Chapter III

Monitoring age dependent changes in Topoisomerase II α and β sensitivity to etoposide and mAMSA
**Introduction**

Topoisomerase II is the major target for many structurally distinct anti cancer agents. The topo II poisons are classified in to different classes of molecules; these are anthracylins (doxorubicin, daunorubicin etc) epipodophillotoxins (etoposide, tenoposide) amino acridines (amsacrine) ellipticines (ellipticinium), metal complexes (cisplatin, copper complexes, ruthenium complexes titanium, cobalt and iron complexes). All these drugs interfere with a single step in the catalytic cycle of topo II through stabilization of cleavage complex i.e. a complex composed of DNA, drug bound to the enzyme. Stabilization of this complex inhibits the cell proliferation, and it is perceived as a lethal signal for the cells. Such a signal induces a cascade of events that allow cell to enter apoptosis/ necrosis. Drugs interfering with the Topo II catalytic activity to convert the enzyme in to a cellular double stand-breaking enzyme thus accumulating double stranded breaks in the genome.

A determinant of the cell sensitivity to topo II interfering drugs is the extent of cleavable complex formation and stabilization in the presence of the drug. The ability of the enzyme to form the cleavage complex and the degree of interaction between the drug and the enzyme influences the cellular sensitivity of the enzyme towards the Topo II interfering drugs. Variation in the topo II molecular form or the capacity to interact with the anticancer drugs can be responsible for the resistance against these drugs. Many mechanisms have been proposed for the resistance of Topo II enzyme to the drugs. Some of the mechanisms are specific for a drug or a class of a drug. Since they require the enzyme for their activation or recognized as a specific target. In addition, MDR gene
products have a role in providing drug resistance in cell. P-glycoprotein associated drug resistance is well studied.

Etoposide and mAMSA are widely used in cancer chemotherapeutics. The central structural region of topoll is implicated in the interaction with the drugs. The present study was taken up to monitor how the molecular form of topoll in brain liver and testis of different age groups can interact with these drugs. This information would be useful to understand the molecular changes occurring in topoll in these tissues during aging.
Methods

Preparation of Tissue extracts:

The brain, liver and testis tissues were dissected out from young, adult and old rats. The tissues were homogenized in extraction buffer (20mM tris HC1 pH 7.5, 0.1mM β-mercaptoethanol, 1mM MgCl₂, 0.1mM EDTA, 5% glycerol, 0.1% triton X-100, 0.5M KC1, 0.5mM PMSF and faigjjil pepstatin and leupeptin.). The homogenate was kept at 4°C for one hour and centrifuged at 1,00,000 g for an hour in an ultracentrifuge. The supernatant containing all the cytosolic and nuclear proteins were used as a source for Topoisomerase II.

Immunoprecipitation of topoisomerase II α and β from tissues.

100µg total protein of extracts were prepared from brain, liver and testis of the young, adult and old age groups were taken in separate eppendorffs for immunoprecipitation of Topoisomerase II α and β. Topoisomerase II α or β antibody (1:1000 dilution in IP buffer containing 100mM Tris HC1 pH 8, 750mM NaCl, 2mM EDTA, and 1mM PMSF, 0.75% Nonidet) was added to each sample. The antigen-antibody mixture was incubated at room temperature for one hour and 25µl of 6% protein A agarose beads were added. The beads are incubated at 4°C for 15 minutes then the beads are spun down and the supernatant was removed. The protein A agarose beads were washed with 0.5% triton X-100. The beads were directly used for monitoring the relaxation activity of topoisomerase II captured by immunoprecipitation.
**DNA Relaxation assays in the presence of topo II poisons**

DNA relaxation by Topoisomerase II involves the change in the linking number of DNA by 2. During relaxation the supercoiled DNA band (form S) disappears and completely relaxed plasmid DNA (form R) appears. Supercoiled plasmid DNA (~ 0.6µg) containing 200 fM of topoll poison is incubated with the immunoprecipitated Topoisomerase II captured on the Protein A agarose beads in relaxation buffer (50mM Tris HCl pH 8.0, 120mM KC1, 0.5mM EDTA, 0.5mM DTT, 10mM MgCl2, 30µg/ml BSA, 1mM ATP) for 30 min at room temperature. The beads were spinned down and the supernatants were collected separately. The reaction was stopped by addition of 10% SDS to the supernatant, and the DNA products were resolved on 1% agarose gel stained with ethidium bromide and photographed.

**DNA Cleavage assay in the presence of Topo II poisons.**

In the presence of topoll poisons, the DNA in the cleavage complex is stabilized in the cleaved form, thus resulting in the appearance of the linearized DNA band due to double strand break in the DNA. The topo II poisons inhibit the religation activity of the Topo II enzyme. This experiment is carried out by incubating ~ 0.6 µg of supercoiled plasmid DNA containing 200 µM of topoll poisons added to immunoprecipitated Topoisomerase II a or β bound to protein A agarose beads (from the brain, liver and testis extracts of young adult and old rats) in relaxation buffer (without ATP) for 30 minutes at room temperature. The beads are spinned down and the supernatant was collected. 2 µl of 500 mM EDTA and 2 ml of 10 % SDS were added to the supernatant to stop the reaction and
the products were resolved on 1 % agarose gel, ethidium bromide stained and photographed.
Results

The results obtained from the relaxation and cleavage assays carried out using the tissue extracts of young adult and old rats are as follows.

Activities of topoisomerase II α and β from tissues

Immunoprecipitated topoisomerase II α and β were assayed for their relaxation activity by incubating with the plasmid DNA. The activities of the topoisomerase II α (figure 21 panel A) and β (figure 21 panel B) have shown variation in different tissues with the α isoform showing negligible activity in the brain tissues of young adult and old rats. In liver and testes tissues, the topo II α activity is found to be high indicating higher levels of the enzyme in these tissues. In liver and testes the enzyme showed age dependent variation with highest activity seen in the young and adult testes tissues. Where as in the liver, there is increase in the topoII α activity with age.

The topo II β activity is found to be high in all the three tissues with variation seen in the brain tissue with age. As reported in chapter 2 the young extracts of brain have shown highest activity of topoisomerase II p. The results of Topo II β activity in testis show a slight decrease in the activity of topoisomerase II p in the adult rat testis when compared to young and old rat testis. No much change is seen in the liver extracts of the three age groups for the topoisomerase II β activity.
Sensitivity of DNA relaxation and cleavage activity of TopoII α and β in the presence of mAMSA.

The relaxation and cleavage activities of immunoprecipitated topoisomerase II α and β in the presence of 200 mM mAMSA was studied. Topoisomerase II α showed complete inhibition of DNA relaxation activity of the enzyme in presence of mAMSA in all the tissue samples. (figure 22 panel A)

The topoisomerase II β in the brain tissue of the adult and old rats have showed less inhibition of the enzyme catalyzed DNA relaxation activity in the presence of drug, this also could be due to decrease in Topo II. Whereas, in the liver extracts of the young rats the presence of mAMSA showed high inhibition of enzyme catalyzed DNA relaxation activity of topo II β with a slight decrease of inhibition in adult and old rats. In the testis extracts, the inhibition of topoisomerase II β activity towards mAMSA was found to be similar in all the three age groups (Figure 22 panel B). When DNA relaxation activities were compared between α and β, the α isoform showed lesser inhibition.

The cleavage assay performed in the presence of mAMSA the liver and the testes extract shows the formation of linear supercoiled DNA (figure 23) suggesting double stranded breaks. Cleavage assay was conducted with the topoisomerase II β in presence of mAMSA; the result showed that there is less linearization of DNA in the old brain extracts in the presence of mAMSA. Whereas the liver and testis extracts show a significant linearization of supercoiled DNA indicating that TopoII of these extracts is enzymatically inhibited by mAMSA (figure 24)
Sensitivity of Topoisomerase II α and β with Etoposide.

The topoisomerase II α and β relaxation and cleavage activities were studied in the presence of 200 μM of etoposide. The DNA relaxation activity of topoisomerase II α in the young rat liver extracts showed lesser inhibition towards the etoposide when compared to the adult and old rats indicating that the inhibition of topo II activity by this drug in the liver increases with age. The testis extracts did not exhibit much of variation in the inhibition with age. (figure 25 panel A).

Etoposide showed lesser inhibition of topo II β in the adult and old rat brain extracts when compared to the young ones. There was no much change observed in the inhibition of TopoII in the liver and the testis extracts of the three age groups. (Figure 25 panel B)

Etoposide showed highest amount of inhibition of topo II α activity showing linearization of the supercoiled DNA with topo II α in the liver and testis extracts of young, adult and old rats. (Figure 26). Where as etoposide showed no linearization of supercoiled DNA with topo II β in the brain extracts indicating least inhibition of the drug, towards topo II β in brain. The liver extracts of all three age groups have shown very high amount of inhibition of TopoII β activity in presence of etoposide. The young and adult testis extracts showed linearization of supercoiled DNA indicating highest level of cleavage reactivity, where as the old extract did not show any linearization indicating less cleavage (figure 27).
Discussion

Etoposide and mAMSA are used for treatment of colon cancer and other types of cancers. These drugs exert cytotoxic effects by stabilizing covalent complexes between topoisomerase II and DNA thus generating DNA double strand breaks which result in cell death. These topo II poisons are used for chemotherapy in patients of all the age groups (young adult and old). The sensitivity of the α and the β isoform may show variation due to their differential interaction of enzyme with the drugs. It is reported that in some tumors the Topo II (3 isoform is predominant. So the variation in the sensitivity of the topo II isoforms towards topoII poisons may result in emergence of resistance to the chemotherapy with these drugs in certain tumors. Further, these drugs may cause non-specific toxicity due to non-targeted enzyme activity.

The present study was carried out to analyze the age dependent changes in the interaction of the topoll isoforms with topoll poisons like etoposide and mAMSA. The α and β isoform of topo II in brain liver and testis tissues (young, adult and old rats) were taken for the inhibitory study with mAMSA and etoposide. The results of the experiments suggest that Topo II α is high in proliferating tissues like liver and testes and is negligible in differentiated tissue like brain (as found in the chapter II). The activity of β isoform of the enzyme is found to be high in all the three tissues. In the brain activity was found to be decreasing with increasing age as studied previously (chapter II).

The variations in inhibition activity of etoposide and mAMSA against activities of topo II isoforms suggest a variation in the sensitivity of these enzymes towards the drugs. The results of the experiment done with topo II α and β from brain liver and testis extracts in the presence of etoposide and mAMSA show that the α isoform of the enzyme is more
sensitive towards these drugs, thus showing high inhibition of the enzyme activity. Where
β isoform showed less inhibition towards etoposide and mAMSA indicating less
sensitivity of this isoform towards these topo II poisons. The sensitivity of the β isoform
in the brain tissue varied with age. The adult and old rat brains showed less inhibition of
topoll (3 suggesting that the sensitivity of this isoform in brain decrease with increasing
age. The exact reason for this is not known. The molecular form of the topoll (3 isoform
in brain may be different that change its interactive ability with etoposide and mAMSA
compared that of the a isoform.

The results of the above study indicating the sensitivities of the isoforms to the two topo
II poisons may be useful for application of these drugs for targeting specific tumors and
chemotherapy in patients of different age groups
Figure 21
Relaxation activity of topoisomerase II α and β from tissues of young, adult and old rats. Panel A and B show relaxation activity of immunoprecipitated topoisomerase II α and β from 100 μg total protein of brain (lanes 3-5), liver (lanes 6-8) and testis (lanes 9-11) of young adult and old rats captured on to Protein A agarose beads was incubated with ~0.6 μg of pRYG plasmid DNA and lane 2 shows DNA with 2 units of Topo II. The products were resolved on 1% agarose gel stained with ethidium bromide and visualized under UV light and photographed.
Figure 21

A

Relaxation activity of Topoisomerase II α in tissues

B

Relaxation activity of Topoisomerase II β in tissues
Figure 22

Relaxation activity of topoisomerase II α and β from tissue extracts in presence of mAMSA: Panel A and B show relaxation activity of immunoprecipitated topoisomerase II α and β from 100 μg total protein of brain (lanes 4-6), liver (lanes 7-9) and testis (lanes 10-12) of young adult and old rats captured on to Protein A agarose beads was incubated with ~0.6 μg of pRYG plasmid DNA in presence of relaxation buffer and 200μM mAMSA. The products were resolved on 1% agarose gel stained with ethidium bromide and visualized under UV light and photographed.
Figure 22

A

Topo II α relaxation activity in presence of mAMSA

B

Topo II β relaxation activity in presence of mAMSA
Figure 23
Cleavage activity of topoisomerase II α and β from tissue extracts in presence of mAMSA. Figure shows relaxation activity of immunoprecipitated topoisomerase II α from 100 μg total protein of brain (lanes 4-6), liver (lanes 7-9) and testis (lanes 10-12) of young adult and old rats captured on to Protein A agarose beads was incubated with ~0.6 μg of pRYG plasmid DNA in presence of cleavage buffer and 200μM mAMSA and proteinase K treated. Lane 1 shows ~0.6 fig of pRYG plasmid DNA and lane 2 shows DNA with 2 units of Topo II and lane 3 shows DNA with 2 units of Topo II in presence of 200μM mAMSA. The products were resolved on 1% agarose gel stained with ethidium bromide and visualized under UV light and photographed. The linear DNA was quantified and the bar diagrams are shown below the gel.
Figure 23

A

Topo II α cleavage activity in presence of mAMSA

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Topo II α cleavage activity

Intensity of linear DNA

1 DNA control
2 topo II control
3 mAMSA control
4 young brain extract
5 adult brain extract
6 old brain extract
7 young liver extract
8 adult liver extract
9 old liver extract
10 young testis extract
11 adult testis extract
12 old testis extract
Figure 24
Cleavage activity of topoisomerase II α and β from tissue extracts in presence of mAMSA. Figure shows relaxation activity of immunoprecipitated topoisomerase II (3 from 100 μg total protein of brain (lanes 4-6), liver (lanes 7-9) and testis (lanes 10-12) of young adult and old rats captured on Protein A agarose beads was incubated with ~0.6 μg of pRYG plasmid DNA in presence of cleavage buffer and 200 μM mAMSA and proteinase K treated. Lane 1 shows ~0.6 μg of pRYG plasmid DNA and lane 2 shows DNA with 2 units of Topo II and lane 3 shows DNA with 2 units of Topo II in presence of 200 μM mAMSA. The products were resolved on 1% agarose gel stained with ethidium bromide and visualized under UV light and photographed. The linear DNA was quantified and the bar diagrams are shown below the gel.
Figure 24

Topo II β cleavage activity in presence of mAMSA

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<td>7 young liver extract</td>
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<td>10 young testis extract</td>
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Intensity of linear DNA

lane no
Relaxation activity of topoisomerase II α and β from tissue extracts in presence of etoposide: Panel A and B show relaxation activity of immunoprecipitated topoisomerase II α and β from 100 μg total protein of brain (lanes 4-6), liver (lanes 7-9) and testis (lanes 10-12) of young adult and old rats captured on to Protein A agarose beads was incubated with ~0.6 μg of pRYG plasmid DNA in presence of relaxation buffer and 200μM etoposide. Lane 1 shows ~0.6 μg of pRYG plasmid DNA and lane 2 shows DNA with 2 units of Topo II and lane 3 shows DNA with 2 units of Topo II in presence of 200μM etoposide. The products were resolved on 1% agarose gel stained with ethidium bromide and visualized under UV light and photographed.
Figure 25

A

Topo II α relaxation activity in presence of etoposide

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Topo II β relaxation activity in presence of etoposide

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S
Figure 26

Cleavage activity of Topoisomerase IIα and β from tissue extracts in presence of etoposide: Figure shows relaxation activity of immunoprecipitated Topoisomerase IIα from 100μg total protein of brain (lanes 4-6), liver (lanes 7-9) and testis (lanes 10-12) of young, adult and old rats captured on to Protein A agarose beads was incubated with ~0.6μg of pRYQ plasmid DNA in the presence of cleavage buffer and 200μM etoposide and proteinase K treated. Lanes 1 shows ~0.6 μg of pRYG plasmid DNA and lane 2 shows DNA with 2 unit of Topo II, Lane 3 shows DNA with 2 units of Topo II in presence of 200μM etoposide. The products were resolved on 1% agarose gel stained with ethidium bromide and visualized under UV light and photographed. The linear DNA was quantified and the bar diagrams are shown below the gel.
Figure 26

A

Topoli a cleavage activity in presence of etoposide

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Cleavage assay with topo II α

Intensity of linear DNA

0  5  10  15  20  25

0  1  2  3  4  5  6  7  8  9  10  11  12  13  14

1 DNA control
2 Topo II control
3 etoposide control
4 young brain extract
5 adult brain extract
6 old brain extract
7 young liver extract
8 adult liver extract
9 old liver extract
10 young testis extract
11 adult testis extract
12 old testis extract
Cleavage activity of Topoisomerase IIα and β from tissue extracts in presence of etoposide: Figure shows relaxation activity of immunoprecipitated Topoisomerase IIβ from 100µg total protein of brain (lanes 4-6), liver (lanes 7-9) and testis (lanes 10-12) of young, adult and old rats captured on Protein A agarose beads was incubated with ~0.6µg of pRYG plasmid DNA in the presence of cleavage buffer and 200µM etoposide and proteinase K treated. Lanes 1 shows ~0.6µg of pRYG plasmid DNA and lane 2 shows DNA with 2 unit of Topo II. Lane 3 shows DNA with 2 units of Topo II in presence of 200µM etoposide. The products were resolved on 1% agarose gel stained with ethidium bromide and visualized under UV light and photographed. The linear DNA was quantified and the bar diagrams are shown below the gel.
Figure 27

Topo II β cleavage activity in presence of etoposide

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Topo II β cleavage activity

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Legend:
- 1 DNA control
- 2 Topo II control
- 3 drug control
- 4 young brain extract
- 5 adult brain extract
- 6 old brain extract
- 7 young liver extract
- 8 adult liver extract
- 9 old liver extract
- 10 young testis extract
- 11 adult testis extract
- 12 old testis extract