Effect of mitoxantrone on proliferation dynamics and cell-cycle progression

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Synopsis

MTX (mitoxantrone), an anti-tumour antibiotic, is known to cause cell death by intercalating the DNA bases. But how it interferes with the cellular proliferation is not well known. Hence, in the present study, we have tried to evaluate the interaction of this drug using proliferation dynamics to gain a better understanding of MTX's antineoplastic action. Inhibition of proliferation by these drugs was detected by evaluating its effect on cell proliferation and growth curve of the cells. MTX was also found to affect the cell viability and, thereby, cell physiology. Typical apoptotic morphologies such as condensation of nuclei and membrane permeabilization were observed through CLSM (confocal laser scanning microscopy) and fluorescence spectroscopy, which implicates commitment to cell death. Cell-cycle distribution was measured by flow cytometric measurements. The analysis demonstrated significant cell-cycle arrest on MTX treatment. Inhibition of lacZ gene expression was also observed on drug treatment, which implicates its interaction with gene expression.

Key words: cell-cycle arrest, colony-forming unit (CFU), gene expression, growth inhibition, mitoxantrone (MTX)

INTRODUCTION

Antineoplastic drugs generally have a narrow therapeutic index and are delivered at doses close to toxicity. Endogenous factors affecting drug response involve genetic predisposition, disease states and other factors that influence absorption, distribution, activation and detoxification of the drug. In particular, the pharmacological activity of any genotoxic anticancer drug is strictly dependent on tumour-specific physiological/biochemical conditions, such as a functional respiratory chain [1,2] and the presence/absence of drug-metabolizing enzymes [3–5]. MTX (mitoxantrone), an analogue of the anthracycline antibiotics, belongs to the anthracenediones, a class of synthetic chemotherapeutic agents. The mode of action of these drugs has not yet been clearly established. Among others, this drug exhibits a range of intracellular effects, the most dominant of which appears to be the induction of DNA damage and apoptosis. Programmed cell death or apoptosis is an essential homoeostatic mechanism in multicellular organisms allowing the elimination of no-longer-needed or seriously damaged cells by an orderly process of cellular disintegration. Depending on the organism, apoptosis involves a typical set of morphological events, including chromatin condensation [6,7], DNA and nuclear fragmentation, cell shrinkage, plasma membrane blebbing, increased numbers of cytosol vacuoles and formation of apoptotic bodies followed by phagocytic digestion [8,9]. It has been demonstrated that molecular pathways leading to the apoptotic phenotype are also present in yeast and that they developed before the evolutionary separation of fungi and metazoan organisms [10,11]. In view of the above background, we initiated our study with the aim of understanding the mechanism of action of MTX. Considering Saccharomyces cerevisiae as an experimental model, we have tried to clarify the basic cellular mechanism mediating the biological action of anticancer treatments and understanding the possible inflection of drug action by a holistic interaction of many cellular systems.

MATERIALS AND METHODS

Materials

2,3,5-Triphenyltetrazolium chloride, PI (propidium iodide), MTX and glutaraldehyde were purchased from Sigma. All solutions were prepared freshly in sterile Milli-Q water or PBS (pH 7.4). Yeast extract, bacto peptone and dextrose were
obtained from Himedia. All other solvents and reagents used were of analytical grade.

**Yeast strain and culture medium**

The experiments were carried out with the haploid yeast *S. cerevisiae* strain from Clontech Laboratories (AH109) mating type *a*. Cells were grown in YPD (yeast extract, peptone and dextrose) broth on orbital shaker at 30°C. Cells were picked from the culture during exponential growth phase at an attenuation (*D*) between 2 and 4 at 700 nm, and cultures were diluted with fresh medium in order to obtain a density of 10^7 cells/ml (*D*<sub>700</sub> = 0.5).

**Growth inhibition**

The inhibition effect of the drug on the growth of the yeast cells was determined. The cells were inoculated at 10<sup>4</sup> cells/ml and, after 2 h incubation at 30°C, cell division inhibition was evaluated as a percentage with respect to control (no drug) for each at each drug concentration.

**Survival assay**

In another set of experiments, yeast cells were incubated overnight in YPD media, inoculated to fresh YPD medium at a *D*<sub>700</sub> of 0.1, incubated at 30°C for 8 h, treated with various concentrations of drug, diluted 1:10000 in sterile deionized water, spread on to YPD plates and CFU (colony-forming units) was determined. CFU of the drug-treated cells were compared with those of untreated cells and expressed as percentage survival frequency (CFU%). The survival ratios were expressed as the means ± S.D. for at least three independent experiments.

**Fluorescence labelling with PI**

To explore the desired aspects with the respective instruments, the untreated control and the tested samples were immediately incubated with PI, an impermeable nucleotide-binding probe. A 5 ml aliquot of 20 mM PI solution in PBS was mixed with a 3 ml portion of the samples. Samples were thoroughly mixed by pipetting up and down several times, and incubated in the dark at 25°C for 15 min.

**Confocal laser scanning microscopy**

Confocal microscopy images were acquired to assess the effect of drugs on normal cell growth. A Leica TCS SP2 confocal system attached to a research Leica DM RXA2 microscope (Leica, Heidelberg, Germany) fitted with a water immersion dipping objective lens (×60) and a Kr–Ar laser was used. The specimens were stained for 1 h with PI (0.2 mg/ml) in a buffer containing 0.1% sodium citrate, 0.1 mg/ml RNase and 0.3% Brij-58. The *λ*<sub>excitation</sub> was 594 nm. A scan speed of 400 lines/s was used to ensure minimum dislocation due to the movement of the cells. Images of the colour were adapted to the 8-bit range of system. An HCX PL APO CS 63.0 × 1.32 NA (numerical aperture) oil UV objective was used with an additional zoom of ×4, resulting in a 512 × 512 image with a pixel size of 0.12 μm. The pinhole size was set at 1AIRY Disc. A series of images through the whole thickness of the biofilm were taken with a step size of 0.04 μm. The Kr–Ar laser was used in the range 30–45 % power, to minimize photobleaching. Each sample was scanned at five randomly selected positions away from the disc edge. Each stack in an experiment was examined and the threshold value that best fits all image stacks of a trial was chosen. The images of control and test samples were averaged and compared.

**Flow cytometry**

A FACS analysis was performed to check for possible cell-cycle redistribution or arrest. Yeast cells were grown to reach exponential phase and were then treated with IC<sub>50</sub> of MTX. After 12 h of treatment, the medium was centrifuged (15700 g for 5 min at 4°C) and aspirated. The cells were washed twice with cold PBS. The cells were then pelleted (15700 g for 5 min at 4°C), rinsed in 0.5 ml of PBS with RNase A (1 mg/ml) and incubated for 1 h at 37°C. Cells were then washed and suspended in pepsin (1 g/l of pepsin in 5.5 mM HCl) for 5 min at room temperature (25°C), then washed once with PBS and resuspended in 1 ml of PI (3 μg/ml in PBS). Then cells were analysed by Coulter Epics Elite flow cytometer (Beckman Coulter France S.A., Paris, France) with 488 nm excitation by an argon-ion laser at 15 mW. Discriminators were set on FSC (forward scatter cell) and SSC (side scatter cell) signals to reduce electronic and small-particle noise. The optical filters were set so that PI fluorescence was measured at 610 nm and BOX fluorescence at 525 nm. The flow rate was 300 cells/s. Each measurement acquired counted for the analysis of 10<sup>5</sup> treated cells. Typically, signals from 10000 cells were acquired and analysed for each sample using the Window Multiple Document Interface for Flow Cytometry (Win MDI) 2.8 software. The flow cytometry results presented in the present study are representative of at least three independent experiments.

**Yeast two-hybrid system**

**Colony lift β-galactosidase assay**

Doubly transformed yeast colonies were grown in the absence and presence of increasing concentrations of the drug. Then, colony lift or filter β-galactosidase assay was performed by picking cells on to the filter paper. Yeast cells were permeabilized by freezing yeast-impregnated filters in liquid nitrogen and thawing at room temperature. The filter was placed over a second filter that was presoaked in 0.1 M PBS (pH 7.4) containing 300 mg/ml
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Liquid β-galactosidase assay
To perform the liquid β-galactosidase assay, 1.5 ml of transformed culture with required incubation of the drugs was centrifuged at 15700 g for 3 min at 4°C and the pellet was washed with 1.5 ml of Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl and 1 mM MgSO₄) having a pH value of 7. The pellet was finally dissolved in 300 µl of Z-buffer. To permeabilize the yeast cells, microcentrifuge tubes containing the yeast pellet with Z-buffer were frozen in liquid nitrogen and thawed at room temperature. Next, 700 µl of Z-buffer containing 50 mM 2-mercaptoethanol was added. To this, 160 µl of o-nitrophenyl β-d-galactoside (10 mg/ml, made in Z-buffer and along with 50 mM 2-mercaptoethanol) was added, and the mixture was kept at 30°C until a yellow colour developed in the mixture. Relative enzymatic activity was determined in three independent experiments and used to calculate the S.D. The samples were processed together and were incubated for the same length of time. To stop the reaction, 400 µl of sodium carbonate (1 M) was added. Reaction tubes were centrifuged at 15 700 g for 10 min, and the supernatant was transferred to cuvettes to calculate β-galactosidase expression levels, which were determined by measuring A₄₂₀ [13].

RESULTS AND DISCUSSION

Cell division inhibition
Exponentially growing S. cerevisiae cells were grown in the presence of increasing amounts of MTX. After 3 h of incubation with the drug, concentration-dependent decrease in cell density was observed (Figure 1A). The decrease in cellular density corresponds to dose-dependent inhibition of cell growth. The concentration at which the cell division diminishes to 50% is 3 µg/ml. We have also evaluated the effect of this drug on the growth curve of the yeast by treating with 4 µg/ml. On comparing the growth curve of treated cells with untreated cells (Figure 1B), we observed that the growth curve of the treated cells was shifted towards the lag phase as compared with the growth curve of the untreated cells. The above two results illustrate the marked inhibition of cell division by the tested drug. The results suggest the probable interference of this drug with vital processes required for duplication and cellular proliferation, which delayed the normal growth phase of the cells.

Cell survival
To detect drug-induced toxicity, the cells were treated with different concentrations of MTX. The cells plated after the 3 h incubation illustrate the effect of the drug on cell physiology. Figure 2 depicts the resultant cytotoxicity on treatment with different concentrations of MTX. The most striking effect was the drastic reduction in the cells’ ability to form colonies on 2 h incubation with the drug, indicating the general loss of viability. We also calculated the drug concentration at which the CFU decreased to 50% and found it to be 8 µg/ml for MTX. Hence, the result shows its potent cytotoxicity and impact on cell physiology.

DNA content
To determine whether the growth inhibition by MTX is correlated with the accumulation of cells at a specific point during its proliferation, we estimated the DNA content of the cells grown with and without the drug. The cells stained with DNA-binding dye were visualized by confocal microscopy (Figures 3A and 3A’). The untreated control cells were found to have a drastically reduced DNA content when compared with the drug-treated cells. The focused fluorescence, suggesting chromatin
condensation, and some dispersed fluorescence signals, indicating some fragmentation in the drug-treated cells, were the common features of an ordinary apoptotic cell, implicating that further exposure of the cells to the drug may result in apoptosis. The DNA content was further analysed by estimating the fluorescence of extracted PI-labelled DNA from cells grown in the absence or presence of MTX. Approx. 50% reduction in relative DNA content was observed in the treated cells.

**Cell integrity**

Since the drug seems to affect the cellular viability and DNA content might be related to the accumulation of dead cells in MTX-treated cultures, we investigated the cell membrane integrity in this experiment. The cells treated with or without the drug were stained with PI and scanned for the fluorescence emission after excitation at 490 nm. Figure 4 illustrates the dose-dependent effect of the fluorescence of PI. The Figure depicts the increase in the fluorescence on increasing the drug concentration. This increase in fluorescence corresponds to the increase in cell injury that is caused by repairable membrane damage in the cellular permeability on treating with the drug. During extended cultivation, the control cells retained impermeability to PI; this further authenticates the drug-induced permeability in the treated cells and shows that it does not relate to the developmental stages of the cell.

Interaction with cellular membranes have been demonstrated to play a crucial role in the cytotoxicity induced by this drug, as membranes are important structural and functional components of all cell types both as boundaries of cells or intracellular compartments and as the environment for enzymes involved in
vital cellular processes. Plasma membrane has been shown to be one of the important targets of the mechanism of action of the anthracyclines. In fact, several activities of the plasma membrane, such as lectin interactions [14], glycoprotein synthesis [15], phospholipid structure and organization [16], fluidity and expression of hormone receptors [17], seem to be modulated by anthracyclines. They proved to be genotoxic only in cellular systems, suggesting that metabolism of the drug is a necessary step leading to DNA damage [18].

Effect of the drug on cell-cycle distribution
To determine whether treatment with the drug resulted in cell-cycle redistribution, the yeast cell lines were treated at their respective IC_{12.5} and IC_{25} dose for 24 h and analysed by flow cytometry as described in the Materials and methods section. Intercalation of the PI in the DNA of the cells in the sample allows for quantification of the number of cells in a specific cell-cycle phase via fluorescence after exposure to an argon laser. Supplementary Figure S1 (see http://www.bioscirep.org/bsr/030/bsr0300375add.htm) represents the events detected by the flow cytometer in the absence or presence of increasing concentration of MTX. The cytograms reported on the right-hand side of the experiments represent an analysis of their respective cell complexity (with side scatter, SSC-H) and cell size (with forward scatter, FSC-H) of the haplodiploid population for cells treated with the drugs are compared. The complexity of the cells seems to decrease down the column on increasing the concentration of the drugs as compared with the untreated cells. This suggests that large numbers of cells are arrested at the initial phases of cell cycle, thereby minimizing the cellular complexity. The almost comparable cell size found with the control and the drug treatment rule out the presence of cell debris in the analysed cell population [19]. Table 1 shows the phase distribution of cells after treatment with MTX, which suggests that the highest number of cells were found in G_1- and S-phase as well as in the G_1- and G_2-phase on treatment with the drug. There was no evidence of apoptotic peaks (left of the G_0/G_1 peak) with any of the drug treatments. The FACS analysis was repeated in triplicate, and the data revealed a statistically significant cell-cycle arrest with the drug (P \leq 0.005). Regulated progression through the cell cycle and its checkpoints is paramount to normal cell proliferation [20]. Deregulation of the cell cycle plays a fundamental role in carcinogenesis. The G_1- and G_2-phase checkpoints are necessary to prevent tumour cells from unmitigated cycling and proliferation. As is seen from our results, MTX promotes cell-cycle arrest and growth inhibition by forcing G_1- and G_2-phase arrest. Arrest of S-phase suggests the blockage of the synthetic phase, thereby minimizing the synthesis and doubling of metabolically important constituents required for proliferation and sustenance of cells. By promoting cell-cycle arrest and subsequent growth inhibition, cells containing damaged DNA are allowed to be checked and possibly undergo apoptosis [21]. The significant increases in cell-cycle arrest that we demonstrated by FACS analysis also suggest an effect on cell-cycle regulatory proteins. Balance between the regulatory proteins (cyclins and cyclin-dependent-kinases) promote cell-cycle transitions. They play an important role in regulation of the G_2/M-phase gap and G_1-phase progression [20]. Hence, the significant increase in cell-cycle arrest on increasing the drug concentration suggests the effect of MTX on the cell-cycle regulatory proteins.

Effect of MTX on lac Z gene expression
Colony lift (filter) assay was carried out to see the effect of MTX on the expression of lacZ gene fused with GAL4 DNA-binding domain. The effect of drug on the gene expression was determined by analysing the blue colour due to \( \beta \)-galactosidase activity. Figure 5(A) shows the representative colony lifts in the absence or presence of the tested drug. The result shows the drastic diminution of the colour on the treatment of cells with 4 \( \mu \)g/ml MTX. The negative control shows no background in the experimental condition of 1 h incubation period with substrate for the present study. The results clearly indicate indication of the drug with gene expression.

Furthermore, we quantify the effect of MTX on enzyme activity by liquid \( \beta \)-galactosidase assay. In this experiment, the cell suspension culture was treated with different concentrations of the drug. The negative (untransformed strain) and positive (transformed strain without drug) controls were set to accurately evaluate the enzyme activity and the mean relative \( \beta \)-galactosidase activities as shown in Figure 5(B), which illustrates the drastic reduction in the enzyme activity on treatment with the drug. The reduction was found to be dose-dependent, that is, activity decreases with an increase in the drug concentration. After the drug treatment, the cells were harvested and the drug washed with PBS to eliminate the possibility of the enzyme inhibition by the drug. Hence, we can correlate the reduction in the enzyme activity on drug treatment with the repression of lac Z gene. Intercalating drugs can alter cell growth not only by producing lesions on DNA but also by preventing the binding of essential transcription factors to DNA, as they may compete with the factors for binding to DNA, provided their cognate sequences coincide. We propose such a mechanism for the inhibition of Gal4p-driven transcription by MTX, since the drug and Gal4p might compete for two crucial CpG steps in the UAS (upstream activation sequence) of the Gal4 promoter [22]. A similar mechanism has been proposed to explain the inhibition of Sp1-activated transcription upon addition of anthracyclines and bisanthracyclines in vivo and in vitro [23,24] or the inhibition of myogenic differentiation in cell cultures by other drugs [25].

In conclusion, we have shown the inhibition of cell proliferation on MTX treatment. The cells exhibit DNA condensation and

<table>
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<th>Drug conc. (( \mu )g/ml)</th>
<th>Cell distribution (%)</th>
<th>( G_1 )</th>
<th>S</th>
<th>( G_2 )</th>
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<td>42</td>
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Figure 5  Effect of MTX on lacZ gene expression

(a) Representative spots for colony lift assay. Expression of the lacZ gene in a yeast two-hybrid system was analysed in the absence or presence of increasing concentration of MTX. The effect of MTX on lacZ gene is shown as a function of galactosidase activity. The host strain is the untransformed cell used as the blank for background/innate colour of the cells and the drug-treated cells were taken as the tested samples. (b) Plot depicts the effect of MTX on the enzyme activity in the absence or presence of drugs, correlating drug-induced gene repression. The values plotted here are corrected by subtracting the blank from the studied samples and are the means ± S.D. values for three independent experiments.

cell membrane permeabilization, which implicates the induction of apoptosis. The results were also suggestive of inhibition by the drug of gene expression. Future work will focus on the identification of any specificity of this inhibition and other details at the transcriptional level. The yeast S. cerevisiae, a versatile eukaryotic in vivo system, was confirmed to be useful in further clarifying basic cellular mechanisms mediating the biological action of anticancer treatments and understanding the possible modulation of drug action by a holistic interaction of many cellular systems. There may, of course, be limitations to the utility of yeast as a mammalian surrogate, owing to differences in the molecular environment and the more complex genetic interactions in mammals. However, S. cerevisiae could be proposed as a cell model in the early studies on drug action to provide rapid screening of the different biological activities of chemicals [26].
**REFERENCES**


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SUPPLEMENTARY ONLINE DATA

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Figure S1 Representative cytograms of the cells grown with and without MTX treatment
(a) Untreated cells used as the control. (b and c) Cells grown with increasing concentration of the drug. Respective bivariate dot plots of cellular complexity (side scatter; SSC-H versus FL2-H) and cell size (forward scatter; FSC-H versus FL2-H) are shown on the right-hand side of the respective cytogram.

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