependent increase of 5-FU was less than that of mitomycin C. Mitomycin C and 5-FU inhibited DNA synthesis dose-dependently. The rate of DNA synthesis at concentration of 1.49 $\mu M$ of mitomycin C ($NR_{90}$ value) was 2% of the control and at concentration of 38.4 $\mu M$ of 5-FU ($NR_{90}$ value) the rate was 20% of the control. Both mitomycin C and 5-FU induced DNA damage at low concentrations below those causing cytotoxicity as determined by NR assay. However, Mitomycin C showed cytotoxic and genotoxic effect at lower concentration than those of 5-FU.*
**Key words:** *Mitomycin C, 5-FU, NR assay, SCE assay, DNA synthesis, Cytotoxicity, Genotoxicity*

## INTRODUCTION

There is currently considerable interest in the use of *in vitro* cytotoxicity assays for the prediction of toxicity *in vivo*. Lately, NR assay has been used to evaluate the acute cytotoxicity of various chemical agents *in vitro*. The NR assay is based on the incorporation of the supravital dye, neutral red (NR) into lysosomes of viable uninjured cells. It is thus possible to distinguish between viable, damaged and dead cells by their ability of NR uptake. To evaluate the various action mechanisms

of chemical agents, it is necessary to compare the cytotoxicity and genotoxicity.

Since the development of differential staining of sister chromatids by fluorescence plus Giemsa (FPG) staining, determination of the frequency of sister chromatid exchange (SCE) has been used to examine chromosomal mutations in cells exposed to chemical mutagens. The chemical mutagenic process is though to be initiated through the modification of normal genes by interaction between the DNA molecule and the mutagen. Inhibition of replicative DNA synthesis is regarded as genotoxicity and it is generally recognized as an indicator of DNA damage. Examining the inhibition of DNA synthesis can provide an explanation for genotoxic effects. Inhibition of DNA synthesis may lead to increased SCE frequency. A great majority of the SCE inducing compounds give rise to DNA damage.

In one study of 5-(1,2-dichlorovinyl) glutathione (DCVG), 5-(1,2,2-trichlorovinyl) glutathione (TCVG) and 5-(1,2,3,4,4-pentachloro butadienyl) glutathione (PCBG) the concentrations which cause cytotoxicity have been shown to be quite different from those causing genotoxicity. It has also been suggested that the cytotoxic and genotoxic activities of adriamycin are due to a complex interactions between several types of cellular damage. So, it would be interesting to compare different cytotoxic and genotoxic effects in the same cell type under similar exposure conditions.

Therefore, the present study compares the cytotoxic and genotoxic effects of two, anticancer agents: mitomycin C, which alkylates DNA, and 5-FU, an antimetabolite. Neutral red (NR) assay, sister chromatid exchange (SCE) assay and measurements of the rate of DNA synthesis provide the bases of comparison.

## MATERIALS AND METHODS

### Cells

Fibroblasts, obtained from newborn rat tail were propagated in Eagle's minimum essetial medium (MEM) supplemented with 10% fetal bovine serum (FBS), 25 U/ml penicillin G, 100 $\mu$g/ml streptomycin. The cells were grown in a humidified atmosphere of 5% $CO_2$ in air.

### Chemicals

Mitomycin C was obtained from Kyoma Chemical Co., Ltd., 5-FU from Sigma Chemical Co., Ltd. and ([methyl-$^3$H]TdR; 68 $Ci$/ml) from Amersham International Ltd., Amersham, U.K.

### NR assay

NR was performed according to the method of Borenfreund and Puerner. Individual well of 24-well tissue culture microtitre plates were inoculated with $5 \times 10^4$ cells/ml. The microtitre plate was incubated for 24 h, after which the medium was removed and replaced with various concentrations of the test agents. After 24 h of incubation, the medium containing the test agents was removed and replaced with 1 ml of medium containing NR (50 µg/ml). The NR containing medium had been preincubated overnight at 37°C and was centrifuged prior to use to remove fine precipitates of dye crystals and incubated another 3 h. Thereafter, the medium was removed and the cells rapidly washed with a fixative (1% formaldehyde-1% $CaCl_2$), followed by addition of 0.2 ml of a mixture of 1% acetic acid-50% ethanol to extract the dye from the cells. After 10 min. at room temperature, the optical

density was measured at 540 nm. The relative cytotoxicities of the test agents were compared to control cultures by calculating the concentrations of test agents needed to reduce the dye absorbance by 10 and 50% ($NR_{90}$ and $NR_{50}$ determination, respectively). All experiments were performed at least 3 times using 4 wells per concentration of test agents.

## Sister chromatid exchange (SCEs) assay

Sister chromatid exchange in fibroblasts was assayed according to the method of Perry and Thompson. Fibroblasts were inoculated in 5 ml growth medium in 25 mm$^2$ tissue culture flasks. After 24 h incubation, 5-bromodeoxyurdine (BrdU) was added and after another 24 h various concentrations of mitomycin C (Sigma) and 5-FU (Sigma) were added respectively. Colcemid (0.1 µg/ml) was added 3-4 h before harvesting. Cells were treated with hypotonic solution and fixed in Carnoy's fixer. After slide preparation, slides were stained with Höechst 33258 for 15 min. and exposed to ultraviolet (UV) light from a mercury lamp for 30 min. and incubated for 15 min. in 2 X SSC. The cells were then stained in 2% Giemsa sol. for 15 min. At least 50 metaphases were scored in each experiment. All cultures were grown for 72 h in the dark to avoid photolysis of the BrdU substituted DNA.

## Rate of DNA synthesis

The rate of DNA synthesis was measured as the degree of the incorporation of [$^3$H] TdR (10 µCi/ml) into DNA during a 1 h of pulse labelling period. At the end of the pulse labelling, the treated monolayered cultures were washed twice with Ca$^{++}$ and Mg$^{++}$ free phosphate buffer saline. Then, 0.05% trypsin-EDTA was added to the dishes and cells were harvested on GF/C glass fibre filters. Finally, the filters were washed with 10% and 5% trichloroacetic acid and absolute ethanol respectively. The radioactivity was determined by liquid scintillation counter.

# RESULTS

The cytotoxicity in NR assay was standardized by expressing absorbance data in the presence of mitomycin C and 5-FU as a percentage of that in controls. For analysis of the cytotoxicity data it is apparently necessary to determine the concentrations of test agents causing initial cytotoxicity ($NR_{90}$ value) and midpoint cytotoxicity ($NR_{50}$ value). The values of $NR_{90}$ and $NR_{50}$ determined were at 1.49 µM and 6.87 µM of mitomycin C, and in the case of 5-FU were at 38.4 µM and 284.4 µM respectively (Fig. 1, Photo 1).

The frequencies of SCE steadily increased with the mitomycin C concentration (Table 1). Marked effects of mitomycin C were evident at concentrations far below the concentraion causing initial cytotoxicity ($NR_{90}$ value). Although weaker than that of mitomycin C, 5-FU also had an effect on induction of sister chromatid exchange (Table 1). The dose dependent increase induced by 5-FU was far less marked than that by mitomycin C (Photo 2).

The rate of DNA synthesis was measured as the incorporation of [$^3$H] TdR into DNA during the 1 h period of pulse labelling after 24 h of mitomycin C and 5-FU treatment respectively. Mitomycin C and 5-FU treatment resulted in a clear
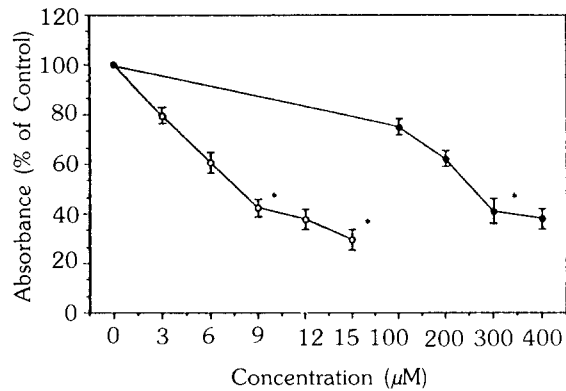
**Fig. 1.** The effect of mitomycin C (—●—) and 5-FU (—○—) on the ability of neutral red uptake. Each point represents the mean from at least three different experiments.
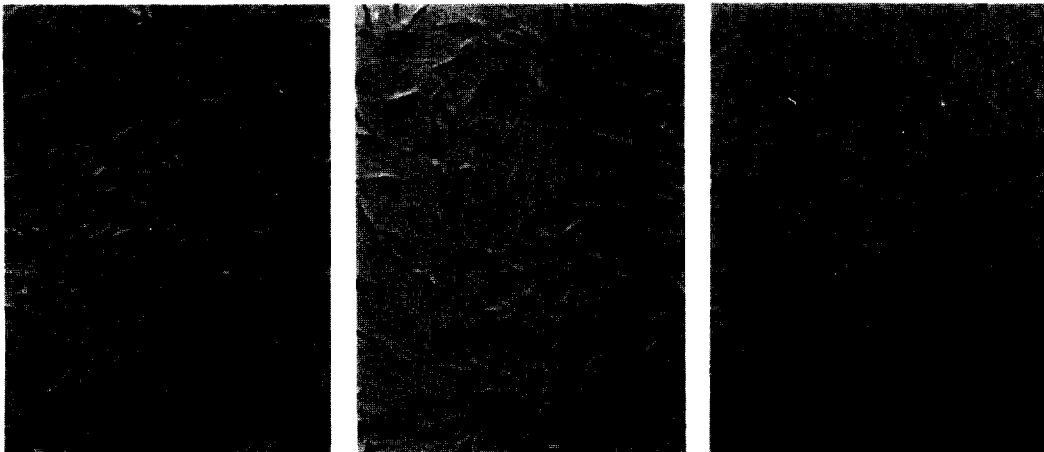*p<0.01.



**Photo. 1.** An inverted photomicrograph of rat fibroblasts cultured in control medium (A) cultured for 24 h in the medium containing 38.4 $\mu$M of 5-FU, $NR_{90}$ value (B) and 6.67 $\mu$M of mitomycin C, $NR_{50}$ value (C). These show the dose-dependent decrease of cell number. magnification ×100.

dose-dependent increase in $[^3H]$ TdR incorporation at concentrations that didn't cause initial cytotoxicity as determined by NR assay. A marked effect of mitomycin C was evident at concentrations of 0.4 μM and above (Fig. 2). Concentrations ≥1. 49 μM of mitomycin C led to 2-3% of the rates of DNA synthesis compared to controls (Fig. 2). In the case of 5-FU, ≥ 284.4 μM concentrations of 5-FU led to 10-12% of the rate of DNA synthesis compared with controls (Fig. 2). Time course induction of DNA synthesis was measured after removal of the media containing the $NR_{90}$ values of mitomycin C and 5-FU (1.49 $\mu$M and 38.4 $\mu$M) respecti-

**Table 1.** The frequency of sister chromatid exchanges (SCEs) in rat fibroblasts induced by mitomycin C and 5-FU

|  | Concentration (µM) | Average No. of SCE/cell |
|---|---|---|
| Mitomycin C | 0 | 4.40 ± 1.43 |
|  | $3 \times 10^{-5}$ | 5.50 ± 2.72 |
|  | $3 \times 10^{-4}$ | 10.40 ± 1.42 |
|  | $3 \times 10^{-3}$ | 10.20 ± 1.54 |
|  | $3 \times 10^{-2}$ | 48.20 ± 4.50 |
| 5-FU | 0 | 4.50 ± 1.45 |
|  | $3 \times 10^{-4}$ | 4.50 ± 1.42 |
|  | $3 \times 10^{-3}$ | 4.50 ± 1.34 |
|  | $3 \times 10^{-2}$ | 5.10 ± 1.89 |
|  | 3 | 6.32 ± 2.17 |
|  | 30 | 8.50 ± 1.79 |



**Photo. 2.** Sister chromatid exchanges (SCEs) in rat fibroblasts treated with 30 µM of 5-FU for 24 h(A) and treated with 0.03 µM of mitomycin C(B). Mitomycin C induced marked increase of SCE far below the $NR_{90}$ value (1.49 µM). magnification ×1000.

vely. Removal of mitomycin C restored the rate of DNA synthesis by as much as 50-60%, compared with the controls (Fig. 3). In contrast, 5-FU treated cells were restored to the control level of DNA synthesis after the removal of 5-FU (Fig. 3).
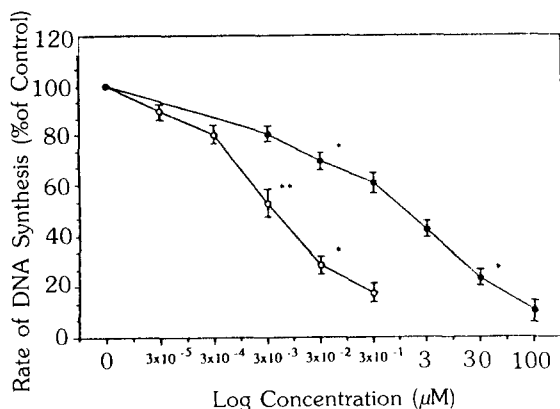
**Fig. 2.** Rate of DNA synthesis in rat fibroblasts with various concentrations of mitomycin C (—●—) and 5-FU (—○—) labelled with [³H] thymidine for an hour at 24 h after exposure to mitomycin C and 5-FU. Results are expressed as a percentage of rates in unexposed controls and each point represents the means of three parallel dishes from one representative experiments.
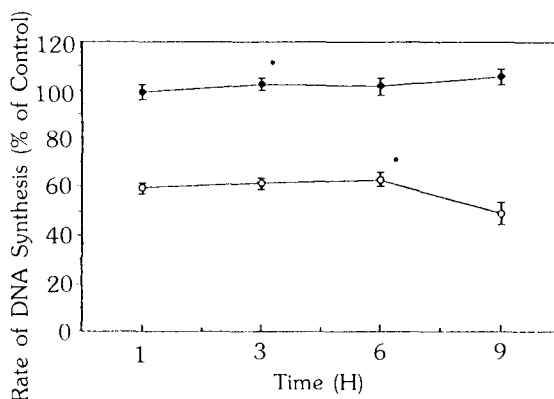*p<0.05   **p<0.01

**Fig. 3.** Time course induction of DNA synthesis in rat fibroblasts removed from mitomycin C (—○—, 1.49 µM, NR₉₀ value) and 5-FU (—●—, 38.4 µM, NR₉₀ value) treated media after 24 h and incubated in fresh media followed by 1 h of pulse labelling period with [³H] TdR.
*p<0.01

## DISCUSSION

The primary significance of the results is found in the correlations observed between cell viability, induction of SCE and inhibition of DNA synthesis in cells treated with mitomycin C and 5-FU respectively. The alkylating agent, mitomycin C, is not dependent upon cell cycle, whereas the antimetabolite, 5-FU is S-phase specific. In this study DNA damage in cells exposed to mitomycin C or 5-FU was investigated using SCE assay, a very sensitive technique for detecting DNA damage. Although the actual biological significance and the precise mechanism of formation of SCE are still not fully known, it is suggested that a great majority of the SCE-inducing compounds also give rise to DNA damage. SCE occurs during DNA synthesis and all SCE inducing agents inhibited DNA replication. Mitomycin C inhibited DNA synthesis and also increased SCE frequency. These results are consistent with the suggestion that inhibition of DNA synthesis may lead to increased SCE frequency. Subsequent investigation of the recovery rate of DNA synthesis in cells removed from mitomycin C containing medium after 24 h of treatment revealed only 50 to 60% of recovery (Fig. 3). Mitomycin C, known as a typical mutagen, is likely to exert its inhibitory effect on DNA synthesis by alkylating DNA directly. This would agree with reports that mitomycin C can induce chromosome damage in mammalian cells. In contrast, a significant increase of SCE was not found after exposure to concentrations of 5-FU which causes inhibition of DNA synthesis effectively (Table 1). 5-FU is likely to exert its inhibitory effect on DNA synthesis by preventing DNA metabolism and inhibiting cells from entering S-phase. When 5-FU is removed after 24 h of treatment, the rate of DNA synthesis was recovered to the control level (Fig. 3). The result support a hypothesis that 5-FU inhibits

DNA synthesis by preventing DNA metabolism.

Neutral red (NR) assay has been used to evaluate the toxicities of a wide range of chemicals. The fact that fibroblasts are damaged by mitomycin C and 5-FU at $NR_{50}$ concentrations provides evidence that assessment of *in vitro* cytotoxicty (as measured by NR assay) is a reliable indicator of the lethal potency of a compound (Photo 1).

In summary, in this study the genotoxic effects of mitomycin C (an alkylating agent) and 5-FU (an antimetabolite) were compare with cytotoxic effects in a range of concentrations. Genotoxicity, measured as the inhibition of DNA synthesis and induction of SCE in treated rat fibroblasts cells, occurs at concentration far lower than those causing cytotoxicity as measured by NR assay. From these results, it is clear that both mitomycin C and 5-FU have genotoxic effects at concentrations far lower than those observed to cause cytotoxicity.

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