Lipid bilayer–methotrexate interactions: A basis for methotrexate neurotoxicity

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The anti-cancer drug, methotrexate has extensive applications in many chemotherapy regimens. However its use has been heavily restricted because of its severe neurotoxic side effects. The present work aims to identify a possible cause for these side effects, by studying the interaction of the drug on a model membrane system mimicking the lipid bilayer architecture of the cell membrane. The investigations led to the conclusion that the non-specific interaction of methotrexate with the lipid bilayer progressively fluidized the membrane architecture that ultimately resulted in its disintegration. The extent of damage caused to the lipid bilayer could be related to the severity of the adverse neuronal impairment caused by the drug.

THE antifolate drug, methotrexate (MTX), is widely used in chemotherapy regimens because of its effectiveness against many cancer cell lines[1,2]. However, MTX therapy often leads to serious neurotoxic side effects[1–3]. Though many theories have been put forward based on protein–MTX interactions, the cause of this neurotoxicity is yet to be ascertained conclusively. Earlier research has established that the lipid bilayer is the primary site of interaction in the case of pesticides and anesthetics[4,5]. Strangely, the drug–lipid bilayer interaction has been an almost neglected aspect in the studies on anti-cancer drugs. The present work focuses on the lipid bilayer–MTX interactions as a possible cause for such neurotoxic side effects. Changes in the electrical parameters of the lipid bilayer system, such as conductance and capacitance, were monitored to determine the extent of drug–membrane interactions. The results have been correlated with the observed clinical toxicities to test the validity of the proposed mechanism. The basis for such research has been derived from the fact that the lipid bilayer construct is the basic structural entity in all cells, regardless of its origin and function[6]. The nerve cell, in particular, has the greatest per cent of lipid[6,7]. Hence the probability of the drug interacting with the lipid part of the cell is more. The model membrane system used in the present study is the bilayer lipid membrane (BLM), which has long been established as a very good model simulating the lipid bilayer architecture of cellular membranes.

The lipid bilayer was formed on a chamfered aperture of outer diameter 1.18 mm, drilled on a 1 mm thick Perspex septum separating two compartments of equal capacity. The phospholipids used for forming the bilayer were extracted from eggs as described elsewhere[8]. The reported composition of egg phospholipids is phosphatidylcholine, 74%; phosphatidylethanolamine, 15%; and a mixture of lysophosphatides, sphingomyelin, plasmolegen and phosphatidylinositol, 11% (ref. 8). A 2% w/v dispersion of the phospholipid mix in *n*-decane was used to form the membrane. Since bilayer experiments are prone to variations, each experiment was repeated 10–12 times to check the reproducibility and the deviation was ± 15%. The readings presented in the report are the average values. The stability of the membranes formed was checked repeatedly and the basal characteristics studied extensively before the drug interaction was studied. The area of the aperture was assumed to be the area of the lipid bilayer, for calculation purposes.

The electrical measurements were made using a digital electrometer (Model 6517, Keithley Instruments, USA) and an LCZ meter (Model 1067, Chen Hwa, Taiwan). Ag/AgCl and calomel electrodes were used for AC and DC electrical measurements, respectively. The entire experimental set-up was shielded from electrical noise and mechanical vibrations. Unbuffered NaCl, NaBr and $MgCl_2$ solutions of different concentrations were used as the bath solutions in either compartment of the experimental chamber, to which different doses of MTX were added in the form of methotrexate sodium from commercially available 2 ml injection vials (ZEXATE, Dabur Pharmaceuticals, India). One ml of the solution contains 25 mg MTX (I.P.), 4.9 mg sodium chloride (I.P.) in sodium hydroxide (to adjust pH) and water, and corresponds to 52.52 μmol of methotrexate sodium.

The electrical characteristics of the membrane did not register an instantaneous change on addition of the drug. It was observed that at least 20 min was required in all cases for a discernible change to be registered in the electrical characteristics of the membrane. Constancy in the electrical measurements was generally achieved within 22–30 min after addition of the drug. The requirement of a delay time for interaction shows that the drug needs some time to interact with the bilayer components.

The initial conductance of the control lipid bilayer membrane is very low due to its highly hydrophobic interior. But when equimolar quantities of MTX were added on either side of the bilayer, the conductance of the system increased gradually. The increase became steeper at MTX doses greater than 400 μM and continued until the membrane ruptured (the drug dose at which the bilayer breaks down is called as the 'lytic dose'). The lytic dose of MTX varied from 900 to 1400 μM depending on the concentration of the bath medium, but remained unaffected by the ionic nature of the medium.

It was observed that the relative per cent increase of membrane current in 0.01 M, 0.1 M and 1 MNaBr bath media for identical drug loads of 500 μM at 40 mV is in

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the ratio 1 : 2 : 4, as shown in Table 1. A similar trend was observed in NaCl and $MgCl_2$ bath media also. The membrane current does not saturate with increasing doses of MTX, but continues to increase until the membrane lyses. This trend is shown in Figure 1. Though the initial values of the membrane current differ, the relative per cent increase of membrane current remained almost the same in all the three media. This implies that the mode of action of MTX on the membrane bilayer is the same in all these media. The nature of the ions only influences the initial value of the membrane current due to differences in the membrane permeability of the ions, which has been attributed to differences in their size and their charge density, by earlier workers[9]. Thus $MgCl_2$ bath exhibits the least conductance, while NaBr exhibits the maximum conductance.

Table 2 shows the dose-dependent changes produced in the dielectric strength of the bilayer. It was observed that the dielectric strength of the lipid bilayer proportionately decreased with increasing MTX loads on the bilayer. The progressive reduction in dielectric strength of the BLM highlights the fluidizing nature of methotrexate. It was also observed that the effect of MTX on membrane current was nearly the same when the drug was added to one side or both sides of the bilayer. These experimental results imply that MTX creates defects on the bilayer matrix through which the small ions could cross to the other side. These 'ion-conducting' defects tend to weaken the compact bilayer architecture as the close packing of the lipid chains are disrupted. The continuous passage of ions through these defects leads to an enhanced ionic pressure on the bilayer. Ueda and coworkers have reported the existence of water molecules at the membrane interface which are associated with the polar head groups of the lipid bilayer through hydrogen bonds[4]. These water molecules are referred to as 'bound' or 'electrostricted' water[4]. They are essential to preserve the structural integrity of the BLM. These bound water molecules can be released from the membrane interface only by external stresses (chemical, mechanical, thermal or electrical)[4]. The accumulation of the bulky MTX molecules at the interface might promote the release of surface-bound water and further intensify the stress on the membrane. These effects synergistically contribute to the observed increase in bilayer current and a reduction in the dielectric strength with increasing MTX doses.

The capacitance measurements showed that MTX increased the bilayer capacitance in a dose-dependent manner, irrespective of the bath medium. However, a slight dip in the capacitance values was noted at drug loads greater than 400 μM. This effect is depicted in the Figure 2. Based on these results, the following mechanism of interaction of MTX with the lipid bilayer could be envisaged.

The lipid bilayer is a compact system that offers a high resistance to the passage of charged species through it. However, this behaviour is altered when MTX is added to the medium. The drug first interacts with the polar head

Table 1. Effect of bath concentration on the methotrexate-induced increase in membrane current

Methotrexate dose (μM)	Conductance per area ($nmho/cm^2$)		
	0.01 M NaBr	0.1 M NaBr	1.0 M NaBr
0	2.6198	9.7386	17.671
100	6.4490	19.194	39.389
200	11.983	40.509	107.90
300	16.613	72.102	210.32
400	20.030	131.29	370.11
500	36.561	197.88	794.40

Applied DC voltage, 40 mV.

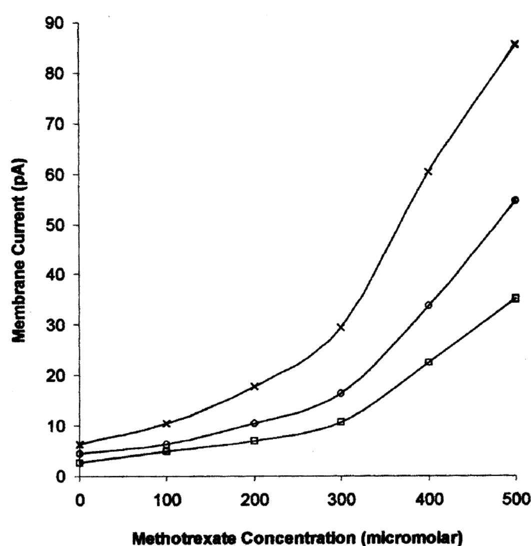

Figure 1. Effect of ionic nature on the methotrexate-induced increase in membrane current. Applied DC voltage, 40 mV; Bath concentration, 0.1 M; Maximum drug load, 500 μM; Crosses, NaBr bath medium; Circles, NaCl bath medium; Squares, $MgCl_2$ bath medium.

Table 2. Dielectric strength of BLM under various drug loads

Methotrexate concentration (μM)	Average breakdown voltage (mV)	Dielectric strength* (V/cm)
0	180	2.571×10^5
200	160	2.229×10^5
400	140	2.000×10^5
600	110	1.571×10^5
800	90	1.286×10^5
1000	70	1.000×10^5

*Dielectric strength of the BLM is calculated from the breakdown voltage using the relationship: Dielectric strength = Breakdown voltage/Membrane thickness. Membrane thickness was assumed to be 70 Å from literature[13,14].
Bath medium, 0.01 M NaCl.

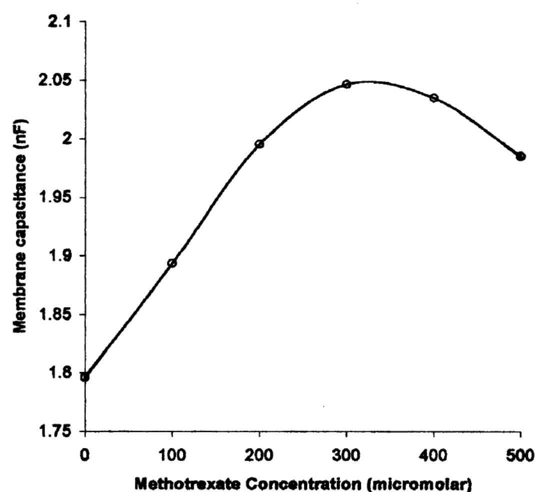

Figure 2. Variation of membrane capacitance with increasing methotrexate doses. Applied R.M.S voltage, 40 mV; Frequency, 40 Hz; Bath medium, 0.01 M NaCl.

groups of the lipid at the bilayer surface. It breaks the hydrogen bonds between the lipid and the interfacial water and consequently the fluidity of the hydrocarbon chains is increased. This increases the bilayer capacitance due to the associated capacitors formed at the surface and a slight increase in the area of the BLM due to the release of 'electrostricted' or surface-bound water. MTX also alters the conductance of the BLM by creating ion-conducting defects. The rate of formation of these defects is dependent on the MTX dose. As the drug dose is increased from 100 μM, MTX progressively accommodates itself within the defects in the bilayer. This breaks the van der Waals' attractive forces between the lipid chains and results in more fluidization of the membrane. Ultimately, the fluidized membrane ruptures unable to withstand the ion flux and the passage of the bulky drug molecules.

The present experiments conducted on a purely lipid model without any specific transport systems, confirm that MTX could not cross the lipid bilayer at low doses, but it is capable of crossing the bilayer at sufficiently high doses. This finding is substantiated by the clinical reports, which have confirmed the presence of significant quantities of MTX in the cerebrospinal fluid after high dose and prolonged MTX therapy[1,10]. This implies that a reasonable quantity of MTX could pass through the lipid bilayer under biological conditions also.

The increase in the membrane current at low doses of MTX is not very high. Hence, the fluidizing effect of MTX on the lipid bilayer will not be manifested at low doses. This explanation is supported by clinical reports of mild neurotoxicity at low doses of MTX[11]. However, MTX progressively penetrates the bilayer at higher doses and fluidizes it, as observed from the present experimental results. Such perturbations could lead to disruption of normal cellular activities, apart from disintegrating the bilayer assembly. Since the drug is able to completely destroy the membrane architecture, the damage caused by the drug to the nerve membrane also is expected to be permanent. This hypothesis is supported by reports on the irreversible nature of MTX neurotoxicity in most cases[12]. Many recent *in vitro* studies have tried to relate the observed MTX neurotoxicity to drug-induced defects in enzyme release[13]. Such possibilities also cannot be ruled out. The MTX–bilayer interactions could modify the conformation of membrane-associated protein (enzyme/receptor) leading to activation or deactivation of the enzymes associated with the membrane. Thus, the direct (disintegration of bilayer) or indirect (alteration in the membrane associated proteins) involvement of MTX–bilayer interactions could be an important cause for the neuronal impairment during MTX therapy.

The drug doses used in the present study are higher than those usually used in standard therapeutic practice. This is to magnify the changes produced in the electrical characteristics of the membrane so that the changes could be followed. The experiments give an insight into the type of interaction of MTX with the purely lipoidal bilayer. MTX would exhibit a similar mechanism of action on the lipid bilayer even in smaller doses. Moreover, the clinical reports suggest that the neurotoxic effects of MTX are cumulative and manifest themselves only after prolonged exposure to the drug. The biological system is extremely sensitive to even minute changes in the ionic environment. Therefore, even the slightest perturbing action of MTX on the lipid bilayer architecture of biological membranes would be magnified and could alter the extremely fragile ion balance in the cell. This could trigger undesired reactions that could possibly result in the observed toxicities.

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Genome-wide expression profile of RNA polymerase II subunit mutant of yeast using microarray technology

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Rpb4, the non-essential core subunit of RNA polymerase II has been assigned a function of regulating stress response in *S. cerevisiae* based mainly on phenotypes associated with its deletion. The actual mechanism has been elusive, although various hypotheses have been put forth. We have shown previously that it plays a significant role in activation of a subset of genes, rather than causing generalized defect in transcription. We used the microarray technology to look at the effect of this RNA polymerase subunit on the expression pattern of the entire *S. cerevisiae* genome. Many surprises emerged when we compared the genome-wide expression patterns of wild type and a mutant lacking the RPB4 gene (*rpb4Δ*) subjected to heat shock. The initial analysis of genes down-regulated in the mutant showed that the co-regulation of genes is not position-dependent, although the locus carrying the deletion had unexpectedly a large cluster of down-regulated genes. We also found that among the known down-regulated genes, a majority is involved in hexose uptake and utilization. We speculate that this could potentially contribute to the slow growth rate of the mutant. Compared to the other components of the transcription machinery, the Rpb4 subunit affects a unique set of genes.

THE most highly regulated step in gene expression is transcription. Many viruses transcribe their genes using highly efficient single polypeptide RNA polymerases. Prokaryotes employ a core RNA polymerase composed of five polypeptides (2α, β, β ′, ω). The specificity of the polymerase is achieved through the sixth subunit designated as sigma. The task of eukaryotic transcription is shared by three different RNA polymerases I, II and III (also called A, B and C). The core RNA polymerase II (RNA pol II), which is composed of 12 subunits, transcribes all messenger RNAs in the cell. All these polymerases interact with many other proteins and together they achieve a highly orchestrated gene expression which is essential for a cell to rapidly adapt to changes in its immediate environment. The number and complexity of these additional factors also increase markedly from viruses to eukaryotes. The very large number of multisubunit complexes which together constitute eukaryotic transcription machinery has been collectively called the transcriptosome (not to be confused with the complete set of expressed RNAs of a cell at a given time–transcriptome)[1,2].

The eukaryotic RNA pol II is highly conserved from yeast to humans. The yeast RNA pol II is assembled from 12 subunits, Rpb1–12. Rpb1–3 makes up the enzymatic core of the polymerase. Some of the smaller subunits provide the structural integrity to the polymerase, while others may act as targets for regulation[3]. We have been studying Rpb4, a 25 kDa peripheral, non-essential protein of the polymerase over the past few years. The deletion of this subunit leaves the cell viable but compromised in survival at extreme temperatures[4]. Molecular genetic experiments have shown that *rpb4Δ* is defective in activation of many regulated promoters *in vitro* and *in vivo*[5–7]. *In vivo*, the stoichiometry of this subunit within the polymerase changes with the growth phase[8,9]. These features taken together suggest that Rpb4 could functionally be the eukaryotic counterpart of the prokaryotic sigma subunit[10]. There are conflicting reports in the literature cited above regarding the role of Rpb4 in transcription. The proposed roles include an effect on basal transcription of almost all genes, a more pronounced effect on activated transcription and a stress-related but gene-specific role, all of which may result in the phenotypes shown by *rpb4Δ* cells. This dilemma can be ultimately solved only by studying the whole genome expression pattern in *rpb4Δ* compared to the wild type. We report here the results of our attempt to study the role of this subunit in the backdrop of transcriptosome – the effect of the deletion of *RPB4* on the genome-wide expression pattern of yeast.

Ever since yeast became the first eukaryote whose genome was completely sequenced, newly available tools have opened up the opportunity to ask many new questions which could not previously be asked. The availability of the whole yeast genome microarray, an ordered assembly of DNA from yeast genes immobilized on a 5 cm × 2 cm area of a glass slide, has added yet another powerful tool to the geneticists' toolbox. Using microarray analyses, Holstege *et al.*[11] have identified the transcriptome of cells in which key components of the

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