A Study on the age dependent changes of Topoisomerase II a and Topoisomerase II β isoforms in rat brain and sensitivity of Topoisomerase II isoforms to Topo II poisons

A thesis submitted for the degree of

Doctor of Philosophy

By

Neelima Konuru

Department of Biochemistry
University of Hyderabad
Hyderabad-500046

July 2004
Enrollment number: 99LBPH11

Department of Biochemistry
School of Life sciences
University of Hyderabad
P.O. central university
Hyderabad-500046
India

Declaration

I hear by declare that the work presented in my thesis is entirely original and was carried out at the department of Biochemistry, university of Hyderabad, under the guidance of DR. Anand K. Kondapi. I further declare that this work has not been submitted before for the award of degree or diploma from nay other institution or university

Date 31-7-2004

Neelima Konuru

Anand K. Kondapi

Certificate

Department of **Biochemistry**
School of Life Sciences
University of Hyderabad
P.O.Central **University**
Hyderabad
India

This is to certify that this thesis entitled **"A** study on the age dependent changes of Topoisomerase **IIα** and Topoisomerase **IIβ** isoforms in rat brain and sensitivity of Topoisomerase II isoforms to Topo **II** poisons" submitted to the University of Hyderabad by Ms Neelima Konuru for the **degree** of doctor of philosophy, is based on the studies carried out by her under my supervision. This has not been submitted before for the award of degree or diploma from any university or institution

Dr.Anand K. Kondapi
Supervisor

2/8/04

Head
Department of Biochemistry

HEAD
DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES,
UNIVERSITY OF HYDERABAD,
HYDERABAD-500 046. (A.P.)

2/8/04

Dean
School of Life Sciences

School of Life Sciences
University of Hyderabad
Hyderabad

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ABBREVIATIONS:

ATP- Adenosyl triphosphate

Topo II- Topoisomerase II

mAMSA- Amsacrine

CKII- casein kinase II

MAP kinase- Mitogen activated kinase

EGL- external germinal layer

PMSF- phenyl methyl sulphonyl fluoride

TBS- tris buffered saline

SDS PAGE - Sodium dodecyl sulphate -polyacrylamide gel electrophoresis

RuBen (dmso) –[(η^6–benzene) dichloro sulfinyl bis (methane)-o- ruthenium (II)]

RuBen Pyr –[(η^6–benzene) (pyridine)-N- ruthenium(II)]

RuBen Apy –[(η^6–benzene) (3-amino pyridine)- N1- ruthenium (II)]

RuBen PyCHO–[(η^6–benzene) (pyridine 3-carboxaldehyde)- N1- ruthenium (II)]

RuBen $PySO_3H$–[(η^6–benzene) (pyridine-3-sulphonic acid)- N1- ruthenium (II)]

RuBen PyOH–[(η^6–benzene) (3-hydroxy pyridine)- N1- ruthenium (II)]

RuBen Nam-[(η^6–benzene) (nicotinamide)- N1 - ruthenium (II)]

RuBen Agu -[(η^6–benzene) (amino guanidine)-N1- ruthenium (II)]

Ruben Aba –[(η^6–benzene) (p-amino benzoic)-O- ruthenium (II)]

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Chapter I

Introduction

Introduction

Types of Topoisomerases

Topoisomerases are nuclear enzymes that catalyze the conversion of one DNA topological isomer to another via concerted breaking and rejoining of DNA helix. Three types of reactions are catalyzed by the topoisomerases. Relaxation of supercoiled DNA, interconversions of between single stranded DNA rings, linking of single stranded rings of complimentary sequences. Topoisomerases in general are of two types. in both prokaryotes and eukaryotes. (Revised by Roca 1995, Wang 1996)

Type I Topoisomerases: These are monomeric enzymes, which are ATP independent; they change the linking number of the DNA in steps of one (linking number is the number of right handed turns that one DNA strand makes around the other in a DNA duplex.).

Type I topoisomerase are of Two types.

Topoisomerase 1 5'

These are proteins with a molecular weight ~97 kDa. These enzymes binds to a single strand of duplex DNA and forms an enzyme- DNA intermediate through a covalent bond between a tyrosine residue of the enzyme and the 5' phosphate at the DNA break point. Functions of these enzymes are partial relaxation of negatively supercoiled DNA and knotting of single stranded DNA rings into a double stranded ring.

E.coli DNA topoisomerase I, III and eukaryotic topoisomerase HI are some of the enzymes in this class.

Topoisomerase I 3': This is a 95-135-kDA protein. It is similar to the 5' enzyme but bind preferentially to double stranded DNA and cleaves a single strand of DNA. It forms a phosphorylated linkage between a tyrosine residue of the enzyme and the 3' phosphate at the break site. The unbroken strand is passed through this break to release the twisting stress on the helix. These enzymes completely relax both positive and negative supercoils in the DNA.

Eukaryotic Topoisomerase I, vaccinia virus Topoisomerase I and topoisomerase V of hyperthemophilic bacteria fall under this category of enzymes.

Type II Topoisomerases: these are essential enzymes for an organism. They are dimeric molecules and are ATP dependent. These enzymes change the linking number of the DNA in steps of 2. They are of two types depending on whether they are prokaryotic or eukaryotic.

DNA Gyrase: This is a prokaryotic Topoisomerase II. Its catalytic activity is same as that of the eukaryotic enzyme.

These enzymes preferentially relax positive supercoils and induce negative supercoiling in the bacterial chromosome and extra chromosomal DNA (plasmids) all the prokaryotic Type II topoisomerases and type IV topoisomerases fall under this category.

Topoisomerase II: These are eukaryotic type II topoisomerases. They have a molecular weight of 160-180 kDa and are highly conserved in all the organisms. This enzyme binds to the duplex DNA and forms double stranded breaks in both the strands, 4 base pairs apart. The 5' broken ends are covalently bonded to two tyrosine residues (one from each monomer) through phosphotyrosyl linkages. Additional interactions restrict free rotation

of the free 3' ends at the break site. A second duplex segment is transported through the break.

This enzyme catalyses the relaxation of supercoils or knotting / unknotting of DNA if the gated and transported segments reside in the same DNA fragment. If they are in different DNA segments the enzyme catalyses their decatanation / catenation.

All the eukaryotic Type II topoisomerase come under this category of enzymes.

Human Topoisomerase: In humans and other mammal's topoisomerase II is reported to be present in 2 isoforms, which are genetically, and biochemically distinct (Drake *et. al.,* 1987). The first type the topoisomerase II a, which is a ~170 kDa protein similar to that of Topoisomerase II of all eukaryotes and performs all the functions of the typical Type II enzymes. (Woessner *et. al.,* 1990). The second one is Topoisomerase 11 (5 which shares a sequence homology of 68% with the a isoform and has a molecular weight of ~180 kDa. The 2 isoforms appear to have evolved from the gene duplication event, which included several flanking markers like retenoic acid receptor a and (3 genes. (Coutts *et. al.,* 1993). The topo II a gene is located on the chromosome 17q 21-22. (-Pflugfleder *et. al.,* 1988) where as the Topo II (3 gene is located on the chromosome 3 p24 (Tan *et. al.,* 1992) The 2 isoforms show distinct nuclear localization patterns and cell cycle expression profiles. The a isoform is shown to be localized in the nucleoplasm. (Woessner *et. al.,* 1990) and it is found to be maximum during the G2/M phase of the cell cycle.(Heck *et. al.,* 1988) The β isoform is found to be localized in the nucleoli (Negri *et. al.,* 1992) and the level of this enzyme is found to be relatively constant throughout the cell cycle (Woessner *et. al.,* 1991).

Differential specific functions for the a and β isoforms could be mediated in a number of ways with their differential localization in the nucleus and altered capacity to interact with the other cellular proteins. The a isoform is shown to play an important role in the chromosome condensation, segregation, replication and transcription. . The function of the β isoform in vivo is unknown. The β isoform of topoisomerase II is detected to be high in the terminally differentiated tissues like brain of the rats and humans. (Tsui *et. al.,* 1993, Watanabe *et. al.,* 1994, Austin and Marsh 1998). It is possible that Topoisomerase II β is involved in an important physiological role in the neuronal activities, presumably in the topological regulation of the genomic DNA

Functions of Topoisomerase II

Topoisomerase II enzyme has an ability to promote topological interconversions of DNA. It plays an important role in various cellular processes such as chromosome segregation, chromosome condensation, replication, transcription, maintaining the genomic integrity and recombinatioa The exact role played by this enzyme in these cellular processes would be described below.

Chromosome segregation

Replication of the dense chromatin results in catenation of replicated chromatids. Decatanation of the interwined daughter chromatids is performed by the type II topoisomerase enzymes in the G2 phase of cell cycle in a way helping the segregation of newly replicated strands during mitosis and meiosis (Ishida *et. al.,* 1994). Mitotic process is inhibited when chromatids are insufficiently decatenated by topoisomerase II sustained

chromatid catenation results in chromosome stress which in turn results in aneuploidy and polyploidy through aberrant mitosis causing genetic instability (DiNardo *et. al.*, 1984, Uemura and Yanagida 1986). The significant role of Topoisomerase II in meiotic chromosome condensation and segregation has been reported in yeast. Arresting the topoisomerase II functioning inhibited the formation of metaphase chromosomes in cells induced to progress from prophase to metaphase resulting in cell cycle arrest and aneuploidy (Holm *et. al,* 1985).

Chromosome condensation and decondensation.

Topoisomerase II helps in condensing the DNA into chromatids and further condensing this chromatin into chromosomes (Adachi *et. al.,* 1991: Wood and Earnshaw, 1990). They are even involved in the decondensation of these structures. This enzyme is shown to interact with proteins of chromosomal scaffold like the SMC proteins (SCII, XCAP-C and XCAP-E) during chromosome condensation (Ma *et. al.,* 1993; Saitoh *et. al.,* 1994). Removal of topoisomerase II activity completely inhibits chromosome assembly and condensation (Hirano and Mitchison, 1993). Cyclic modifications of the enzyme such as phosphorylation and dephosphorylation may be involved in the DNA condensation and decondensation.

Replication and transcription

The unwinding of the DNA stands during the replication and transcription generate positive supercoils ahead of the advancing fork and negative supercoiling behind the fork, which results in torsional stress in the DNA (Lockshon and Morris, 1983; Liu and

Wang, 1987). Topoisomerases help in relaxing the positive and negative supercoiling in the DNA allowing successful completion of these ongoing processes. This function of Topoisomerases is termed as swivelase activity, which is equally, exhibited by both type I and type II topoisomerases. Studies of Topoisomerase II Inhibition suggest that this enzyme is required for initiation and late elongation of replication.

Maintenance of chromosomal structure

Topoisomerase II is an abundant chromosomal protein in eukaryotes (Swedlow *et. al.,* 1993; Earnshaw and Heck 1985). Experimental evidence suggests that topoisomerase II plays a key role in chromosomal scaffolding. Immunolocalisazion studies showed that Topoisomerase II localizes along the central axis region that traversed the length of each chromatid including the kinetochore. Topoisomerase II acts as an anchor joining the A-T rich DNA sequences called the scaffold attachment regions (SAR) (Gasser and Laemmli, 1987) found at the base of the chromosomal loops to the nuclear scaffold. Consensus sequences for topoisomerase II DNA cleavage *in vitro* is found in the SAR sequences. Topoisomerase II is the major non-histone protein found in the nuclear scaffold (Hirano and mitchison 1993).

Recombination and Genomic integrity

Topoisomerase II together with topoisomerase I is involved in suppressing the mitotic recombination in the rDNA clusters of S.cerevisiea. It was suggested that the movement of transcriptional machinery across repetitive DNA sequences creates a topological situation favoring recombination and subsequent excision of these sequences.

Topoisomerases are required to suppress this phenomenon and maintain genomic integrity (Kim and Wang 1989).

Structural and functional domains of Topoisomerase II.

Protease digestion of the topoisomerase II showed 3 distinct regions of this polypeptide. N terminal region homologous to the B subunit of the bacterial DNA gyrase, central region homologous to the gyrase A subunit and the c- terminal region characterized by clusters of charged amino acids.

N- terminal region contains consensus sequence for the ATP binding site and has the ability to hydrolyze ATP *in vitro.* This domain dimerises in the presence of nonhydrolisable ATP analogue. So it is shown to contain sequences, which direct protein-protein interactions. The central region of the enzyme has 3 amino acid motifs EGDSA, PLRGK, IMTDJD. EGDSA and PLRGK correspond to the loops found in the structure of resolvase a topoisomerase related protein. Mutations in these regions decrease the activity of the enzyme. PLRGK is the region associated with the binding of drugs to the protein / DNA complexes. The active site tyrosine, which binds covalently to the DNA, is found at the C- terminal of the central region. The beginning of the central region is highly susceptible lo proteases. These regions are not conserved. Three different roles are ascertained to the C-terminal region of Topoisomerase II. These are nuclear localization, dimerization and regulation of the enzyme activity.

Nuclear localization:

Several nuclear localization signals are present through out the Topoisomerase II enzymes. Sites specific for targeting Topoisomerase II to the nucleus are defined in the c-terminal region.

Dimerization.:

Eukaryotic Topoisomerase II acts as a homodimer and therefore must contain defined sequences, which direct dimerization of the individual promoters. The residues between Lys-1137 and Gly-1166 are found to be vital for enzyme activity. This region is implicated in the dimerization based on the primary sequence analysis.

Regulation of Enzyme activity:

Phosphorylation of topoisomerase II plays an important role in the enzyme activity and the responsiveness of the enzyme to various inhibitors. Phosphorylation is important in

determining the specific activity and in regulating the mitotic functions of Topoisomerase II. Protein kinase C and casein kinase II are the major phosphorylating enzymes for Topoisomerase II (Ackerman *et. al*, 1985, 1988; Devore *et. al.*, 1992). The specific activity of Topoisomerase II increases 2-3 fold as a result of phosphorylation thus enhancing the rate of ATP hydrolysis. (Corbett *et. al.*, 1992, 1993b; Takano *et. al.*, 1991)

Cell cycle regulation of phosphorylation:

Phosphorylation of topoisomerase II is regulated with respect to cell cycle position and cellular growth state in eukaryotes. Topoisomerase II phosphorylation is higher in mitotic cells than in G1 cells. No phosphorylation occurs in the absence of casein kinase II. Topoisomerase II expression and phosphorylation is maximal during the G2/M phase of cell cycle this is brought about by the mitotic kinases called mitogen-activated kinase (MAP kinase) .The phosphorylation of Topoisomerase II by these kinases is regulated by the master controller of mitotic events, the $p34^{cdc2}$ kinase.

Sites of Phosphorylation:

C-terminal region is the major target for the regulatory phosphorylation of Topoisomerase II additional phosphorylation sites are also found on the N-terminal region.

Phosphorylation and antineoplastic drug resistance:

Phosphorylation alters the susceptibility of eukaryotic Topoisomerase II to inhibition by anticancer drugs. Phosphorylation by casein kinase II and protein kinase C leads to

attenuation of the effects of etoposide and mAMSA in stabilizing the Topoisomerase II cleavable complexes. Hyperphosphorylation is one of the path way for the cells to overcome the cytotoxic effects of Topoisomerase II inhibitors.

Catalytic function of Topoisomerase II

Topoisomerase II alters the topological state of nucleic acids by passing an intact helix **of** DNA through a transient double stranded break, which is generated, in a separate DNA helix. The double strand DNA passage reaction requires ATP hydrolysis and magnesium. The catalytic mechanism involves various steps. Each catalytic cycle removes two negative supercoils from DNA. The steps involved in the catalytic cycle of Topoisomerse II are described below.

Substrate binding and recognition

Topoisomerase II initiates the catalytic cycle by binding to its nucleic acid substrate. This protein DNA interaction is determined by nucleotide sequence and the topological structure of the DNA. Nucleic acid sequence defines the sites of enzyme mediated DNA cleavage and catalytic activity. DNA sequences recognized by Topoisomerase II are asymmetric. Topological state of DNA modulates the level of Topoisomerase II binding. The enzyme preferentially interacts with supercoiled nucleic acids over relaxed molecules. It binds to DNA at regions of helix-helix juxtaposition. Such DNA crossovers are more prevalent in super coiled DNA .The enzyme binds to DNA even in the absence of a divalent cation. Recognition of DNA crossovers is independent of enzymatic activity.

Pre-strand passage DNA cleavage/ religation: Topoisomerase II after recognition of the nucleic acid substrate establishes a double stranded DNA cleavage/ religation equilibrium. DNA cleavage and religation requires the presence of a divalent cation upon hydrolysis of the DNA, Topoisomerases become covalently attached to both newly generated 5' termini via phosphotyrosine bonds. Topoisomerase II makes a staggered break in DNA, which results in 4- base 5' overhangs. The enzyme pre-strand passage DNA cleavage/ religation equilibrium is an important target for many anti neoplastic drugs Topoisomerase II targeted drugs. These drugs alter the enzyme's utilization of DNA cleavage sites.

Double stranded DNA passage:

Topoisomerase II after creating a double stranded break passes a **separate** double stranded segment of DNA through the break. This strand passage activity is dependent on binding of Mg ATP in non-hydrolysable form. This enzyme mediated DNA strand passage activity converts a negative supercoil DNA into a positive super helical twist. Topoisomerase II removes the two super helical twists per catalytic cycle. And it undergoes major conformational changes.

Post strand passage DNA cleavage/ religation.

Topoisomerase II establishes DNA cleavage/ religation equilibrium after strand passage event. The post strand passage equilibrium lies more towards the cleavage event. There is a decrease in the religation activity when compared to the pre strand passage equilibrium. The post strand passage enzyme DNA cleavage complex is also a target for the *in vitro*

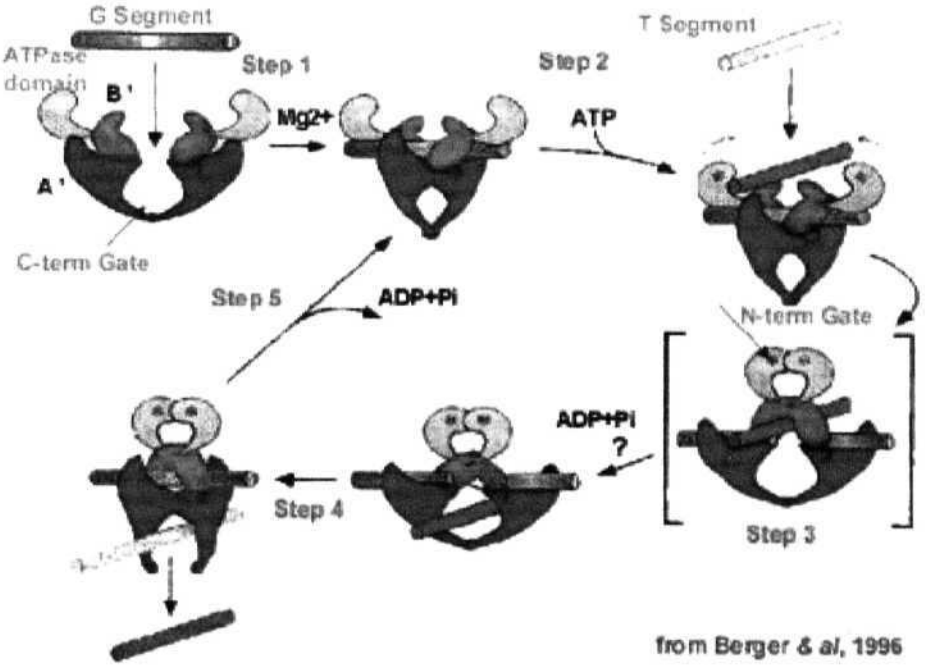
action of anti neoplastic drugs both pre and post strand passage cleavage complexes are physiological targets for anti-neoplastic drugs.

ATP hydrolysis.

Topoisomerase II hydrolyses ATP to ADP and inorganic phosphate. This activity is greatly stimulated by the presence of DNA. The catalytic activity of topoisomerase II is strictly coupled to ATP hydrolysis.

Enzyme turnover:

Enzyme turn over is the process by which topoisomerase II regains its ability to initiate a new round of catalysis this is an ATP dependent process. The process of enzyme turn over regenerates the active conformation of topoisomerase II and confers upon the enzyme the ability to dissociate from its DNA product.



from Berger & al, 1996

Topoisomerase II as anti cancer drug target:

Topoisomerase II is a successful drug target because of its indispensable functions in the cellular events. Inhibitors of this enzyme have become central parts of both primary and adjuvant chemotherapy for neoplastic diseases. The strand passage event in the catalytic cycle of Topoisomerases, which generates double stranded breaks in the DNA, is targeted by many of the Topoisomerase II inhibitors. These inhibitors prevent the religation event in the cleavage/ religation reaction of the strand passage **activity.** Increase in the physiological concentration of the DNA breaks bring many deleterious effects on the genetic material (Corbett and Osheroff 1993, Anderson and Berger 1994, Ferguson and Baguley 1994)

The well known anti cancer drugs like etoposide and amsacrine (m-AMSA) act on the cleavage / religation activity of the enzyme (Nelson *et. al.,* 1984, Chen *et. al,* 1984). These drugs allow DNA cleavage by the enzyme but block the DNA religation event. This particular group of compounds are known as Topoisomerase II poisons, where as the topoisomerase II inhibitors interfere with the enzyme turnover number (Smith 1990). These topoisomerase II poisons interact bi-directionally with the DNA and the enzyme or with the enzyme alone. (When the enzyme is bound to the DNA). The drug bound Topoisomerase II cleaves the DNA as per the normal catalytic activity. At this point the transient intermediate of the covalently linked enzyme cleaved DNA complex is frozen by the drug. This ternary complex consisting of the enzyme- drug- DNA complex is called the cleavage complex. (Liu 1989, Smith 1990). Formation of this complex disrupts the DNA cleavage/ religation equilibrium shifts towards the DNA cleavage and the enzyme does not bring religation of the DNA. This results in the accumulation of double

stranded breaks in the DNA, which are covalently linked to topoisomerase II (Ammon-Osheroff 1995).

The topoisomerase II enzyme is over expressed in cancer cells (Hsiang *et. al,* 1988; Tricoli *et. al.,* 1985; Bodley *et. al.,* 1987) and when these cancer cells are treated with Topoisomerase II poisons they accumulate the topoisomerase II induced DNA cleavage complexes (Potmesil and Kohn 1991; Slichenmyer *et. al,* 1993; Sinha 1995) Replication and transcription complexes in the region of the breaks split up these cleavage complexes which will expose the DNA breaks. These DNA breaks become the targets for the DNA repair and recombination pathways. This in turn stimulates the sister chromatid exchange, large deletions / insertions, translocations and large chromosomal aberrations (Corbett and Osheroff 1993; Chen and Liu 1994; Anderson and Berger 1994; Ferguson and Baguley 1994). When these genetic aberrations accumulate at high concentrations they trigger apoptotic and necrotic events (Liu 1994;l Pommeir *et. al.,* 1994; Beck *et. al,* 1994).

Antagonism of Topoisomerase II:

Topoisomerase II is the target for number of structurally different compounds (Chen and Liu 1994). These compounds are categorized in to two classes viz. topoisomerase II poisons and Topoisomerase II inhibitors.

Topoisomerase II poisons

Topoisomerase II poisons are defined as drugs that stabilize the cleavable complex to cause DNA breaks. They are called poisons because they convert this essential nuclear enzyme into a lethal poison.

During the catalytic cycle of topoisomerase II gaps in the form of Cleavable complexes before are after the DNA processing are produced which are attacked by the topoisomerase II poisons.

Topoisomerase II poisons are again divided into two classes of substances.

DNA intercalating Topoisomerase II poisons:

These molecules possess a domain which intercalates with the **DNA** and a domain for enzymatic interaction.. Through this bi-directional interaction they form enzyme-drug- DNA ternary complexes called cleavage complexes.

Amsacrine (mAMSA), adriamycin, ellipticine are examples of this group of drugs.

DNA non-intercalating topoisomerase II poisons:

These molecules also form the ternary complex or the cleavage complexes but do not intercalate with DNA, they interact with the enzyme and may or may not interact with the DNA with out intercalation.

Examples of this group of compounds are etoposide and tenoposide.

Topoisomerase II inhibitors

This class of drugs binds to the enzyme and inhibits the catalytic activity of the enzyme with out forming the cleavage complex rather interferes with the DNA dependent ATP hydrolysis of the enzyme. These compounds do not induce DNA breaks and may even prevent their formation. They act at any stage of the catalytic cycle of the topoisomerase II enzyme other than the cleavable complex. Either by interference with the Topoisomerase II binding to the DNA or by locking the DNA bound enzyme in the shape of a closed clamp.

Example for such a class of compound is Fostriecin.

Another class of compounds is drugs that interfere with the ATP hydrolysis reaction of topoisomerase II. ATP hydrolysis causes a conformational change in the enzyme, which opens a molecular clamp for a double stranded DNA segment through the distal (C-terminal) end of the enzyme. Drugs, which inhibit ATP hydrolysis by the enzyme, do not allow the passage of the DNA segment through this clamp.

Examples of this class of drugs are Novobiocin, Coumeromycin, and Amonafide.

Topoisomerase II targeting in current clinical use.

COMPOUND NAME	Conc. Of topo II a & β Inhibition (μM)	Application
TOPO II POISONS		
	Intercalaters	
Amamsacrine	100 / 100	-Acute leukemia
Doxorubicin	0.56/0.56	.-Multiple myeloma, lung, breast, Bladder cancers, leukemia etc
Mitoxantrone	1.8/0.56	- Acute leukemia
RuBen dmso	500	- to be studied
RuBen Apy	250	- to be studied
	Non Intercalaters(non DNA binding)	
Etoposide	32/ 100	- lung, testicular, ovarian cancers
Tenoposide	100/ > 100	-Small cell lung cancer
Substituted Ferrosine	300	-to be studied
TOPO II INHIBITORS		
ICRF 187 (Dexrazoxane)	56 / > 100	Central nervous system Metastasis
ICRF 159(Razoxane)	5.6 / > 100	- Central nervous system . Metastasis
Aclarubicin	--	-Acute myeloid leukemia

DNA Repair and aging:

Aging is a very complex phenomenon, involving multiple mechanisms at different levels. DNA damage contributes to age related changes aging is supposed to be caused by accumulation of random DNA damage in somatic cells and tissues. Genetic damage is suggested to be one of the major causes for aging of cells and organisms. This is because cellular DNA is constantly exposed to exogenous and endogenous DNA damaging agents. DNA damaging agents cause a time dependent accumulation of damage in the genome. When cellular defense and repair systems do not counteract it the resulting unrepaired and persisting DNA damage or products of low fidelity DNA repair interfere with DNA transcription and replication.

Fibroblasts and various other non-transformed cells lose their replicative potential after number of population doubling and enter a state of replicative senescence. This is related to *in vitro* aging. The exhaustion of replicative potential of certain cell T lymphocytes may contribute towards immunosenescence and hence human aging.

DNA damage in replicating cells, which is not immediately, repaired either causes transient cell cycle arrest or replicative senescence or ultimately apoptosis. DNA damaging would triggers senescence prematurely.

RecQ helicases and Aging.

DNA metabolic pathways have a common requirement that is separation of the complimentary strands of DNA duplex by the action of helicases. Helicases utilize the energy derived from the hydrolysis of ATP to perform essential roles in the progress of DNA replication, transcription, genetic recombination and DNA repair. Recent evidences

indicate that mutations in genes encoding members of a particular class of helicases called the Rec Q family of helicases are responsible for genomic instability, disorders associated with several premature aging or cancer.

Rec Q family of proteins are highly conserved in both prokaryotes and eukaryotes. The highly conserved domain comprises of 450 amino acids polypeptide that includes seven motifs found in many classes of DNA and RNA helicases. Among these the ATP binding sequence and DEAH box that is required for ATP hydrolysis is one. Rec 0 family of helicases are 3'-5' exonucleases that translocates in the 3'-5' direction, utilize the energy derived from NTP hydrolysis into mechanical energy and separate the complimentary strands of the DNA.

Defects in Rec Q helicases in humans lead to genomic instability disorders associated with cancer disposition and premature aging like blooms syndrome, Werner's syndrome and Rothmund Thompson syndrome. These disorders caused by mutations in the BLM, WRN and recQ4 genes respectively. Despite of the structural and biochemical similarities between the BLM, WRN and RecQ4 proteins the phenotypes are significantly different.

Blooms syndrome is characterized by growth deficiency, immunodeficiency, infertility, susceptibility to diabetes, and susceptibility to cancers. Blooms syndrome shows high frequency of genetic recombination events particularly sister chromatid exchange and elevated somatic mutations.

Werner's syndrome causes age associated disorders like puberty, graying and thinning of hair, bilateral cataract formation, type II diabetes mellitus, hypogonadism, ulceration of legs, osteoporosis and atherosclerosis. Soft tissues are prone to cancers like sarcomas and osteosarcomas in 47% of patient's death are due to cancer of cardiovascular disease.

Werner syndrome cells display variegated translocation, mosaisism, increased illegimate recombination, high frequency of large chromosomal deletions and retarded cell cycle progression.

Rothmund-Thompson syndrome shows growth deficiency, photosensitivity with poikilodermatous skin changes, cataract, early graying and loss of hair and increase in some cancer incidence. These cells show Trans isomerization and increased frequency of chromosomal aberrations.

WRN protein plays a role in DNA replication and cellular responses to DNA damage. The following observations support to this function of WRN protein.

1. WRN translocates to sites of replication/ repair when DNA replication is blocked.
2. WRN co purifies with DNA replication complexes and interacts with proliferating cell nuclear antigen and Topoisomerases.
3. Single stranded DNA binding proteins like replication protein A interacts with WRN and its presence increases WRN helicase activity.
4. Werner's Syndrome cells show a protracted S phase.
5. WRN interacts with DNA polymerase (, which is required for DNA replication.

WRN protein plays a key role in the cellular pathways like DNA replication, Recombination, Apoptosis Transcription. Recent studies suggest that WRN may also participate in pathways at the telomeric ends. WS cells display some defects in telomeric metabolism including increased rates of telomere shortening. (Schulz *et al* 1996) and deficiencies in repair at the telomeres (Kruk *et. al.,* 1995). Expression of telomerase in WS syndrome prevents premature replicative senescence (Wyllie *et. al.,* 2000) and reduces hypersensitivity to 4 NQO (Hisama *et. al.,* 2000). There is an

evidence for WRN localization at telomeric ends in some human fibroblast cell line in vivo (Johnson *et. al,* 2001) WRN yeast homologue is shown to participate in a telomerase independent mechanism of telomere lengthening in yeast (Johnson *et. al,* .2001). So WRN may function at telomeres by resolving secondary structure in order to allow access to replication, repair and / or telomere lengthening machinery.

Topoisomerases **and** helicases

DNA double helix that carries the genetic information undergoes various conformational changes due to the topological changes, which arise during various cellular functions. Two steps are performed to separate the 2 strands of the DNA releave the topological problems.

1. Disruption of the Hydrogen bonds between the two strands which is Performed by a set of enzymes called DNA helicases (Lohman 1993)
2. Elimination of all the topological stress created in between the two strands performed by the DNA Topoisomerases (Wang 1996)

These observations suggest that DNA helicases and topoisomerase are both required to provide the swivel mechanism for the DNA

The interaction of helicases with topoisomerase suggests cooperation between these two classes of enzymes in many aspects of DNA mechanisms. Like progression of DNA replication \fork, segregation of newly replicated chromosomes, disruption of nucleosomal structure (during transcription) DNA supercoiling and finally DNA recombination repair and genomic stability.

Progression of replication fork:

Topological problems arising at the replication fork are solved by 2 mechanisms. In the first mechanism positive supercoils arising in front of the replication fork.. In this model the helicases and topoisomerase form a part of the large replication complex and the replicating DNA is translocated through the protein complex. The helicase activity separates the 2 strands and the topoisomerase activity allow the relaxation of the positive supercoiling... In a second mechanism the DNA duplexes untangle (Chhampoux and Been 1980, Forterre *et. al.,* 1980) at the replication fork to remove the positive supercoiling at the replication fork. This is achieved by the topoisomerase II working along with the helicases bring about double stranded breaks in the DNA at the end of the replication fork following Segregation of newly replicated chromosomes.

Chromosome segregation presents a major topological problem in both prokaryotes and eukaryotes (Sudin and varsgavsky 1980). Failure to solve this problem results in breakage of chromosomes, non-disjunction, and eventually cell death (Holm *et. al.,* 1989, Uemura *et. al.,* 1987, Spell and Holm 1994), a role of topoisomerase II in the untangling of DNA has been well recognized (DiNardo *et. al.,* 1984, Adams *et. al.,* 1992). When the two replication forks meet the tracking DNA helicases approach each other and double helical turn is converted into a intertwining between two daughter strands. Topoisomerases remove these interwinings by double stranded breaking, passing and rejoining activity (Wang 1996). Thus the helicases and the topoisomerase working at the replication fork bring about chromosomal segregation. A Rec Q helicase called Sgs1 has been identified in eukaryotes, which interacts with

the Topoisomerase II and helps in chromosome segregation Pathway. Mutations in the Sgs 1 like proteins in mammals the BLM and the WRN helicases cause chromosomal breakage, translocation, and intra and inter chromosomal strand exchange.

Disruption of the nucleosomal structure.

Replication and transcription require the DNA to be accessible to the enzymatic machineries involved in these processes. The nucleosomal structure is disrupted for this purpose and reassembled after passage of a transcription complex (Bonne- Andea et. al., 1990, Adams and Workman 1993) Nucleosomal disruption occurs via the positive supercoiling produced by the helicase tracking through DNA duplex in the presence of a topoisomerase helicases locally separates the DNA strands in front of the topoisomerases. The positive supercoiling is absorbed by nucleosomal disruption, while the negative supercoiling is removed by the topoisomerases.

The removal of the nucleosome also applies to repair, transcription and recombinatioa Helicases are systematically associated with the repair and transcription complexes

Recombination and Genomic stability:

DNA helicases play an important role in genetic recombination and genomic stability. Topoisomerases may be necessary to promote recombination by their strand-tranferase activity by allowing formation of plectonemically wound recombination intermediates.

Helicases and topoisomerase plays collective role in recombination and genomic stability. A recent repot showed that helicases transform a frozen intermediate of DNA bound to topoisomerase II into a permanent double strand break *in vitro*. The helicases displace the non-covalently bound 3' ends generating a double strand break up on dissociation of topoisomerase II subunits.

Rec Q helicase and Topoisomerases:

Rec Q helicases and Topoisomerases are both required for the genomic stability, particularly to prevent promiscuous genetic recombination. A recent study suggests that these enzymes can catalyze the linking of plasmid DNA and suggests a novel mechanism for the control of recombination. The maintenance of the genomic stability is essential for cell survival. Repair mechanisms for DNA damage evolved by the cells help in eliminating potentially cytotoxic and mutagenic changes to the genomic DNA. In-appropriate execution of repair can contribute to genomic instability. Genetic recombination is one such process, which helps in removal of certain DNA lesions and faithful Meiotic division. Recombination is initiated by pairing of single stranded DNA with homologous double stranded molecule leading to strand exchange. DNA replication and transcription processes generate regions of single stranded DNA that may act as a substrate for the initiation of recombination. A mechanism involving Helicases and topoisomerase is shown to regulate the recombination processes.

Rec Q helicases are required for the initiation of homologous recombination and for the suppression of the illegitimate recombination. (Drake *et. al.,)* Which occur between DNA segments containing little or no homology.

Mutation of the type I and type II topoiosmerases result in increased rate of homologous recombination. This is due to the accumulation of torsional stress in the heavily transcribed rDNA Topoisomerase mutants display a reduced growth rate and hyper recombination, that help repeat sequences through out the genome. The recent evidence of the strand passage activity of Rec Q and Topo III provides an evidence for the functional interaction between the two classes of proteins. It was shown that Rec Q could unwind covalently closed plasmid DNA that requires a single stranded DNA region for unwinding. It was shown that Rec Q helicases could unwind covalently closed plasmid DNA that requires a single stranded DNA region for unwinding. The strand passage activity of Rec Q helicases and topo III is associated with a type II Topoisomerases that are another class of enzymes involved in regulating levels of genetic recombination. Topoisomerases catalyses the passing of the intact DNA molecules through traasient DNA breaks. These enzymes are involved in regulating levels of genetic recombination. Topoisomerases catalyses the passing of the intact DNA molecules through transient DNA breaks. These enzymes are important for adjusting the level of DNA supercoiling during the cellular processes that involve the separation of complementary strands by helicases. They are also involved in removing the knots and catenenes.

Aims of the work:

Eukaryotic Topoisomerase II present in two isoforms a and p. These two isoforms posses similar catalytic activity but their actual function is not clearly understood. Topo II a present in proliferating cells and topo II β present in all cell types Since the function of Topo II a is well characterized for its role in cell division cycle, such as G2 phase and mitotic phase (Woessner et al., 1991), while the function of Topo II P is not clearly understood. Some reports suggest that topo II β moves away from the nucleus during mitosis and shows a reticulocyte distribution, these observations suggest that topo II β may be involved in some function other than cell division. One of the possible functions of topo II β may be its association with the DNA repair activity. All cell types have the potential to undergo damage and induced for repair. Among the various tissues, the cells in the brain tissue has low frequency of replication and highly prone to DNA damage. The damaged brain cells are rescued through induction of various repair activities. Such a repair activities are very active in young brain tissue and which may diminish during aging. Hence we have chosen aging brain tissue as a model to understand how the activity of topo II isoform is associated with brain tissue during aging? Such information would be useful for further evaluation of the function of Topo II p in brain. This question was addressed in chapter 2 with the following objective.

OBJECTIVE 1: Analysis of topoisomerase 11 a and β in aging rat brain:

Further to understand whether the molecular activity of topo II isoforms is varied in aging in different tissues. We have taken up to study the sensitivity of topo II isoforms against two topo II poisons etoposide and mAMSA.this information would be useful to understand the variations in the molecular form of Topo 11 in aging not only in brain but also in other tissue. This is addressed in chapter 3 with the following objective.

OBJECTIVE 2: Monitoring the age dependent sensitivity of topoisomerase II to etoposide and mAMSA.

Our laboratory is working on developing ogranometallic compounds against topo II with a goal to achieve potential Topo II specific poisons and understanding the structural requirements of coordinated ligands for Topo II poisoning. We have synthesized six derivative of Ruthenium benzene and analyzed how them structural variation contribute to Topo II poisoning. This was addressed in chapter 4 with the following objective.

OBJECTIVE 3: Development of Ruthenium derivatives as Topo II poisons.

Chapter II

Analysis of age dependent changes of Topoisomerase II α and β in Rat brain

In this chapter we report our results of the age-associated changes of the protein and activity levels of the Topoisomerase II **a** and β isoforms in different regions of brain. The changes in the levels of Topoisomerase II at the cellular level were also studied in neurons and astrocytes from whole brain, cerebellum, and cerebral cortex.

Experimental methods:

Sample: Wister rats young (<50 gms), adult (~300 gms) and old (>400 gms) we used 5 rats for each experiment.

Preparation of tissue extracts:

Whole tissue extracts were prepared from young (<10 days), adult (~6 months), old (> 2 years) rats by sacrificing them through decapitating and collecting the brain, liver and testes separately. The tissue was homogenized in extraction buffer (20mM tris HC1 pH 7.5, 0.1mM β-mercaptoethanol, lmM $MgCl_2$, 0.1mM EDTA, 5% glycerol, 0.1% triton X-100, 0.5M KCl, 0.5mM PMSF and 1μg/μl 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.

Isolation of neurons and astrocytes

Neurons and astrocytes were isolated from brain tissue of rats that are collected through decapitating. The brains tissue was placed in isolation medium. They were cleared of blood vessels and minced well. Young rat brains were incubated with medium; adult and

old brains were incubated with medium containing 0.1% trypsin for 30 min. After incubation trypsin containing medium was decanted and 0.1% trypsin inhibitor was added and left on ice for 10 min. Tissue was passed through 103, 80 and 48μm nylon meshes and washed with isolation medium thrice. Before wash the tissue was minced well on the mesh using a flat glass rod. Filtrate was centrifuged at 800g for 15 min the supernatant is decanted and the pellet was resuspended in 7% ficoll medium and centrifuged at 300g for 10 min. The pellet containing the neurons was suspended in *2%* ficoll medium. The supernatant containing the astrocytes was diluted with 2% ficoll medium. Then centrifuged at 1100g for 10 min. Astrocyte pellet was suspended in 2% ficoll isolation medium. Both the suspensioas were centrifuged at 1500g for 10 min and the pellets are further washed with medium without ficoll followed by washing with **PBS.** The final pellet containing the neurons and the astrocytes was suspended in appropriate volume of extraction buffer vortexed and was kept at -20°C overnight. To make lysates, the suspensions were sonicated and spinned down at l,00,000g for 1 hour. Purity of neurons was conformed by immunostaing with NSE and astrocyted with GFAP.

Immunoprecipitation of Topoisomerase II isoforms.

Brain extracts was prepared from whole brain, cerebellum, cerebral cortex and midbrain regions of brain from 5 separate animals of the young, adult and old age groups were taken in separate eppendorffs for immunoprecipitation of Topoisomerase II **a** and β with respective monoclonal antibodies (1:1000 dilution in IP buffer containing 100mM tris HCl pH 8, 750mM NaCl, 2mM EDTA, lmM 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, and the beads are spinned down at 1000 rpm for 10 minutes and the supernatant was removed. The protein A agarose beads were washed with 0.5% triton X. The beads were directly used for monitoring the relaxation activity topoisomerase II captured by immunoprecipitation. Each experiment was repeated 3 times to verify reproducibility of the results.

DNA relaxation assay

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 forms of plasmid DNA (form R) appears. ~ 0.6µg of supercoiled plasmid DNA is incubated with the immuopecipitated Topoisomerase II captured on to the Protein A agarose beads in relaxation buffer (50mM tris HC1 pH 8.0,120mM KC1, 0.5mM EDTA, 0.5mM DTT, 10mM $MgCl_2$, 30µg/ml BSA, lmM ATP) for 30 min. The beads were spinned down and the supernatants were collected separately. The reaction was stopped by adding 2 µl of 10% SDS and the DNA products were resolved on 1% agarose gel stained with ethidium bromide and photographed.

Immunoblotting analysis.

75µg of total protein from the extracts were electrophoresed on a 7.5 % SDS PAGE and transferred on to PVDF membrane for immunoblotting analysis. Blot was incubated with 5% NFDM in TBS (lOmM tris and 15mM NaCl) to block the non-epitopic binding. The membrane was thoroughly washed using TBS containing 0.15% Tween 20 and incubated

with Topoisomerase II a or Topoisomerase II β monoclonal antibodies (1:1000 dilution in TBS) for 30 min at room temperature. The membrane was washed thrice and incubated with the Alkaline phosphatase conjugated Anti mouse IgG anti body (1:2000 dilution in TBS) for 60 minutes at room temperature and washed with TBS containg 0.15 % tween. The blots are developed using NBT- BCIP substrates in TBS. The protein levels were normalized with beta-actin controls.

Phosphorylation of Topoisomerase II

Topoisomerase II (3 from 50-µg total proteins of whole brain and cerebellum extracts was phosphorylated with 5 µCi of γ^{32}P-ATP in 1x kinase buffer (10% glycerol 0.1M EDTA and 50 mM $Mgcl_2$) the mixture is incubated at 37°C for 1 hour. Topo II (3 antibody was added to the above mixture. After incubation at 37°C for 30 minutes, 20 µl of protein A agarose was added. The lysates were incubated at 4°C for 15 minutes with intermittent shaking, then centrifuged at 2000 rpm. The sediment was washed twice with PBS and eluted with 20 µl of 5% trichloroacetic acid (TCA). 10 µi of eluate was spotted on Whatman no.1 filter paper discs and ^{32}P was measured with a Wallac 1400 DSA scintillation counter. Each experiment was in triplicate and all data points represent an average of results from triplicate experiments.

Immunoflourescence.

The cerebellar regions of young adult and old rats, cerebral cortex and midbrain of young rats were dissected out from brains of Westar strain of rats and placed in medium containing 10mM potassium phosphate, 10 % glucose and 8% fructose. Frozen sections

~4 μM of the cerebellum from the three different age groups were taken in a cryostat and mounted on glass slides. The sections were fixed by acetone treatment for 1 hour. The sections were treated with ethanol for 10' followed by washes with double distilled water and PBS. Then the sections were treated with 0.3% triton X 100 in PBS for 3'. The nonspecific sites on the sections were blocked with 1% BSA in PBS and incubated for 1 hour at room temperature. After washing with PBS twice, the sections were then incubated with Topoisomerase II, neuronal markers and isotopic control antibodies diluted in PBS containing 1% BSA for one hour and then washed with PBS twice with PBS. The sections were then incubated with secondary antibodies (FITC conjugated goat anti mouse IgG secondary antibodies for Topo II and fluorescence red conjugated goat anti rabbit IgG secondary antibodies for markers) for one hour and washed with PBS twice. The isolated cells also stained with same antibodies, the slides were then observed under fluorescence microscope and photographed.

Double immunofluorescence staining:

Neuronal cells and astrocytes were isolated from the young, adult and old rat cerebellum (protocol: as mentioned earlier). Cells were washed in phosphate buffer saline (PBS) at 400xg for 5 minutes. Pellet was resuspended in PBS (5x 10^6 cells/ml). 15 μl were applied to each adhesion slide. Then cells were fixed with ethanol incubated for 20 minutes. The slides were washed three times with PBS contains 0.3 % triton X-100. Blocked with *2%* fetal bovine serum for 10 minutes, washed thrice with PBS contains 0.1% tween-20. Then incubated with Topo II **a** and (3: monoclonal antibodies for 1 hour. Washed thrice with PBS. Then incubated with NSE monoclonal antibodies (goat anti

rabbit IgG) for neuronal cells and GFAP (goat anti rabbit IgG) for astrocytes incubated for 1 hour at room temperature. Washed thrice with PBS. Then incubated with FITC conjugated secondary antibodies for Topo II **a** and (3 incubated for 30 minutes at room temperature, washed thrice with PBS and incubated with fluorescence-red conjugated secondary antibodies (anti rabbit) for NSE and GFAP for 30 minutes. Washed thrice with PBS. Then slides were observed under fluorescent microscope and taken photographs

Sub- cellular localization of Topo II a and β:

Neuronal cells were isolated from the cerebellum of young rat (1 day old) pups (protocol: as mentioned earlier). Five million cells were taken; these cells were washed with ice-cold phosphate buffer saline at 1500 rpm for 10 minutes. Supernatant was discarded and the pellet was resuspended in 10 volumes of Dounce buffer (10mM Tris- Hcl, 1.5 mM Mgcl2, 10mM Kcl, 0.2mM PMSF, 0.5 mM DTT.), kept on ice for 10 minutes, then homogenized with Dounce homogenizer and centrifuged at 1500 rpm for 10 minutes. Pellet contains nuclear proteins and supernatant contains cytosolic proteins. Then followed western blotting with corresponding antibodies. We have used actin antibodies for cytosolic and tubulin antibodies for nuclear fractions as internal controls.

Results:

The levels of the two isoforms of Topoisomerase II were analyzed in whole brain, and three regions of brain viz. cerebellum, cerebral cortex and midbrain. The levels of this enzyme were also mentioned in the brain cells *viz* neurons and astrocytes.

The protein levels of the Topoisomerase II a and (3 were analyzed by relaxation assays performed using immunoprecipitated enzyme and immunoblotting analysis of the Protein resolved by SDS-PAGE analysis.

The results observed from the experiments conducted using the extracts made from brain tissues is presented here under.

Topoisomerase II in whole brain:

Whole brain extracts were prepared as mentioned above. Relaxation assays were performed using immuno-precipitated Topoisomerase II a and β from whole brain extracts of young adult and old rats. The results showed varying levels of both the isoforms. Topoisomerase II a activity was found to be low in all the three age groups of rats (figure 1 panel A), where as the (3 isoform showed significant relaxation of the supercoiled plasmid DNA (Figure 1 panel B). The relaxation activity of the Topoisomerase II (3 was found to be maximum in the young brain extracts and decreased with age with lowest activity in the old rats brain extracts. There is an age dependent decrease in the Topoisomerase II (3 activity in the whole brain extracts.

Immunoblotting analysis performed using monoclonal antibodies for both the isoforms of Topoisomerase II showed minimal protein levels for the Topoisomerase II a (figure 2 panel A) in all the three age groups of rats. While the Topoisomearse II β levels were

found to be higher in the brain extracts. In accordance with the observations in the relaxation assays the Topoisomerase II |3 levels were found to be highest in the young brain extracts then a decrease was seen with increasing age with the lowest protein is observed in the old rats. (Figure 2 Panel B). The loading control for the total protein was verified by immunoblotting for actin protein in the total bain extracts (Fig 2 panel c)

To check whether the Topoisomerase II isofoms possess a region specific expression, the above experiments were carried out with extracts made from 3 regions of brain viz. cerebellum, cerebral cortex and midbrain.

Topoisomerase II in cerebellum.

The experiments were performed with extracts prepared from the cerebellar region of brain of young adult and old rats. The results observed from DNA relaxation experiments shows that the activity of topoisomerase II a isoform is low (figure 3 panel A). The activity of topoII a shows a marginal change in all the three age groups. There was no significant age dependent change in activity of Topoisomerase II a in cerebellum. This correlates with the low protein level of topoisomerase II a in young and adult cerebellum, though disappear in old rat cerebellum was observed in the western blot analysis (Fig 4 panel A). These observations are in correlation with the results observed in the whole brain extracts showing that there is low topoisomerase II a in brain and cerebellum. While topoisomerase II β isoform in the cerebellum showed very high DNA relaxation activity (figure 3 panel B). The western blot analysis showed that cerebellar region is rich in topoisomerase II (3 isoform (figure 4 panel B). The activity of β isoform in the three age groups showed slight variation, with the maximum activity shown by the

extracts made from the young rats. The enzymatic activity decreased with age, the old rat cerebellar extracts showed the minimal relaxation activity.

The western blot analysis of Topoisomerase II β protein level correlate well with the catalytic activity of the enzyme (Figure 4 panel B). The young cerebellar extracts showed a higher level of the protein where as the adult cerebellum and old cerebellum showed decrease in the protein level.

Topoisomerase II in cerebral cortex:

Cerebral cortex extracts were prepared with same procedure as followed with whole brain and the cerebellum. These extracts were used for the immnoprecipitation of either of the isoforms of the topoisomerase II. The relaxation activity of the topoisomerase II a (figure 5 panel A) is significant in the cerebral cortex region of brain of all the three age groups viz. young adult and old. Further the topoisomerase II a activity increased with aging. The Topo II a protein level was shown in fig 6, panel A by the western blot analysis (figure 6 panel A) in the topoisomerase II a in cerebral cortex. The results of protein level was show low, the activity increase that we observed, could be due to increase in active form of enzyme through phosphorylation.

The relaxation activity with the topoisomerase II β in young rats cerebral cortex (figure 5 panel B) showed very low enzymatic activity, while in adult and old rats we observed marginal increase in DNA supercoiling, the increase in supercoiling could be due to some modifications of the enzyme in adult and old rats. Hence the DNA relaxation activity of the enzyme was low in the cerebral cortex extracts, of all the three age groups viz.the young adult and old rats. The western blot analysis (figure 6 panel B) of the

Topoisomerase II β protein in cerebral cortex show in all the three age groups was low. Young rat cerebral cortex shows slightly higher protein level compared to adult and old. These observations were compared well with that of the enzymatic activity. Hence Topo II **a** activity in cerebral cortex is significant, while Topo II (3 shows a moderate activity.

Topoisomerase II in midbrain

Topoisomerase II isoforms **a** and β were analyzed in the midbrain extracts prepared from the young, adult and old rats. Relaxation assay performed with the immunoprecipitated topoisomerase II **a** and β (figure 7 panel A and B) revealed that both enzymes are found to be in low levels in the midbrain tissue of the rat brain. Young rat midbrain shows significant activity, while adult and old rat midbrain do not shows appreciable activity. The protein levels of Topoisomerase II α and β was also analyzed using Western blotting analysis (figure 8 panel A and B).

Statistical analysis of the enzymatic activity of Topoisomerase II a isoform:

Test of significance of similarity was conducted between densities of supercoiled DNA in control with that of Young, Adult and Old groups. In the first step Analysis of variance test (ANOVA), was conducted to find if the "mean" of Control, Young, Adult and Old are similar or different. The results of ANOVA (Snedecor, 1934) shows that the Topo II **a** activity in whole brain and mid brain are similar between control and other groups, while Topo II β activity in cerebellum and cerebral cortex is different between control and other groups (table 1). To test for significance of difference between supercoiled DNA in the control and that in Young, Adult and Old we conducted the Dunnet's test

(Dunnett 1955) for comparison of "mean" of control with experimental group. Dunnet's analysis shows that only cerebral cortex exhibits a significant difference of Topo II **a** between control "mean" and the "mean" of Young, adult and old, where as in other regions, there is similarity between the control "mean" with that of any two regions. Based on the above statistical analysis we conclude that cerebral cortex possess significant and distinct Topo II a activity. To understand the age dependent changes of Topo II a we have further analyzed relation between age and Topo II a activity in cerebral cortex using linear regression. The results of the regression analysis were reported in table two suggests that Topo II a activity significantly increased in cerebral cortex with age and population regression is linear.

Statistical analysis of the enzymatic activity of Topoisomerase 11 (3 isoform:

Topo II (3 activity in different regions of brain in young, adult and old rats was analyzed using statistical methods, the tests were conducted to know whether there is any significant difference in the activity of Topo II (3 between age groups. Analysis of variance test (ANOVA) was conducted to find significance of similarity between the supercoiled DNA in control and with that young, adult and old. The result shows that TopoII (3 activity is significantly different from control in whole brain, cerebellum and cerebral cortex, while it is same in midbrain. The results shows that TopoII (3 in whole brain and cerebellum is significantly different from that in the control in all groups, where as in TopoII |3 in cerebral cortex and midbrain is same as that in the control. To analyze the age-dependent changes of Topo II β activity in whole brain and cerebellum we have conducted linear regression. Linear regression analysis of Topo II β in whole

brain and cerebellum in relation to the age is –0.148 and –0.045 respectively. Analysis of variance of linear regression between the groups suggests that the decreasing TopoII (3 activity with age in whole brain and cerebellum is significant. The analysis of population regression suggests that population regression of Topo II β activity in whole brain and cerebellum in not linear. This non-linearity could be due to the higher activity of Topo II |3 in these regions.

Cellular levels of Topoisomerase II β in brain:

Topoisomearse II (3 was analyzed in two different types of cells of the brain tissue namely neurons and astrocyrtes. The purity of the isolated neurons and astrocytes confirmed using corresponding antibodies, GFAP for astrocytes and NSE (neuronal specific enolase) for neurons. The immunoprecipitated topoisomerase II β from whole brain, cerebellar and cerebral cortex neuronal extracts was analyzed for the DNA relaxation activity of the enzyme The results in the Figure 9 shows that the neuronal extracts from whole brain (lanes 3-5) and cerebellum (lanes 6-8) contain high activity of topoisomerase II (3 in terms of relaxation of the plasmid DNA. The relaxation activity of the cerebellar neuronal extracts is found to be more when compared to whole brain and in contrast to this the neuronal extracts of the cerebral cortex (Figure 9 panel B) did not possess topoisomerase II |3 activity indicating very low or negligible amounts of Topoisomerase II (3 in neurons of cerebral cortex. The DNA relaxation activity observed in the whole brain and cerebellar neurons showed a decrease in enzymatic activity with increasing age, the lowest activity is seen in whole brain and cerebellar neurons of old age group rats.

Analysis of phosphorylation of TopoII β in brain extracts.

To know whether the phosphorylated status of the protein can be correlated with the enzymatic activity TopoII (3 phosphorylation was analyzed in whole brain and cerebellum extracts of young adult and old rats. The results of these experiments show that topoisomerase II (3 phosphorylation is higher in whole brain and cerebellum extracts of young rats (Figure 10) when compared to the adult and old rats suggesting that the activity of topo II (3 may be regulated through phosphorylation in cerebellum. Similar analysis in astrocytes showed no activity of both topo II **a** and topo II |3 in all the age groups (data not shown),

Immunohistochemical analysis of Topoisomerase II a and (3

The Immunohistochemical analysis was carried out to confirm the results obtained by the previous studies. The analysis carried out in the sections obtained from cerebellum (Young, adult and old) cerebral cortex (young), midbrain (young). The Topoisomerase II **a** analyzed in the cerebellum sections of the three age groups showed very low amounts of immunoflourescence (figure 11), while the topoisomerase II (3 showed very high amounts of immunoflourescence in the cerebellum sections, the fluorescence was shown to decrease with increasing age, highest amount of fluorescence seen in the young rat cerebellar section (figure 12). The isotopic controls were carried out to confirm that there is no non-specific binding of the Topoisomerase II monoclonal antibodies (Figl6). The cerebral cortex and the midbrain sections analyzed using topoisomerase II **a** antibody showed very low amount of immunofluorescence (Figure 13). The analysis of topo II (3 immunoflourescence in the neurons isolated from cerebellum of young, adult and old

rats showed highest amount of Topo II (3 **in** the young cerebellar neurons (figure 14). The neurons were analyzed for the standard neuronal markers to confirm that the cell type taken for TopoII |3 analysis was neurons (Fig 15 A). Though some slides were developed with both the antibodies (figure 17), we could not take dual fluorescent photographs due to lack of such facility.

Sub cellular localization of Topoisomerase II

Topoisomerase II **a** and β were analyzed in the nuclear and cytosolic fractions of cerebellar neurons. The nuclear fractions show higher protein level than the cytosolic fraction (fig 19 A and B), which confirms the fact that topoisomerase II isoforms are nuclear enzymes.

Topo II a and β in astrocytes:

The immunofluorescence analysis of Topo II **a** and β in aslrocytes shows that both are present in negligible levels (figure 18). This data was supported by western blot analysis (figure 20)

Discussion:

In the present investigation we showed that Topo II β isoform is predominant in rat brain tissue especially in cerebellum, while lower activity of Topo II a is exhibited in cerebral cortex. The Topo IIβ activity in whole brain and cerebellum showed an age-dependent decrease, while the activity of Topo II a isoform showed an age-dependent increase in cerebral cortex. These findings suggest the involvement of TopoII β isoform in some functions of cerebellum, while Topo 11 a may be involved in some functions of cerebral cortex. Since sub-cellular localization studies showed TopoII a and β associated with nuclei, the activity of en/.yme may be associated with genetic rearrangements in nuclei. The studies of Tsutsui et al (1993) on TopoII isoform in developing rat brain showed that TopoII a transcript is higher in developing rat brain embryos day 10 to 2 day new-born, which rapidly decrease Lifter 4th week. TopoII β transcript was present in through out embryonic and post-natal stages. TopoII a was absent in outer mitotic zone of the external granule layer, while TopoII [3 isoform was detected in entire cortical region. Terminally differentiated neuronal nuclei were shown to exhibit continuous TopoII β expression, while the activity of DNA polymerase alpha decrease in a negligible level (Tutsui et al 1986). Transgenic studies of Xia yang et al (2000) using Topo II p deficient mouse model showed that mouse fail to develop and die with severe neuronal dysfunction, suggesting that Topo II β is essential for survival and function of brain. The presence of higher level of Topo II β in non-proliferating cells was also reported by Hadlaczky et al 1988, Roca et al 1989. Holden et al 1990. Nuclear DNA Topo II levels in quail oviduct was shown to enhance by 70% with age (Schroder et al 1989,1990). They

also showed that TopoII activity in quail oviduct nuclei was significantly increased with age through posttranslational modification of enzyme by matrix bound poly (ADP - ribose) synthase and protein kinase. These observations suggest that TopoII activity may be regulated through modification of enzyme; through phosphorylation or ADP riboxylation etc. changes in rate of dephosphorylation of Topo II (3 and Topo II a would affect the activity of Topo II isoforms during development and function of brain. These findings suggest that Topo II a involved in DNA replication or related activity during cell division cycle (Ishemiel et al 1992), (Uemurall et al 1987), while TopoII β may play a role in recombination and DNA repair (Zum et al 1992). Though Topoisomerase II is shown to be involved in DNA repair activity but its exact molecular function is not clearly understood. Large number of studies of DNA repair activity of Topo II was based on action of Topo II poisons. It would be interesting to study the exact function of TopoII in DNA repair. Brain is one of the tissue in which large number of cells are terminally differentiated and damage associated DNA repair activity is high. In ageing brain tissue the DNA repair ability of cells known to diminish, leading to accumulation of un-repaired lesions during aging. Various neurodegenerative diseases are implicated due to defects in DNA repair pathways. Hence it is important to study molecular markers associated with brain ageing that have potential role in the DNA repair status that would be helpful to maintain cellilar-ageing process. Hence we propose whether Topo II (3 in cerebellum and Topo II a in cerebral cortex can be further investigated for them use as potential markers. Also it would be interesting to identify the exact function of Topo II β in cerebellum and Topo II a in cerebral cortex.

Enhancement of rate of dephosphorylation of Topo II β and a isoforms would affect the enzymatic activity thus impairs cellular development and function of brain, suggesting that Topo II phosphorylation associated activity has important role in development. It needs to be studied whether decrease in Topo II β activity is associated with diminishing some Topo II β associated DNA repair activity. Since Topo II a predominantly associated with proliferating cells, it would be important to understand the significance of Topo II a in cerebral cortex, where any mechanism of neuronal precursor cells replenishment or cell development is associated with activity of Topo II a in cerebral cortex. to replace damaged cells.

Figure 1

Relaxation activity of topoisomerase II a and (3 from brain extracts of young adult and old rats. Panel A and B show relaxation activity of immunoprecipitated topoisomerase II a and β from 100 μg total protein of young (lanes3-7). adult (lanes 8-12) and old (lanes13-18) rats captured on to Protein A agarose beads was incubated with ~0.6 μg of pRYG plasmid DNA in presence of relaxation buffer. Lane 1 shows ~0.6 μg of pRYG plasmid DNA and lane 2 shows DNA with 2 units of Topo II. The super coiled DNA was quantified and the bar diagrams are shown in the figure.

FIGURE 1

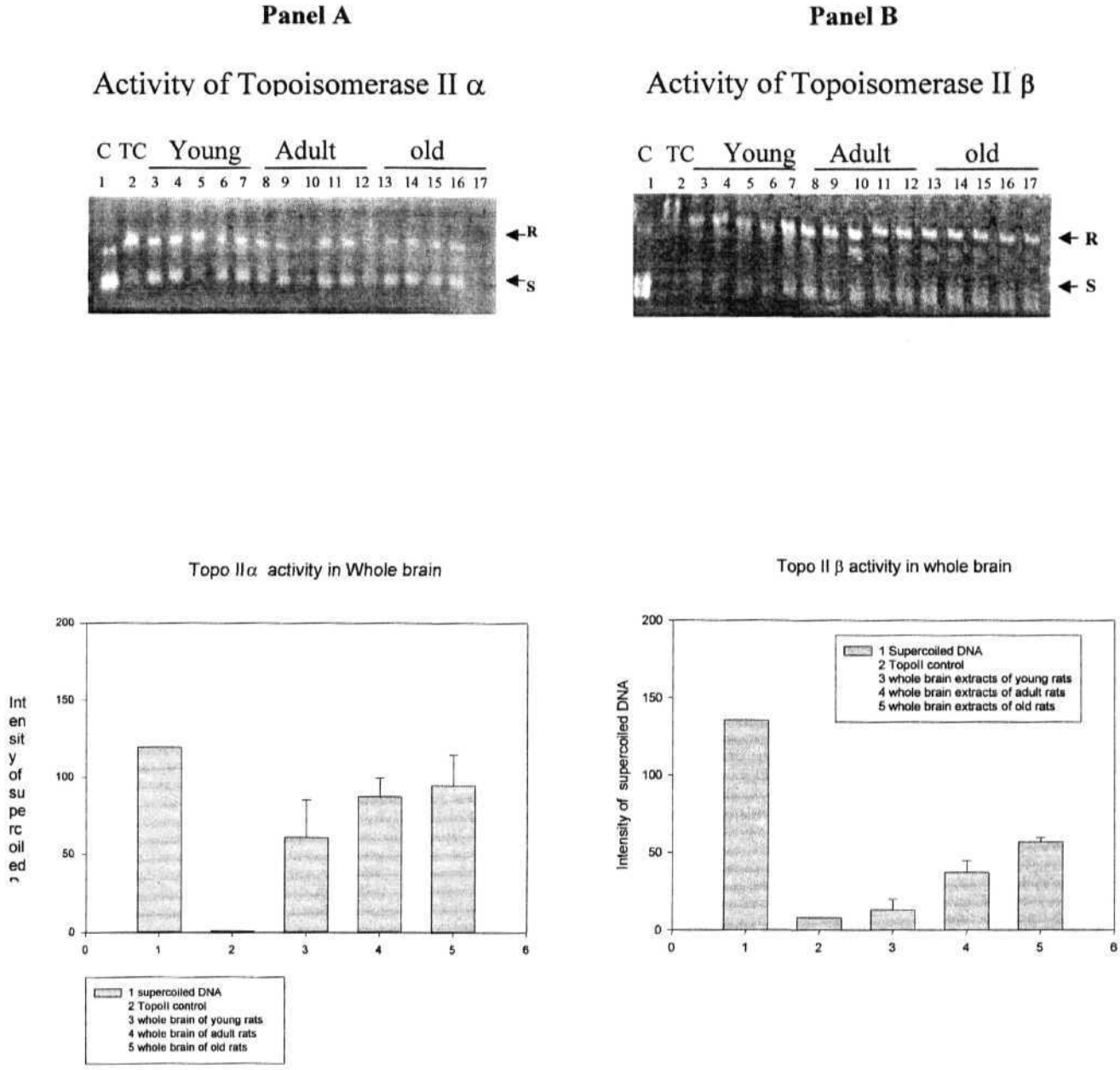

Panel A
Activity of Topoisomerase II α
C TC Young Adult old
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
R
S
Panel B
Activity of Topoisomerase II β
C TC Young Adult old
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
R
S
Topo IIα activity in Whole brain
Intensity of supercoiled
200
150
100
50
0
0 1 2 3 4 5 6
1 supercoiled DNA
2 TopoII control
3 whole brain of young rats
4 whole brain of adult rats
5 whole brain of old rats
Topo II β activity in whole brain
Intensity of supercoiled DNA
200
150
100
50
0
0 1 2 3 4 5 6
1 Supercoiled DNA
2 TopoII control
3 whole brain extracts of young rats
4 whole brain extracts of adult rats
5 whole brain extracts of old rats

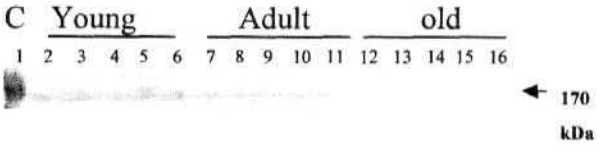
Figure 2

Immunoblotting analyses of topoisomerase II a and (3 from whole brain extracts of young adult and old rats with Topo II a and (3 antibodies. Panel A and B show Immunoblots of TopoII a and (3 from 75 μg total protein of young (lanes2-6), adult (lanes 7-11) and old (lanes12-16) rats. Lane 1 shows Topo II control with 25μg. panel C shows actin control in whole brain.

FIGURE 2

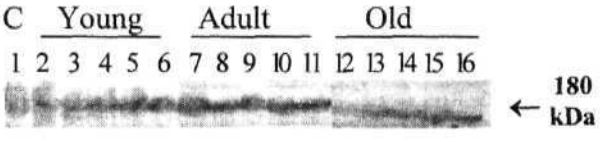
A

Topoisomerase II a in whole brain



B

Topoisomerase II β in whole brain



C

Actin control in whole brain

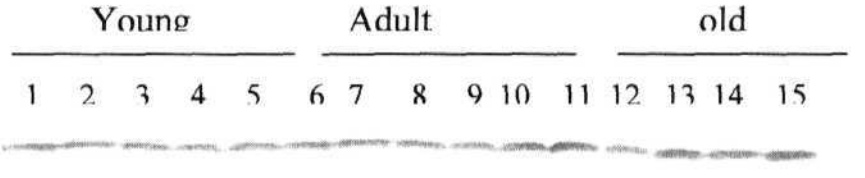


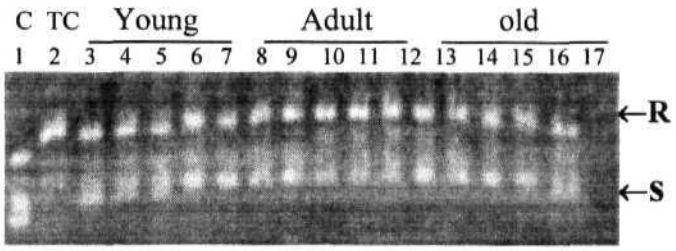
Figure 3

Relaxation activity of topoisomerase II a and |3 from cerebellum extracts of young adult and old rats.: Panel A and B show relaxation activity of immunoprecipitated topoisomerase II a and β from 100 μg total protein of young (lanes3-7).adult (lanes 8-12) and old (lanes13-18) rats captured on to Protein A agarose beads was incubated with ~0.6 μg of pRYG plasmid DNA in presence of relaxation buffer . Lane 1 shows ~0.6 μg of pRYG plasmid DNA and lane 2 shows DNA with 2 units of Topo II. The super coiled DNA was quantified and the bar diagrams are shown in the figure.

FIGURE 3

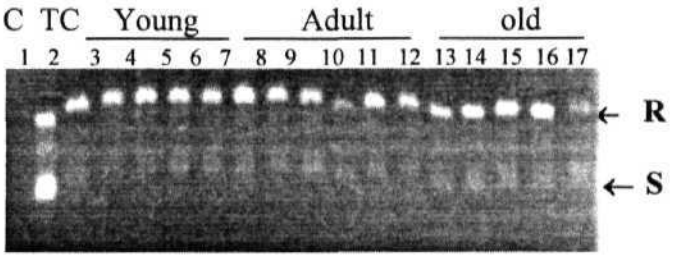
Panel A

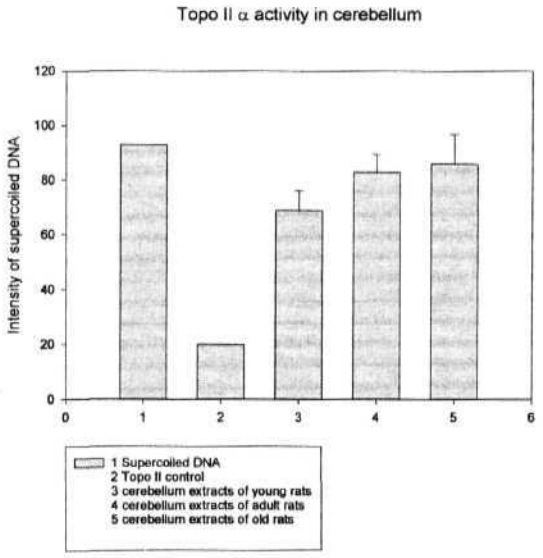
Topoisomerase II a activity



Panel B

Topoisomerase II β activity





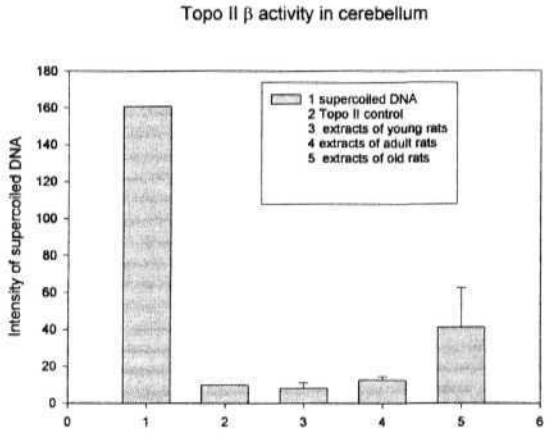


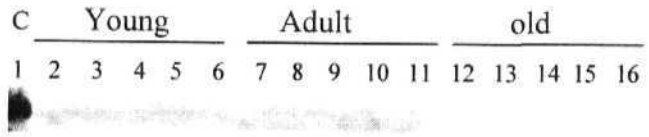
Figure 4

Immunoblotting analyses of topoisomerase II a and β from cerebellum extracts of young adult and old rats with Topo II a and (3 antibodies. Panel A and B shows Immunoblots of Topoll α and β from 75 μg total protein of young (lanes2-6), adult (lanes 7-11) and old (lanes12-16) rats. Lane 1 shows Topo II control with 25μg. panel C shows actin control in cerebellum.

FIGURE 4

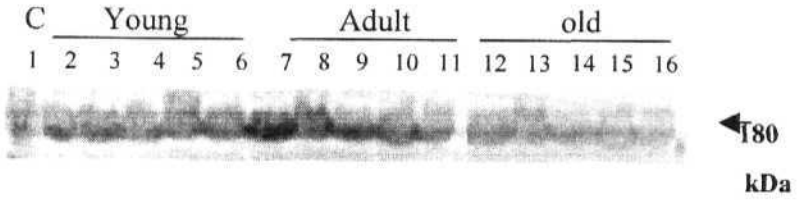
A

Topoisomerase II a in cerebellum



B

Topoisomerase IIβ in cerebellum



C

Actin control in cerebellum

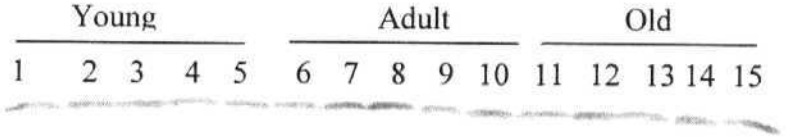


Figure 5

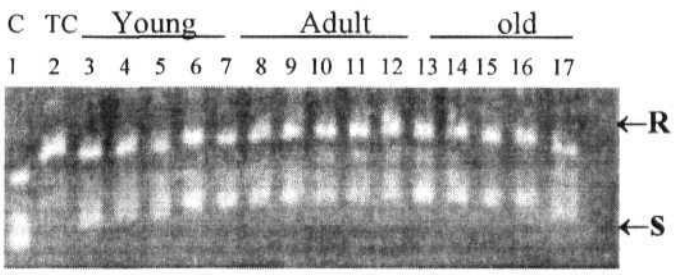
Relaxation activity of topoisomerase II a and β from cerebral cortex extracts of young adult and old rats.: Panel A and B show relaxation activity of immunoprecipitated topoisomerase II a and β from 100 μg total protein of young (lanes3-7).adult (lanes 8-12) and old (lanes 13-18) rats captured on to Protein A agarose beads was incubated with ~0.6 μg of pRYG plasmid DNA in presence of relaxation buffer . Lane 1 shows ~0.6 μg of pRYG plasmid DNA and lane 2 shows DNA with 2 units of Topo II. The super coiled DNA was quantified and the bar diagrams are shown in the figure.

FIGURE 5

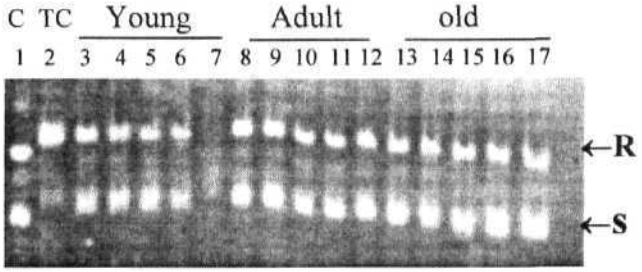
Panel A

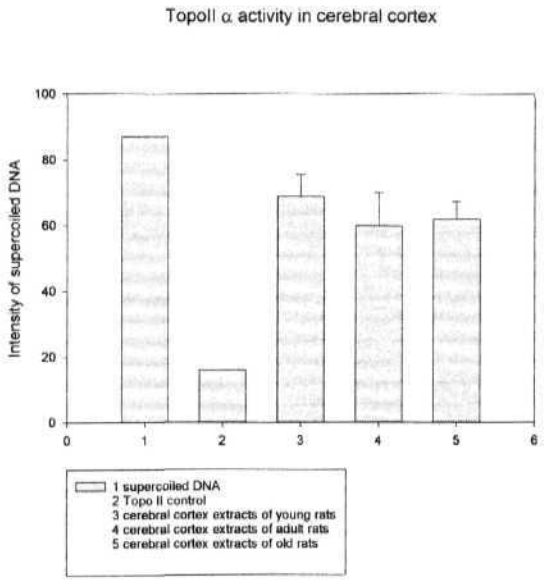
Panel B

Topoisomerase II a activity



Topoisomerase II p activity





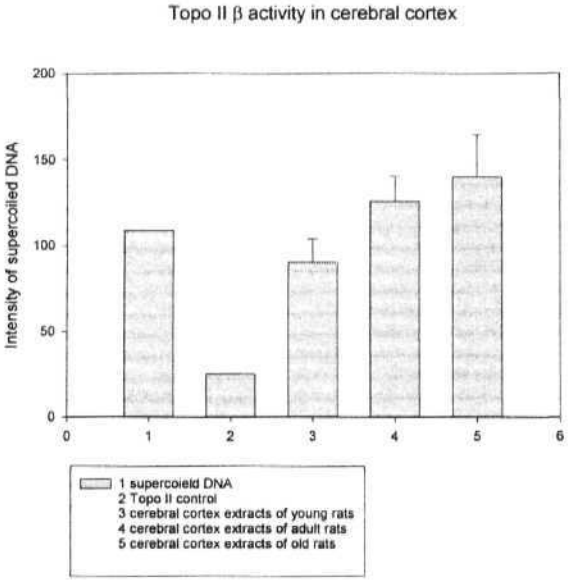


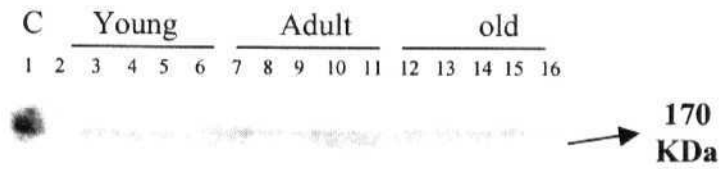
Figure 6

Immunoblotting analyses of topoisomerase II a and β from cerebral cortex extracts of young adult and old rats with Topo II a and β antibodies. Panel A and B show Immunoblots of TopoII a and β from 75 μg total protein of young (lanes2-6), adult (lanes 7-11) and old (lanes 12-16) rats. Lane 1 shows Topo II control with 25μg. panel C shows actin control in cerebral cortex.

FIGURE 6

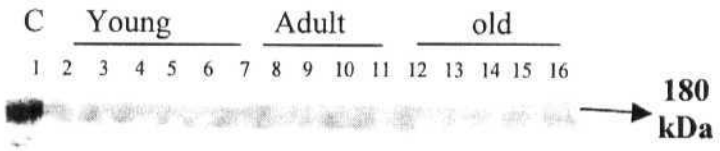
A

Topoisomerase II α in cerebral cortex



B

Topoisomerase II β in cerebral cortex



C

Actin control in cerebral cortex

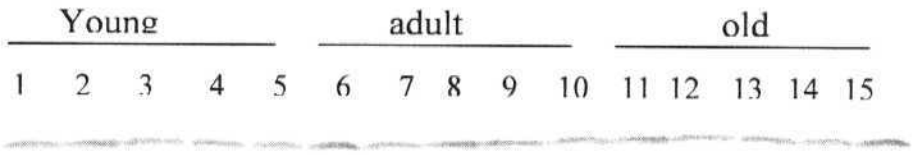


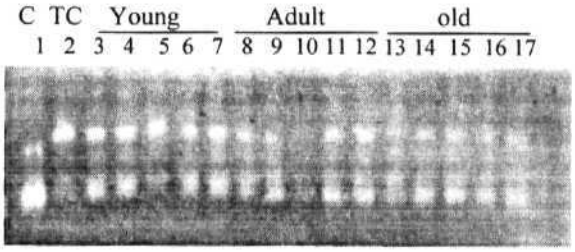
Figure 7

Relaxation activity of topoisomerase II a and β from midbrain extracts of young adult and old rats. Panel A and B show relaxation activity of irnmunoprecipitated topoisomerase II a and β from 100 μg total protein of young (lanes3-7). adult (lanes 8-12) and old (lanes 13-18) rats captured on to Protein A agarose beads was incubated with ~0.6 μg of pRŸG plasmid DNA in presence of relaxation buffer . Lane 1 shows ~0.6 μg of pRYG plasmid **DNA** and lane 2 shows **DNA with** 2 units of Topo II. The super coiled DNA was quantified and the bar diagrams are shown in the figure.

FIGURE 7

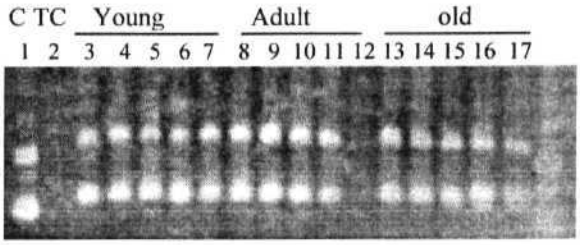
Panel A

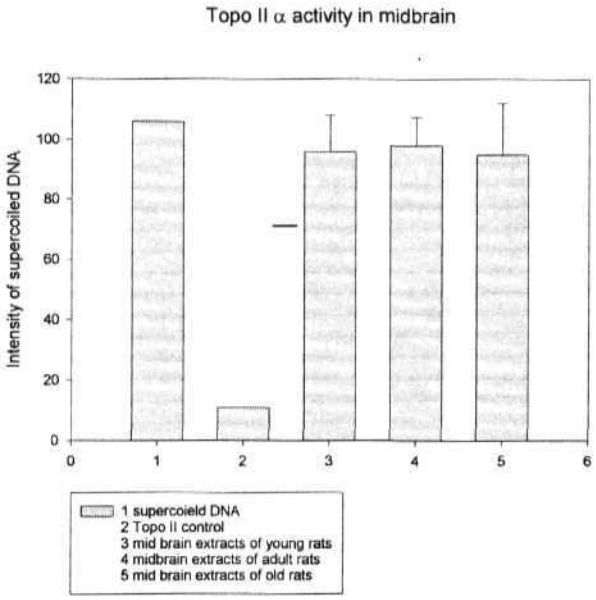
Topoisomerase II a activity



Panel B

Topoisomerase II β activity





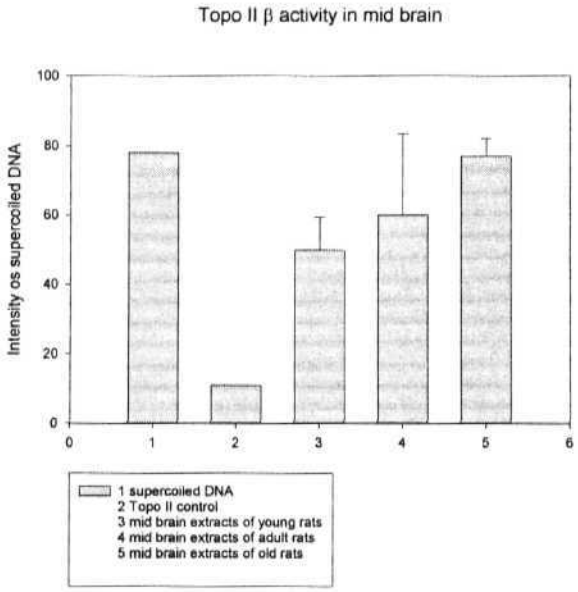


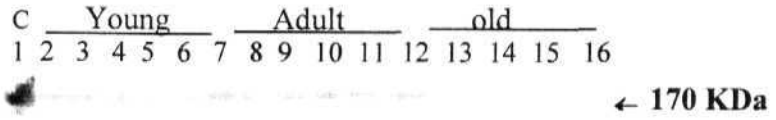
Figure 8

Immunoblotting analyses of topoisomerase II a and β from midbrain extracts of young adult and old rats with Topo II α and β antibodies. Panel A and B show Immunoblots of TopoII a and β from 75 μg total protein of young (lanes2-6), adult (lanes 7-11) and old (lanes12-16) rats. Lane 1 shows Topo II control with 25μg. panel C shows actin control in mid brain.

Figure 8

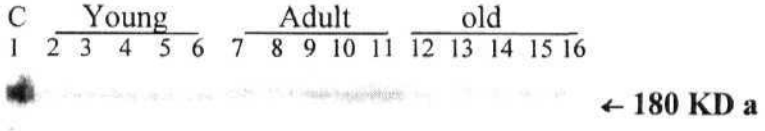
A

Topoisomerase II a in mid brain



B

Topoisomerase II β in midbrain



C

Actin control in midbrain

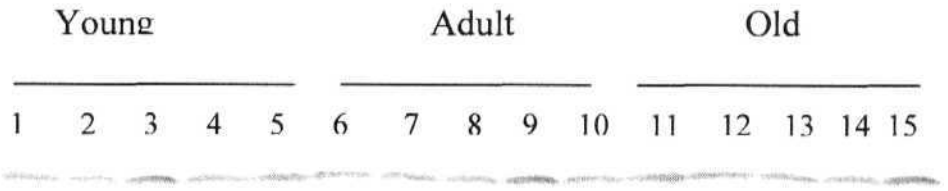


Figure 9

Relaxation activity of Topoisomerase II β from neuronal cells. Topoisomerase IIβ was immunoprecipitated 100μg total protein of neuronal extracts prepared from isolated neurons of whole brain (panel A lanes 3-4). Cerebellum (panel A lanes 6-8) and cerebral cortex (panel B lanes 3-5) of young, adult and old rats and captured onto protein A agarose beads. These beads were incubated with ~0.6μg of pRYG plasmid DNA in presence of relaxation buffer. The products were resolved on 1% agarose gel stained with ethidium bromide and visualized under UV light and photographed. The supercoiled DNA was quantified and the bar diagrams are shown in the figure.

Figure 9

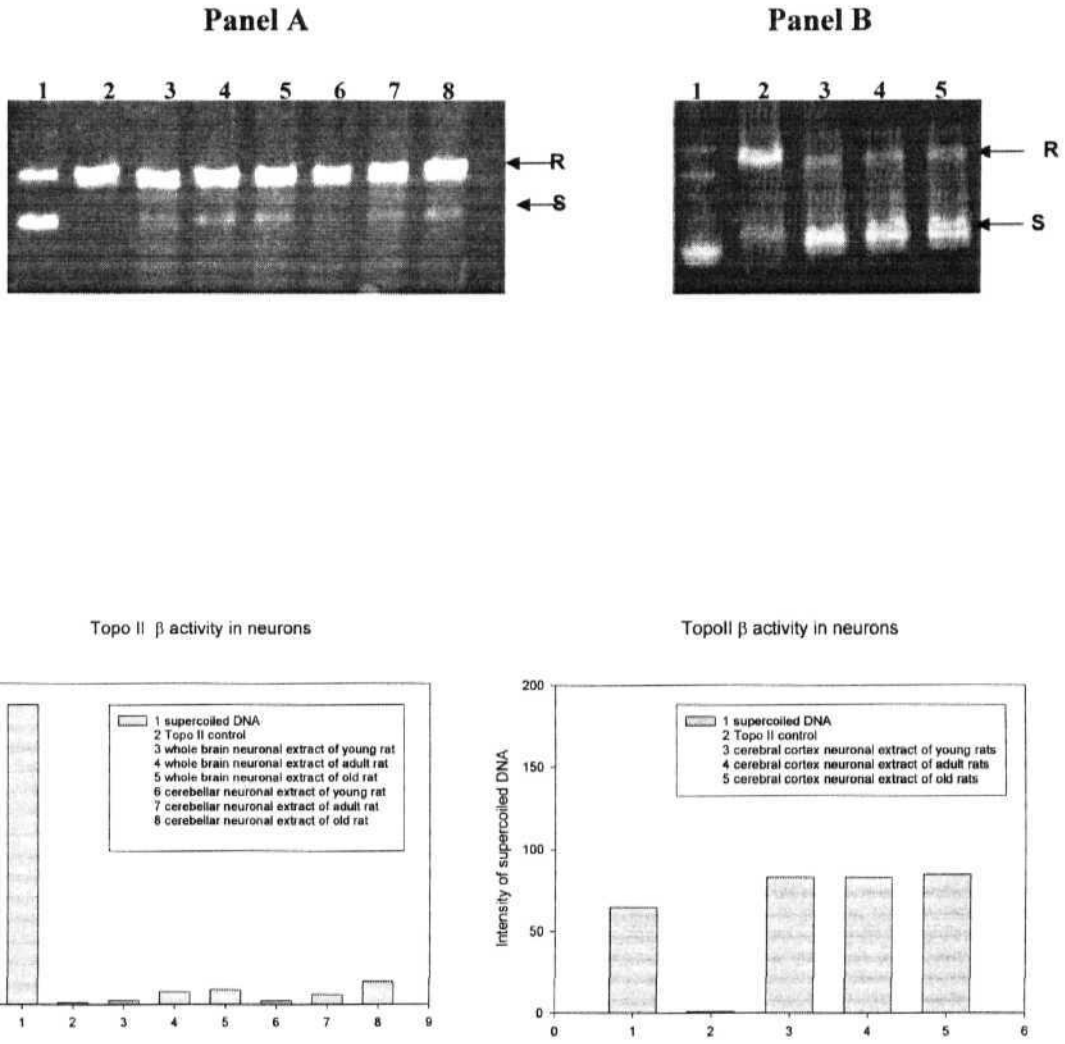

Panel A
1 2 3 4 5 6 7 8
R
S
Panel B
1 2 3 4 5
R
S
Topo II β activity in neurons
Intensity of supercoiled DNA
1 supercoiled DNA
2 Topo II control
3 whole brain neuronal extract of young rat
4 whole brain neuronal extract of adult rat
5 whole brain neuronal extract of old rat
6 cerebellar neuronal extract of young rat
7 cerebellar neuronal extract of adult rat
8 cerebellar neuronal extract of old rat
TopoII β activity in neurons
Intensity of supercoiled DNA
1 supercoiled DNA
2 Topo II control
3 cerebral cortex neuronal extract of young rats
4 cerebral cortex neuronal extract of adult rats
5 cerebral cortex neuronal extract of old rats

Figure 10

Phosphorylation of Topoisomerase IIβ in brain extracts:

Topo IIβ from 50μg total protein of whole brain (lane 2-4), and cerebellum (lanes 5-7) of young, adult and old rats were phosphorylated with 32P ATP in presence of kinase buffer. 25μg of Topo II (lane 1) was used as a control. The phosphorylated enzyme captured on to the protein A agarose beads was TCA eluted and spotted on to whatman filter papers and the radioactivity was counted using a Wallace scintillation counter

FIGURE 10

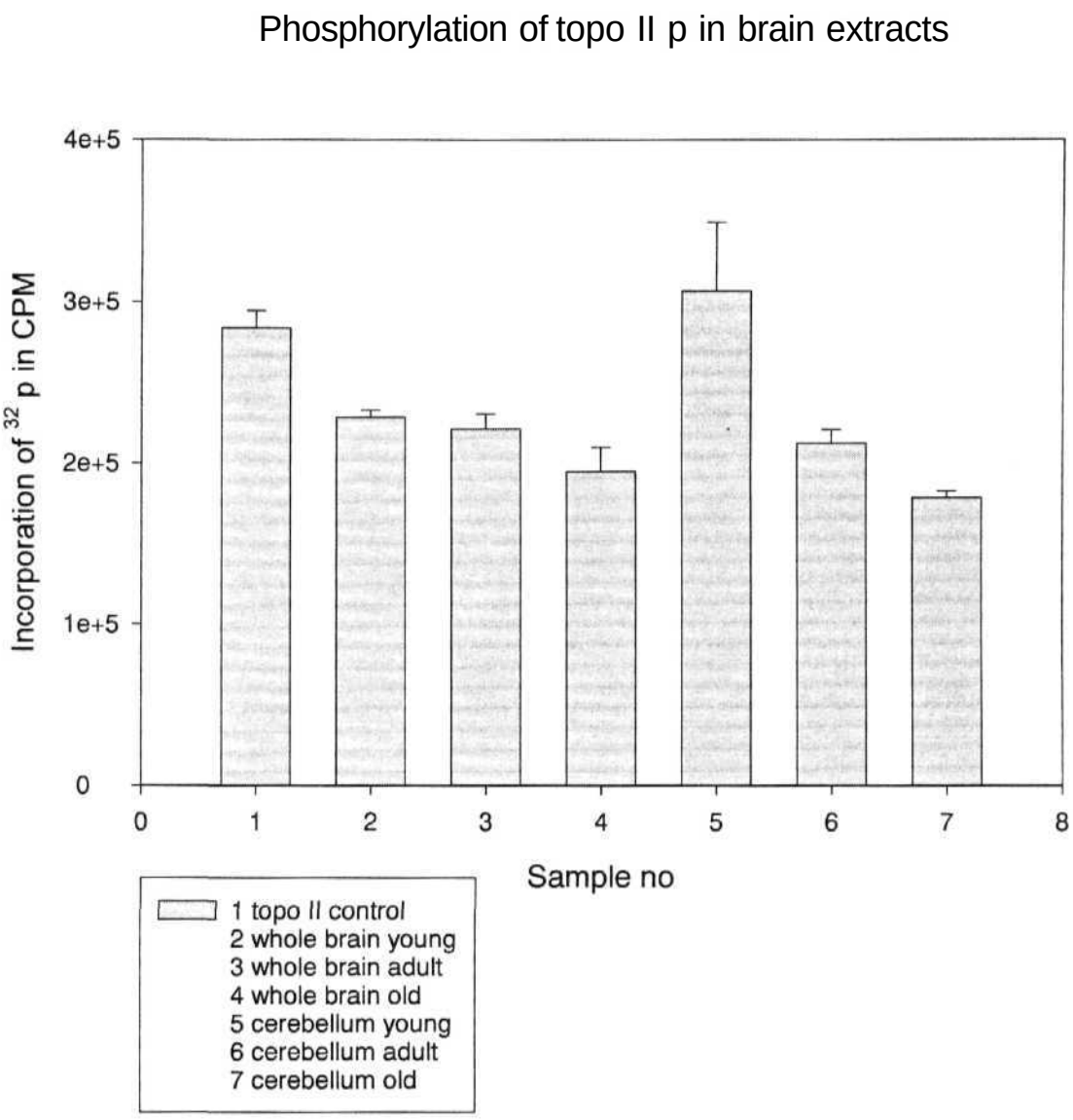

Phosphorylation of topo II p in brain extracts
Incorporation of 32 p in CPM
4e+5
3e+5
2e+5
1e+5
0
0
1
2
3
4
5
6
7
8
Sample no
1 topo II control
2 whole brain young
3 whole brain adult
4 whole brain old
5 cerebellum young
6 cerebellum adult
7 cerebellum old

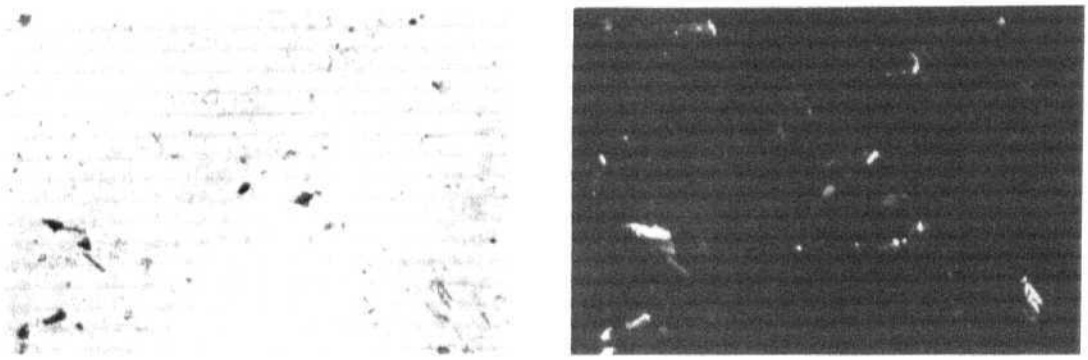
Figure 11

Topo IIα localization in cerebral section.

Frozen cerebellar sections ~4μM of the cerebellum from the three different age groups were taken in a cryostat and mounted on a glass slides. The sections were treated with ethanol for 10 min followed by washes with double distilled water and PBS. The sections were pearmeablized with 0.3% Triton X-100 and incubated with Topo IIα antibodies followed by FITC conjugated secondary antibody for 1 hour and washed with PBS twice. The slides were observed under a fluorescence microscope. The slides were labeled appropriately

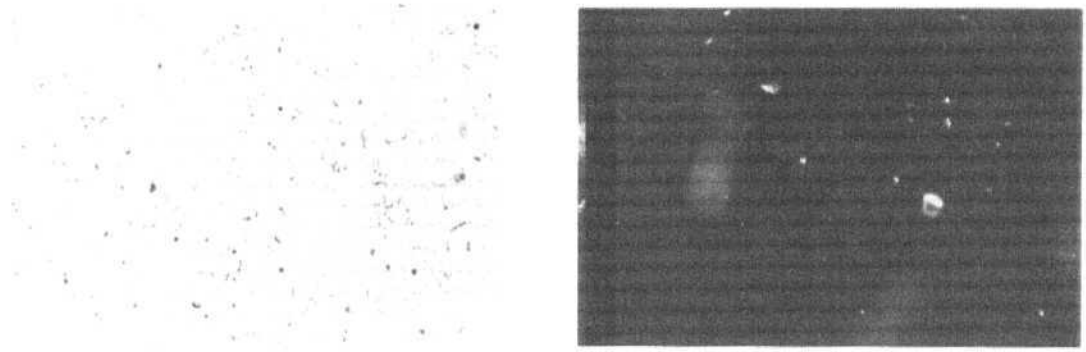
FIGURE 11

Topoisomerase II a in cerebellum of young rat brain



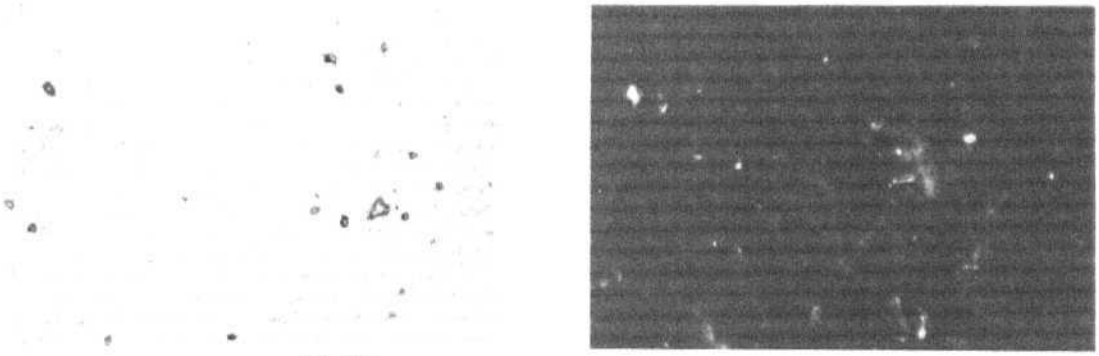
Visible **Fluorescence**

Topoisomerase II α in cerebellum of adult rat brain



Visible **Fluorescence**

Topoisomerase II α in cerebellum of old rat brain



Visible **Fluorescence**

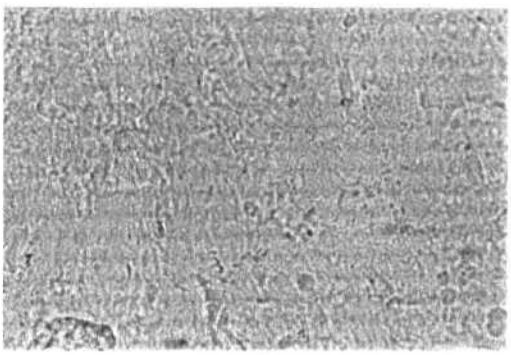
Figure 12

Topo II (3 localization in ceEbral section.

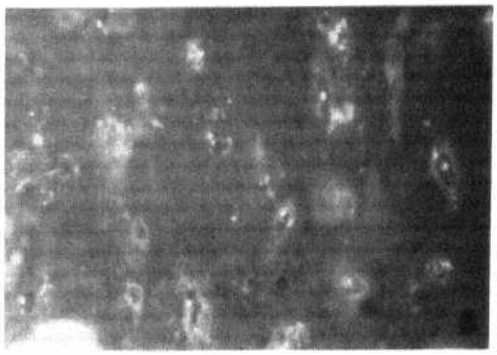
Frozen cerebellar sections ~4μM of the cerebellum from the three different age groups were taken in a cryostat and mounted on a glass slides. The sections were treated with ethanol for 10 min followed by washes with double distilled water and PBS. The sections were pearmeablized with 0.3% Triton X-100 and incubated with Topo IIβ antibodies followed by FITC conjugated secondary antibody for 1 hour and washed with PBS twice. The slides were observed under a fluorescence microscope. The slides were labeled appropriately.

FIGURE 12

Topoisomerase II β in cerebellum of young rat brain

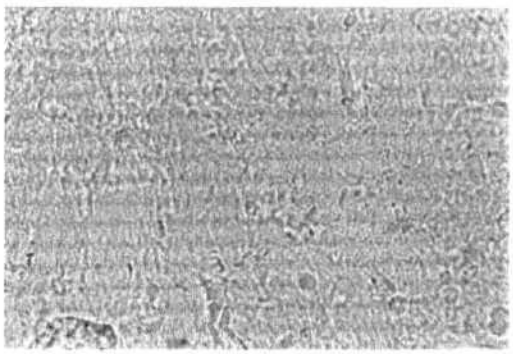


Visible

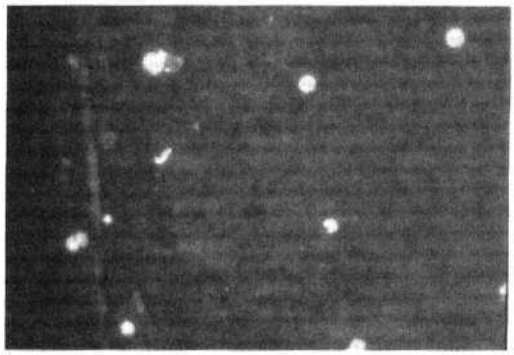


Fluorescence

Topoisomerase II p in cerebellum of adult rat brain

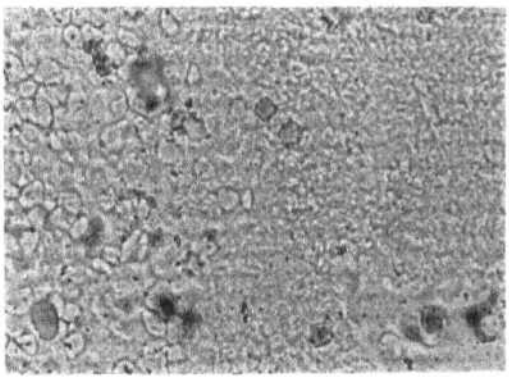


Visible

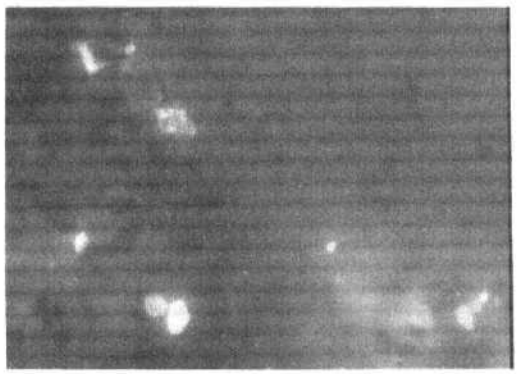


Fluorescence

Topoisomerase II p in cerebellum of old rat brain



Visible



Fluorescence

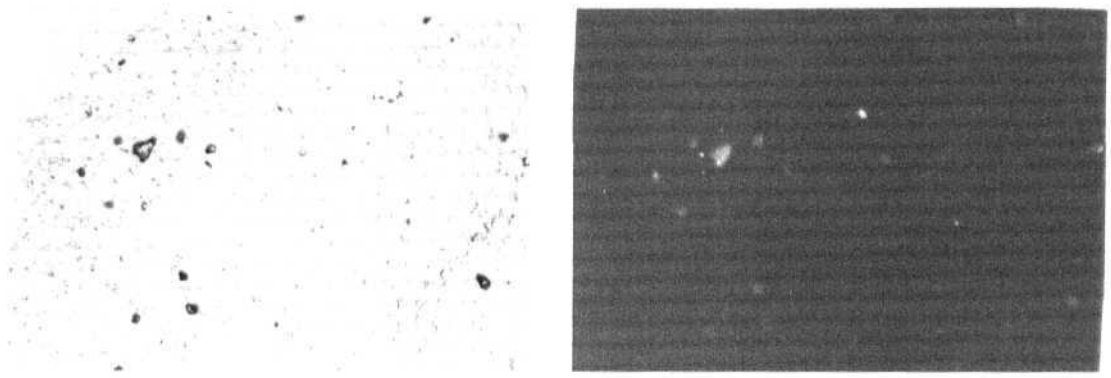
Figure 13

Topo II localization in cerebral cortex and mid brain

Frozen cerebral cortex and mid brain sections ~4μM of the cerebellum from young rats were taken in a cryostat and mounted on a glass slides. The sections were treated with ethanol for 10 min followed by washes with double distilled water and PBS. The sections were pearmeablized with 0.3% Triton X-100 and incubated with Topo II β antibodies followed by FITC conjugated secondary antibody for 1 hour and washed with PBS twice. The slides were observed under a fluorescence microscope. The slides were labeled appropriately.

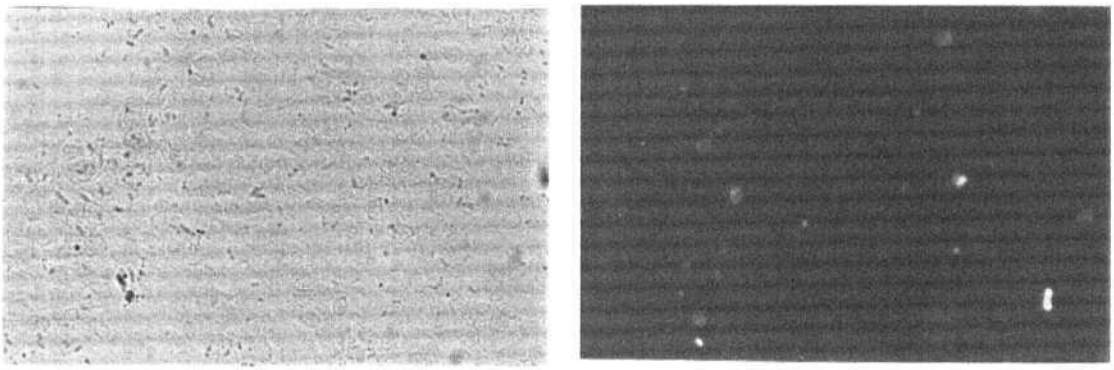
FIGURE 13

Topoisomerase II β in cerebral cortex of young rat brain



Visible Fluorescence

Topoisomerase II p in young rat mid brain



Visible Fluorescence

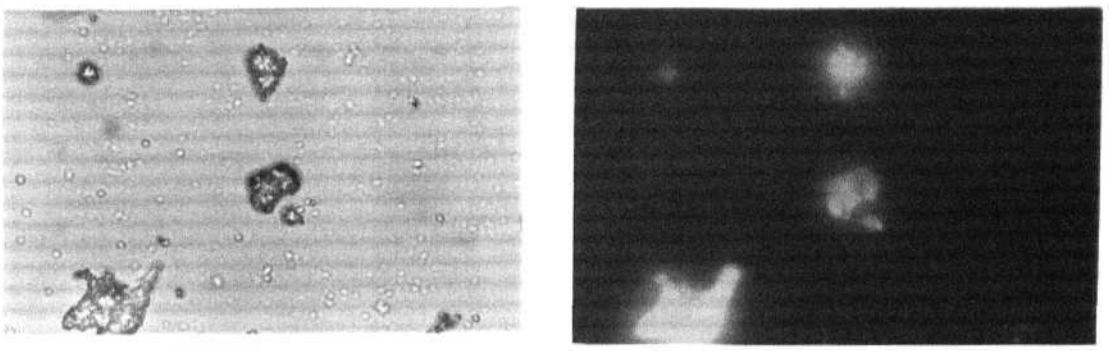
Figure 14.

Topo II localizations in neuronal cells:

Neuronal cells were isolated form cerebellum of rats at 3 different age groups and cytospinned. The monolayers were fixed with ethanol on glass slides. The cells were permeablized with 0.3% Triton X-100 and incubated with Topoisomerase IIβ monoclonal antibody followed by FITC conjugated secondary antibody for 1 hour and washed with PBS twice. The slides were observed under a fluorescence microscope. The slides were labeled appropriately.

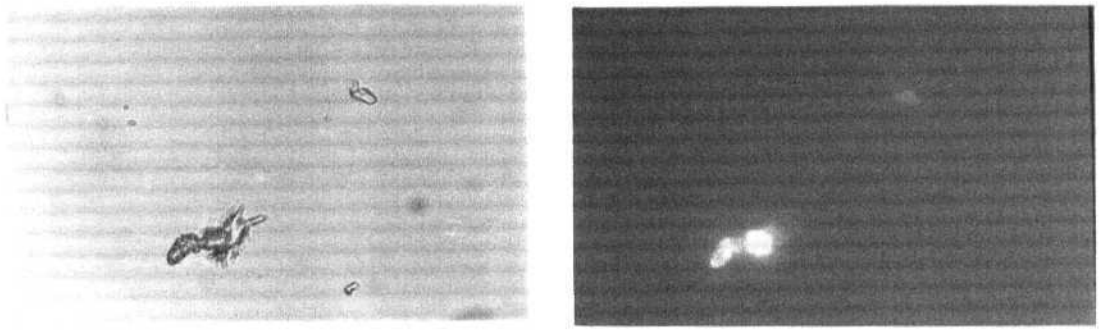
FIGURE 14

Topoisomerase II p in neurons from cerebellum of young rat



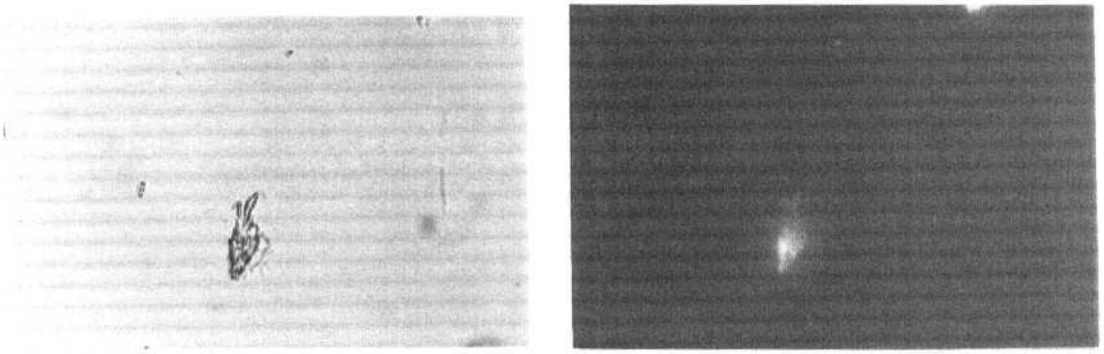
Visible Fluorescence

Topoisomerase II p in neurons from cerebellum of adult rat



Visible Fluorescence

Topoisomerase II p in neurons from cerebellum of old rat



Visible Fluorescence

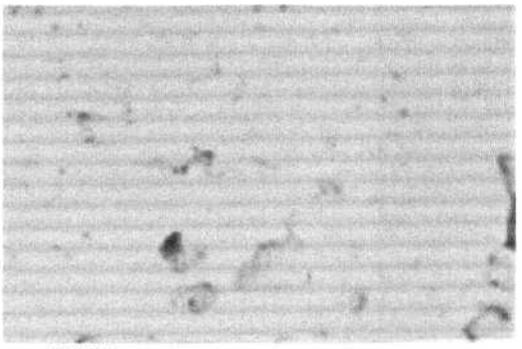
Figure 15

Neurons and astrocytes staining with markers:

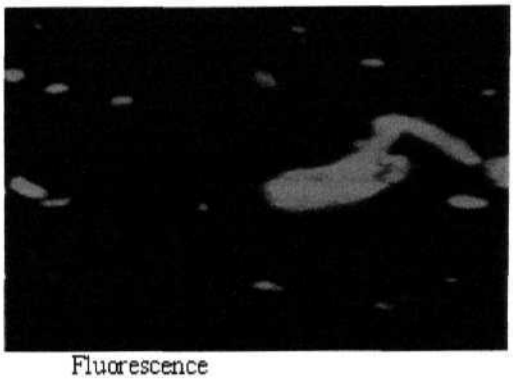
The isolated **cells** were cytospinned on glass slides and fixed with ethanol. Then blocked with 2% fetal bovine serum and incubated with neuronal specific enolase for neurons and **GFAP** for astrocytes followed by flouresene red conjugated secondary antibody for 1 hour. Then washed twice with PBS. The slides were observed under fluorescence microscope and photographed. The slides were appropriately labeled.

Figure 15

NSE marker for neuronal cells

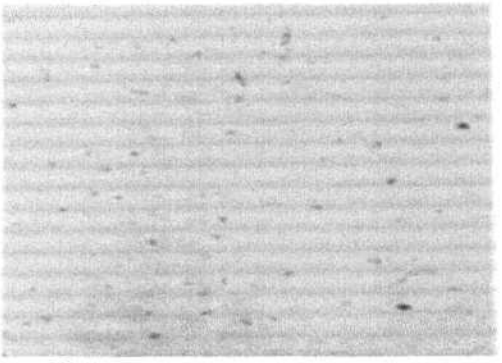


Visible

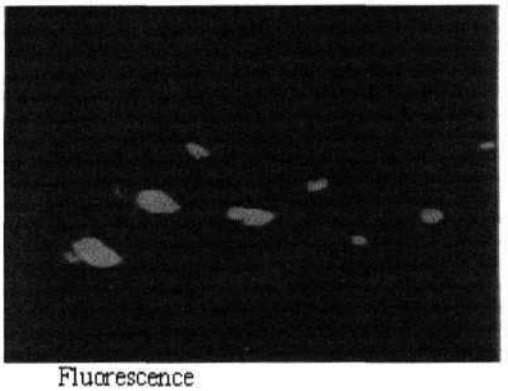


Fluorescence

GFAP marker for astrocytes



Visible



Fluorescence

Figure 16

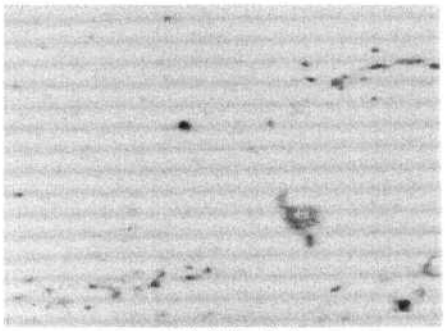
Isotypic controls for neurons and sections:

The cerebellar sections and neurons were fixed with ethanol on glass slide. Then permealized with 0.3% triton X- 100 and washed thrice with PBS. Then incubated with purified mouse IgG antibodies followed by FITC conjugated secondary antibodies for 1 hour. Then washed twice with PBS. The slides were observed under fluorescence microscope and photographed. The slides were appropriately labeled.

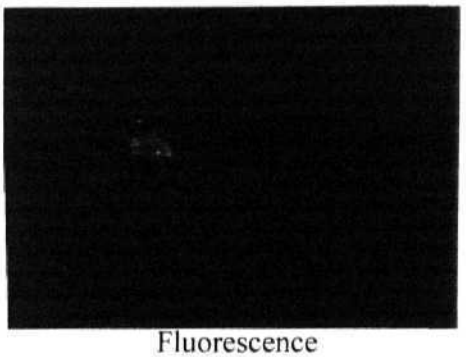
FIGURE 16

A

Isotypic control in neurons



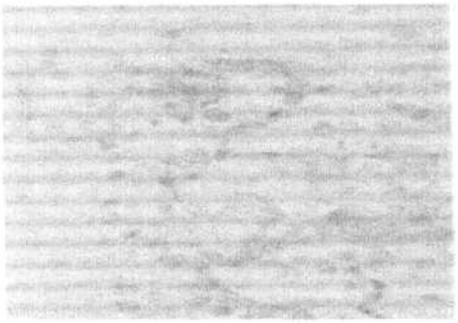
Visible light



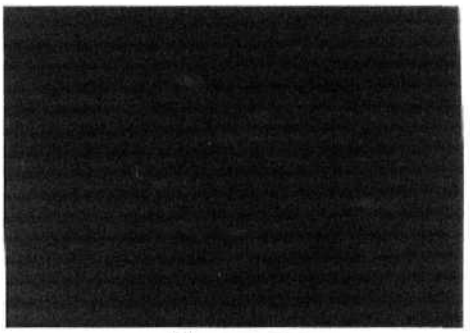
Fluorescence

B

Isotypic control in tissue sections



Visible light



Fluorescence

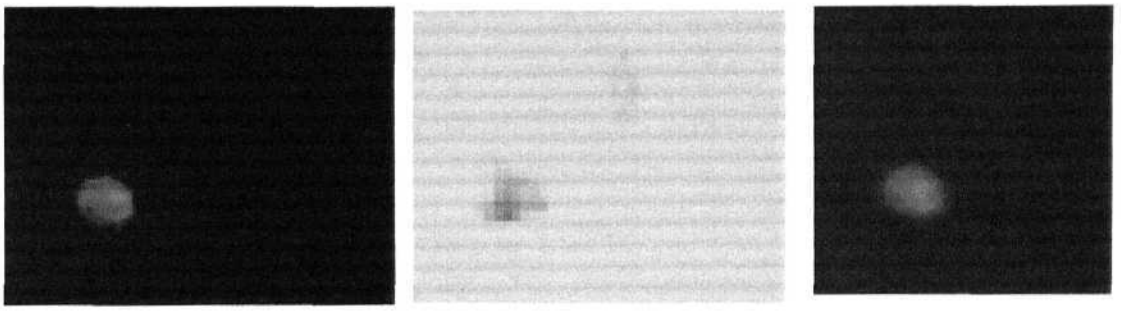
Figure 17

Double immunofluorescence of neurons:

Neurons isolated form young rat cerebellum and cytospinned on to the glass slide, then fixed with ethanol and permeablized using 0 3% Triton X-100. Then incubated with NSC Abs against neuronal specific enolase for 1 hour, and washed twice with PBS. Then incubated with Topo II (3 followed by fluorecene- red and FITC conjugated secondary antibodies. Then washed twice with PBS. The slides were observed under fluorescence microscope and photographed. The slides were appropriately labeled.

Figure 17

Double immunostaining of neurons with NSE and Topo II β Abs



NSE fluorescence | Visible | Topo II β fluoresce

Figure 18

Double immunostaing of astrocytes:

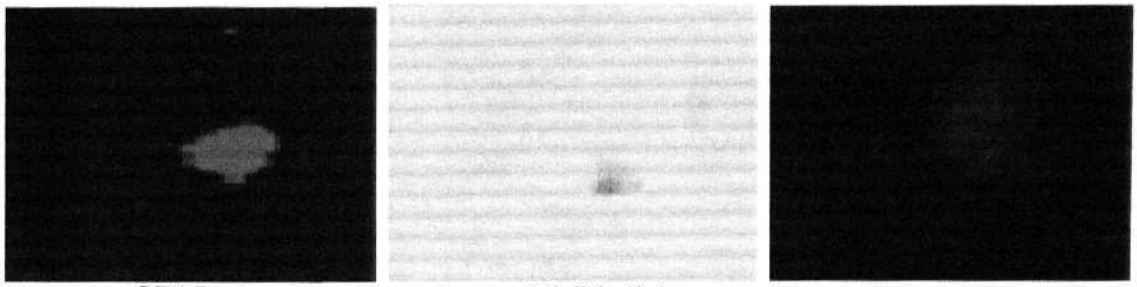
Astrocytes w&re isolated from young rat brain and cytospinned on to the glass slide. Then fixed with ethanol and permealized with 0.3% triton X-lOO.then slides were double immunostained with GFAP and Topo II a primary antibodies followed by incubation with FITC and Fluorescene- red conjugated secondary antibodies (Panel A and B) then washed twice with PBS.

Topo II localization in astrocytes: Isolated cells were fixed with ethanol on glass slides and fixed with 0.3% triton X-100. Then incubated with Topo II a and β monoclonal antibodies followed by FITC conjugated secondary antibodies for 1 hour. Then washed twice with PBS. The slides were observed under fluorescence microscope and photographed (Panel C and D). The slides were appropriately labeled.

Figure 18

A

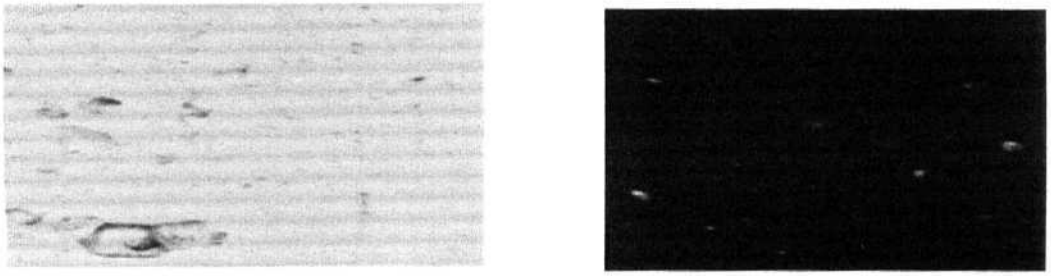
Double immuno staining of astrocytes with GFAP and Topo II a Ab



GFAP Visible light Topo II α fluorescence

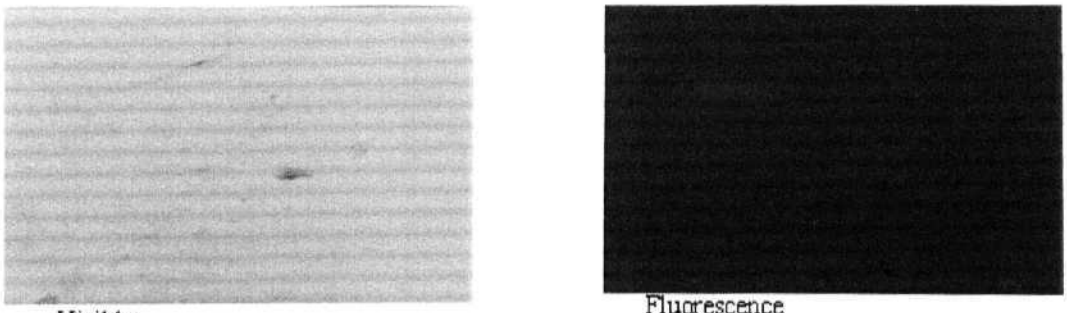
B

Topo II α in young rat cerebellar astrocytes



Visible Fluorescence

Topo II β in young rat cerebellar astrocytes



Visible Fluorescence

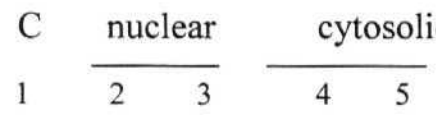
Figure 19

Sub-cellular localization of Topo II a and (3 in neuronal cells with TopoII a and β antibodies. Panel A lanes 2 and 3 shows Topo II (3 in nuclear fraction and lanes 4 and 5 shows Topo II (3 in cytosolic fraction. In panel B lanes 2 and 3 shows Topo II a in nuclear fraction and 4 and 5 shows cytosolic fraction. Panel c shows tubulin and actin controls.

FIGURE 19

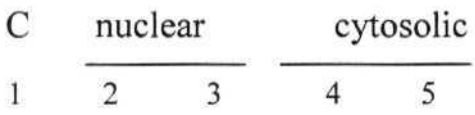
A

Sub cellular localization of Topo II β



B

Sub cellular localization of Topo II α



C

Tubulin and actin controls for nuclear and cytosolic fractions

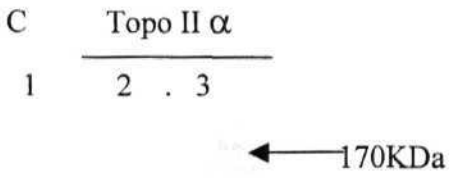
Figure 20

Immunoblotting analyses of topoisomerase II a and |3 in astrocytes with Topo II a and |3 antibodies. Panel A and B show Immunoblots of TopoII a and (3 from 75 μg total protein lane 1 in panel A shows Topo II a control with 25μg. lane 1 in panel B shows Topo II β control with 25 μg.

FIGURE 20

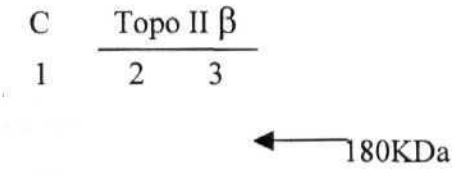
A

Topo II α in astrocytes of young rat cerebellum



B

Topo II β in astrocytes of young rat cerebellum



C

Actin control

Table 1. Statistical analysis of variations of Topo II a and β

[1] Testing the significance of difference of supercoiled DNA among control, young, adult and old, cerebral cortex

[2] Testing the significance of difference of supercoiled DNA among young, adult and old, cerebral cortex

μc= control, μY = mean of young, μA = mean of adult, μo = mean of old

	Analysis of Variance (ANOVA)						Dunnet's test
	4 Groups[1] ($F^2$16= 3.24)			3 Groups[2] (F^2 12 = 3.89)			
	F	RESULT	r^2 %	F	RESULT	r^2 %	
α Whole brain	-7.94	μc=μy=μa =μo	- 58%	-14.95	μy=μa =μo	- 159%	μc≠μy μc=μa μc =μo
α Cerebellum	7.74	μc≠μy=μa =μo	59%	4.7	μy=μa =μo	44%	μc≠μy μc=μa μc =μo
α Cerebral cortex	13.65	μc≠μy=μa =μo	71.9%	1.52	μy=μa =μo	20%	μc≠μy μc≠μa μc≠ μo
α Mid brain	0.295	μc=μy=μa ≠μo	5.2%	0.02	μy=μa =μo	0.3%	μc=μy μc=μa μc =μo
β Whole brain	402.69	μc≠μa≠μy ≠μo	98.7%	51.26	μy≠μo ≠μa	90%	μc≠μy μc≠μa μc≠μo
β Cerebellum	17.6	μc≠μy≠μa ≠μo	97%	8.26	μa≠μo ≠μy	57.9%	μc≠μy μc≠μa μc≠μo
β Cerebral cortex	7.63	μc≠μy≠μa ≠μo	58.9%	8.13	μy≠μo ≠μa	57.4%	μc=μy μc=μa μc =μo
β Midbrain	-123.8	μc=μy=μa =μo	15.94%	-0.02	μy=μa =μo		μc≠μy μc=μa μc =μo

Table 2: Regression analysis of age dependent changes of activity of Topo II isoforms

		Regression coefficient	Coefficient of determination	Analysis of variation	Population R linear
Topo II α activity	Cerebral cortex	0.028	0.47	F= 11.46 F0.05 (1); 1, 13 = 4.65 Regression is $\beta \neq 0$	F= 3.73 F0.05= 4.65 Population regression is linear
Topo II β activity	Whole brain	- 0.148	2.36	F= - 22.54 F0.05 (1),1,13 =4.65 Regression is $\beta \neq 0$	F= - 7.91 F0.05 (1) 1, 13 = 4.65 Population regression is not linear
Topo II β activity	Cerebellum	- 0.045	0.997	F= - 4649.13 F0.05 = 4.65 Regression is $\beta \neq 0$	F= 17444 F0.05= 4.65 Population regression is not linear

Chapter III

Monitoring age dependent changes in Topoisomerase II a 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 respoasible 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 theses 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, lmM $MgCl_2$, O.lmM EDTA, 5% glycerol, 0.1% triton X- 100, 0.5M KC1, 0.5mM PMSF and fytgjjil 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 a 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 **a** and |3. Topoisomerase II **a** or (3 antibody (1:1000 dilution in IP buffer containing 100mM Tris HC1 pH 8, 750mM NaCl, 2mM EDTA, and lmM 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 spinned 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 fxM of topoll poison is incubated with the immunoprecipitated Topoisomerase II captured on to the Protein A agarose beads in relaxation buffer (50mM Tris HC1 pH 8.0,120mM KC1, 0.5mM EDTA, 0.5mM DTT, 10mM $MgCl_2$, 30μg/ml BSA, lmM 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 a and β from tissues

Immunoprecipitated topoisomerase II a and β were assayed for their relaxation activity by incubating with the plasmid DNA. The activities of the topoisomerase II a (figure 21 panel A) and β (figure 21 panel B) have shown variation in different tissues with the **a** isoform showing negligible activity in the brain tissues of young adult and old rats. In liver and testes tissues, the topo II a 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 a 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 a and β in the presence of mAMSA.

The relaxation and cleavage activities of immunoprecipitated topoisomerase II **a** and (3 in the presence of 200 mM mAMSA was studied. Topoisomerase 11 a 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. Where as, in the liver extracts of the young rats the presence of mAMSA showed high inhibition of enzyme catalyzed DNA relaxation activity of topo II p 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 **a** and β, the a 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. Where as 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 a and β with Etoposide.

The topoisomerase II **a** and β relaxation and cleavage activities were studied in the presence of 200 μM of etoposide. The DNA relaxation activity of topoisomerase II a 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 a activity showing linearization of the supercoiled DNA with topo II **a** 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 a 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 a 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 a 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 a and β from brain liver and testis extracts in the presence of etoposide and mAMSA show that the a 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 seasitivity 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

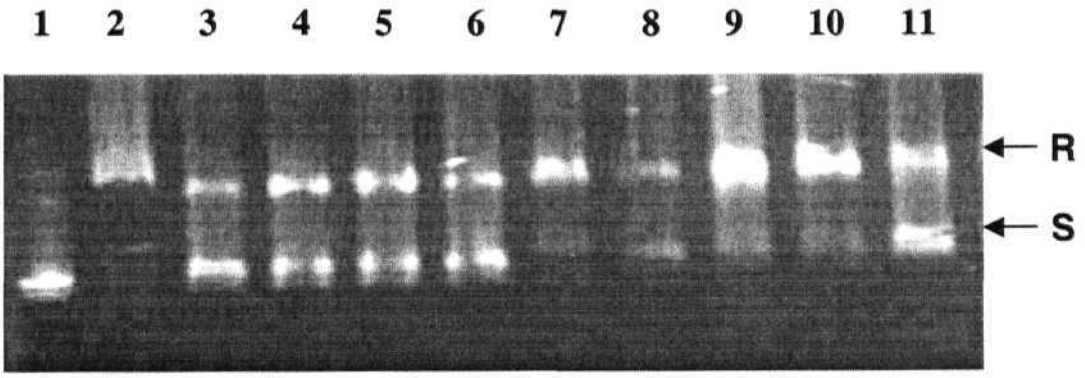
Figure 21

Relaxation activity of topoisomerase II a and (3 from tissues of young, adult and old rats. Panel A and B show relaxation activity of immunoprecipitated topoisomerase II a and β from 100 μg total protein of brain (lanes3-5), liver (lanes 6-8) and testis (lanes9-11) 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 a in tissues



B

Relaxation activity of Topoisomerase II p in tissues

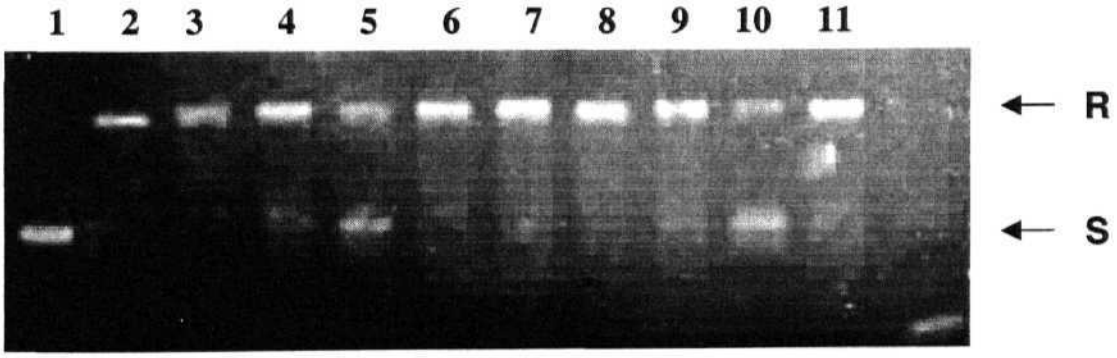
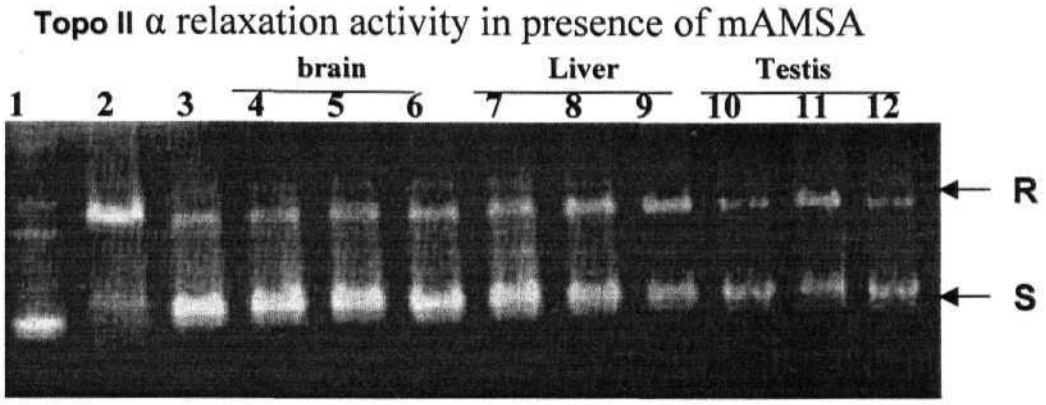


Figure 22

Relaxation activity of topoisomerase II a and β from tissue extracts in presence of mAMSA: Panel A and B show relaxation activity of immunoprecipitated topoisomerase II a and β from 100 μg total protein of brain (lanes 4-6), liver (lanes 7-9) and testis (laneslO-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



B

Topo II β relaxation activity in presence of mAMSA

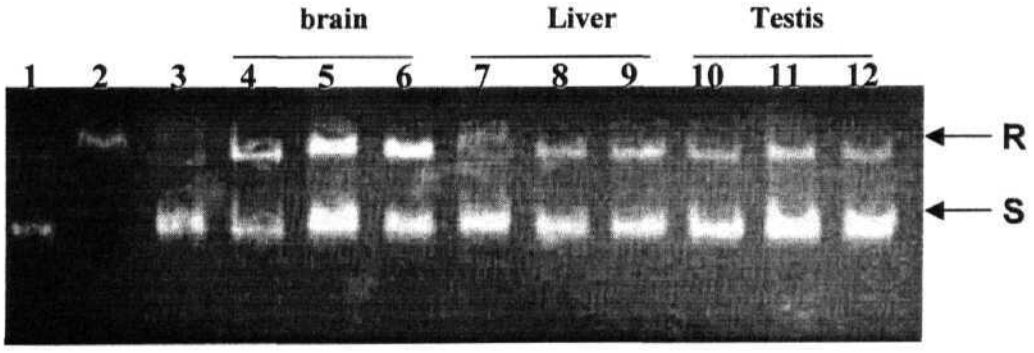
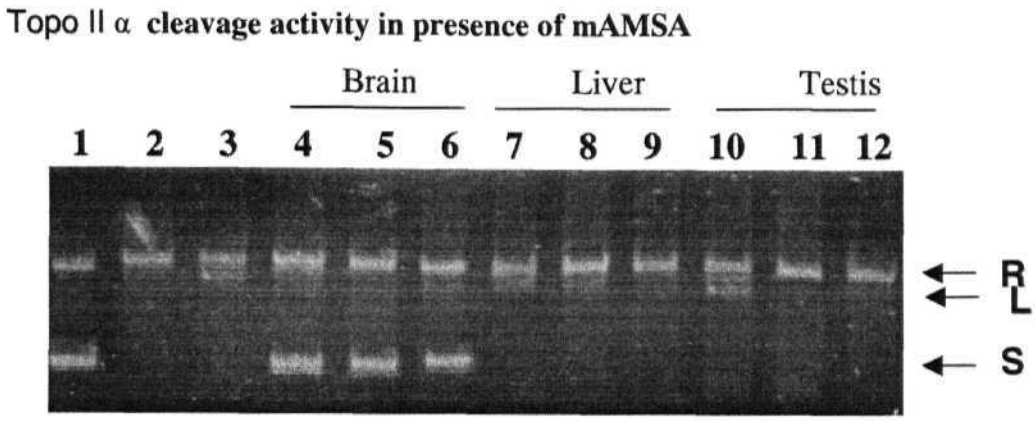


Figure 23

Cleavage activity of topoisomerase II a and β from tissue extracts in presence of mAMSA. Figure shows relaxation activity of immunoprecipitated topoisomerase II a from 100 μg total protein of brain (lanes4-6), 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
Brain
Liver
Testis
1 2 3 4 5 6 7 8 9 10 11 12
R
L
S

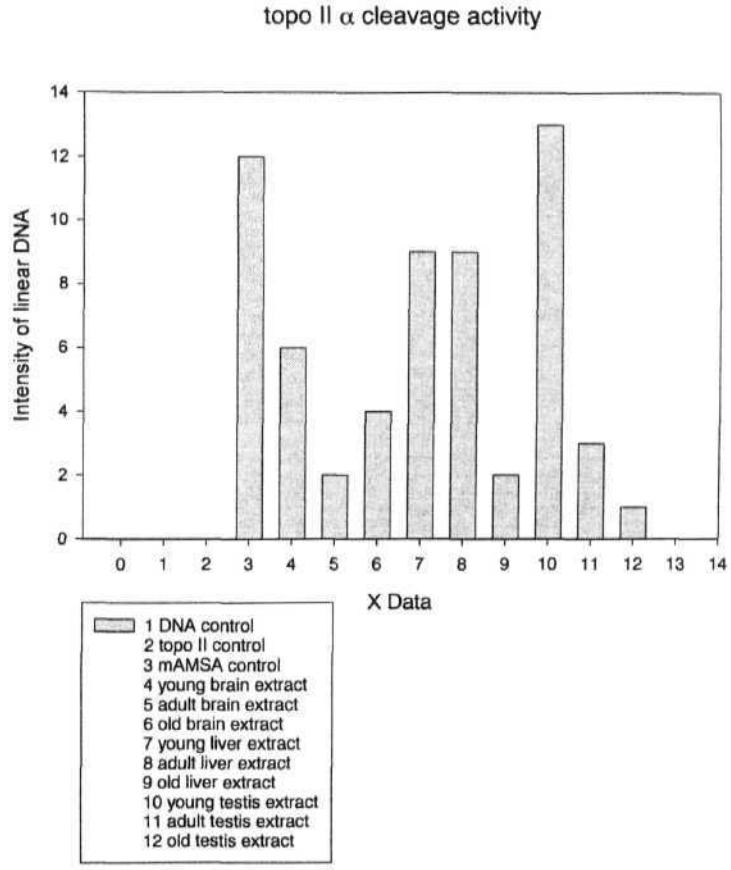

topo II α cleavage activity
Intensity of linear DNA
X Data
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1 DNA control
2 topo II control
3 mAMSA control
4 young brain extract
5 adult brain extract
6 old brain extract
7 young liver extract
8 adult liver extract
9 old liver extract
10 young testis extract
11 adult testis extract
12 old testis extract

Figure 24

Cleavage activity of topoisomerase II a 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 (lanes4-6), liver (lanes 7-9) and testis (lanes10-12) 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 200fiM 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

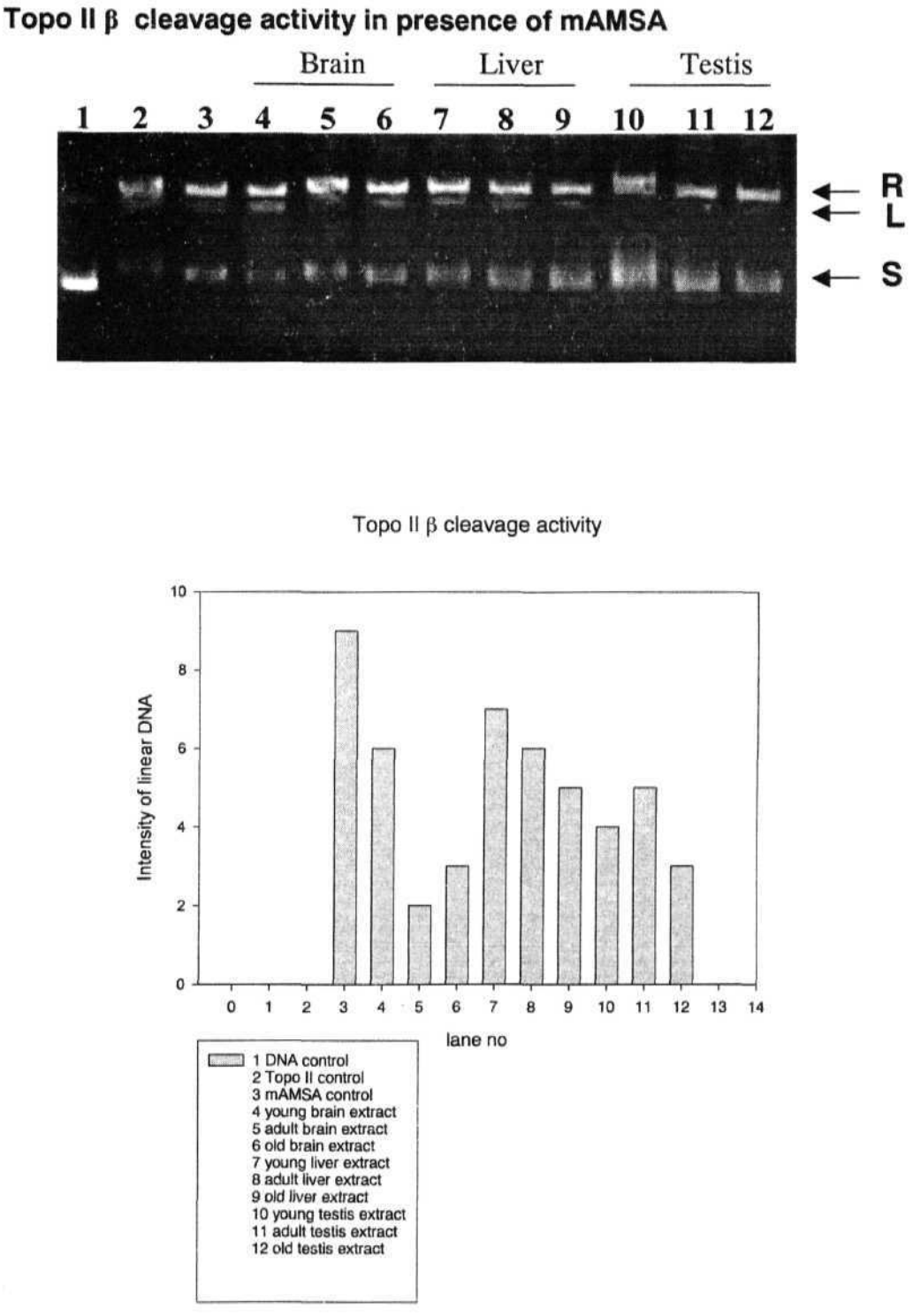
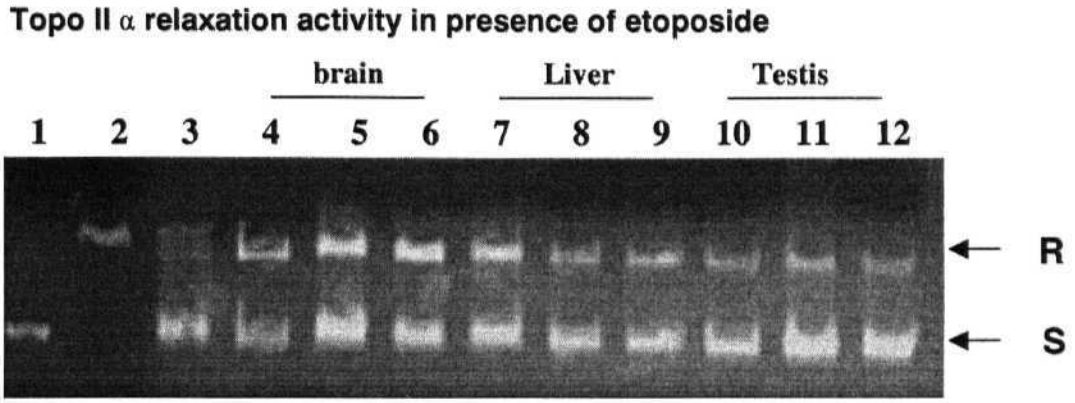

Topo II β cleavage activity in presence of mAMSA
Brain
Liver
Testis
1 2 3 4 5 6 7 8 9 10 11 12
R
L
S
Topo II β cleavage activity
Intensity of linear DNA
0 2 4 6 8 10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
lane no
1 DNA control
2 Topo II control
3 mAMSA control
4 young brain extract
5 adult brain extract
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 25

Relaxation activity of topoisomerase II a and β from tissue extracts in presence of etoposide: Panel A and B show relaxation activity of immunoprecipitated topoisomerase II a and (3 from 100 μg total protein of brain (lanes4-6), liver (lanes 7-9) and testis (lanes10-12) of young adult and old rats captured on to Protein A agarose beads was incubated with ~0.6 (xg of pRYG plasmid DNA in presence of relaxation buffer and 200μM etoposide.. Lane 1 shows ~0.6 (xg 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 200fxM of 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
brain
Liver
Testis
1 2 3 4 5 6 7 8 9 10 11 12
R
S

B

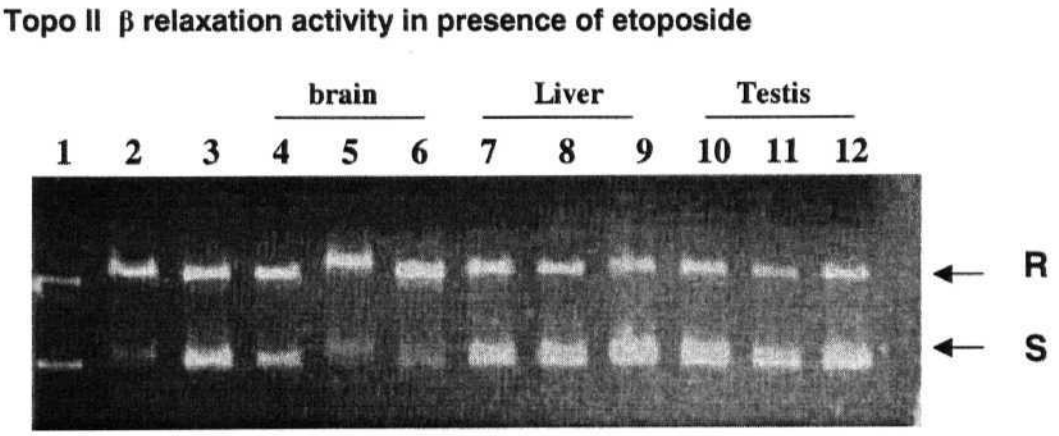

Topo II β relaxation activity in presence of etoposide
brain
Liver
Testis
1 2 3 4 5 6 7 8 9 10 11 12
R
S

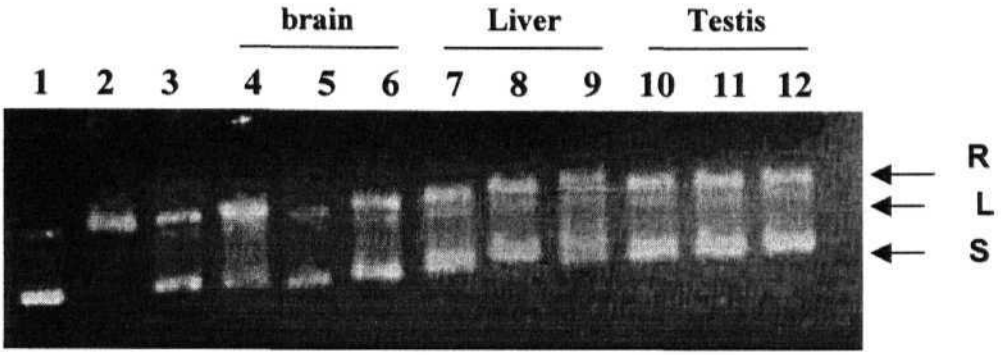
Figure 26

Cleavage activity of Topoisomerase IIα and β from tissue extracts in presence of etoposide: Figure shows relaxation activity of immunoprecipated 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 pRYG 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



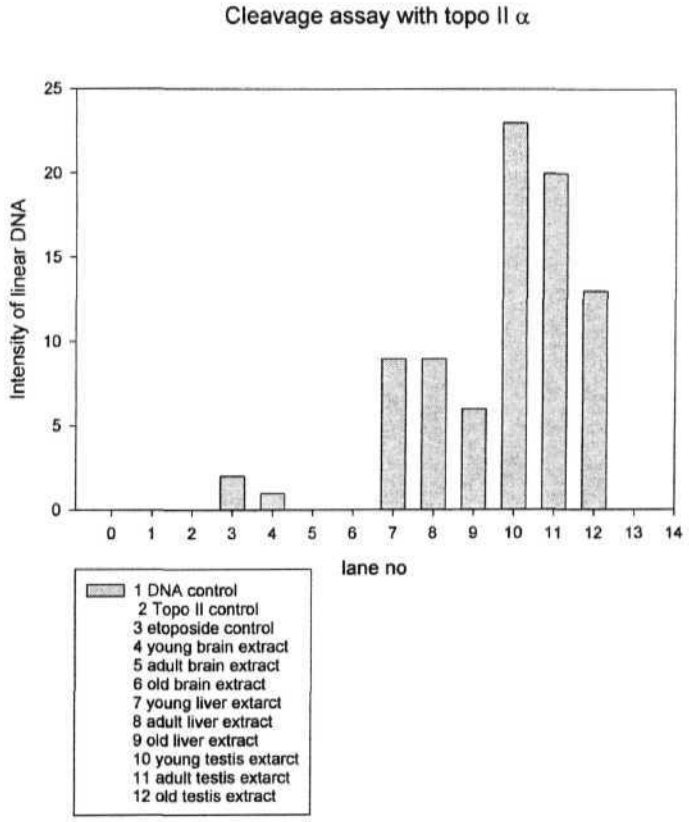
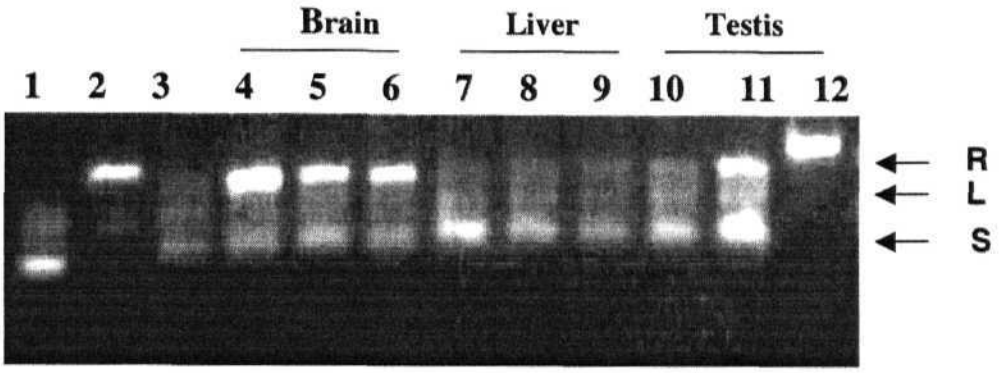


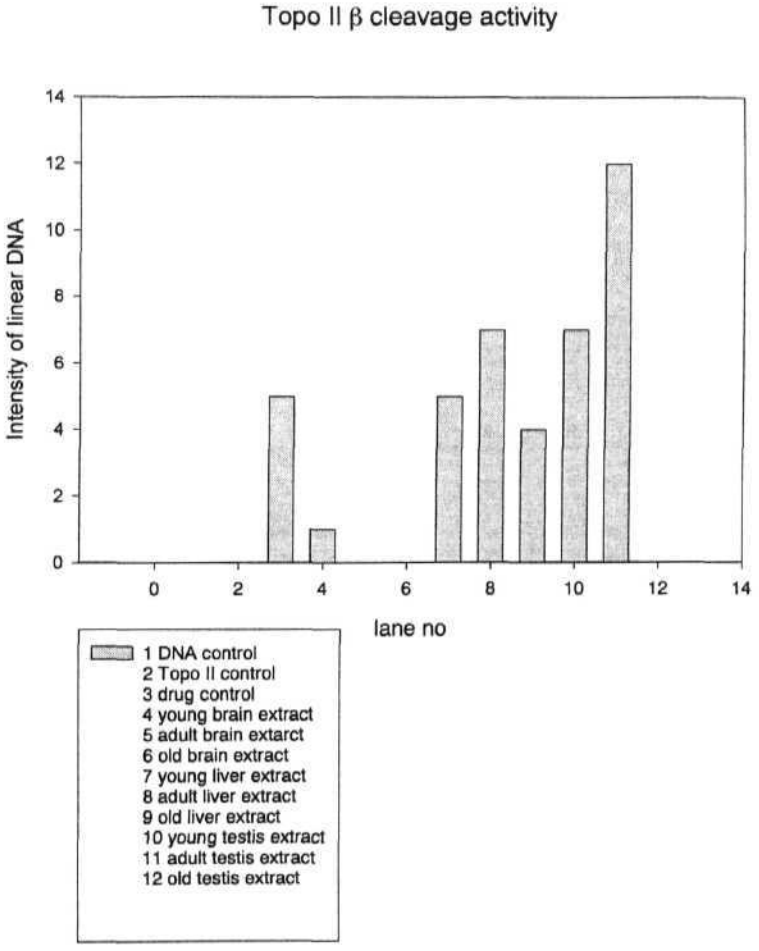
Figure 27

Cleavage activity of Topoisomerase IIα and β from tissue extracts in presence of etoposide: Figure shows relaxation activity of immunoprecipated 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 to 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 ~0.6 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 27

Topo II β cleavage activity in presence of etoposide





Chapter IV

Development of Ruthenium derivatives as topoisomerase II poisons.

Topoisomerase II is being reverd as one of potential target for cancer chemotherapy:

Introduction:

Topoisomerases II is one of the nuclear enzyme responsible for the topological maintenance of the cellular DNA inter conversions in the DNA are the major target for many anticancer drugs belonging to different classes of compounds like epipodophillotoxins (etoposide, tenoposide), anthracyclins (doxorubicin, daunorubicin), amino-acridines (amsacrine), ellipticines (ellipcinium), anthracene-diones (mitoxantrone) etc. These compounds interfere with one of the steps in the catalytic cycle of Topoisomerase II and stabilize the cleavable complex; the transient intermediate have double stranded nicked DNA covalently bound to the enzyme. The cleavable complex is a state of intense fragility of the DNA molecule.

Metal complexes gained importance as anticancer drugs with the discovery of cisplatin. This particular discovery provoked the discovery of the anticancer activity of complexes of other metals. Early transition and late transition series of periodic table have been vigorously tested for their anti cancer activity. Although very few of them matched the efficacy of cisplatin, some of the non-platinum compounds were active against tumors that were unresponsive to cisplatin and other anticancer drugs. Some of the non platinum metal complexes which showed promising anti cancer activity are Spiro germanium a germanium complex, gallium nitrate, titanium metal complexes like titanocene dichloride and budotitane, ruthenium complexes such as Trans-indazolium [bisindazole] tetrachloro Ruthenate and Imidazolium trans- imidazole dimethylsulfoxide tetrachlororuthenate (NAMI-A) complex. These complexes have shown to possess distinct interaction with

DNA, RNA, and protein, which define their anticancer activities. For example Gallium salts interfere with the ribonuclotide reductase activity and inhibit the DNA nucleotide synthesis (Waller 1996). Titanocene complexes and Ruthenium complexes are shown to interact with type IV collagenolytic activity which corresponds to an increase in the extra cellular matrix components in tumor parenchyma (Maragoudakis *et. al,* 1994). This hinders metastasis formation and blood flow to the tumors (Sava *et. al.,* 1996, Morgunova *et. al,* 1999).

Among the metals used in the anticancer metal complexes, ruthenium shows unique properties. It has strong complex forming activity with numerous ligands. Ample studies indicate that most ruthenium complexes bind covalently to DNA via the N-7 atom of purines and cause cytotoxicity by inhibiting cellular DNA synthesis (Kopf *et. al,* 1994, Haiduc et al). The DNA binding property of the Ruthenium complexes has been associated with their anticancer activity. Also ruthenium complexes make use of various biological mechanisms for transport and macromolecular binding and they coordinate with various biological macromolecules. This feature helps in the development of ruthenium complexes that interact with specific biological molecules to bring about targeted anticancer activity. Many of the ruthenium complexes appear to be transported in blood through transferrin. (80%) and to a lesser extent through albumin (Srivastavastava *et. al.,* 1989, Kratz *et. al,* 1994). Tumor localization of Ruthenium, Gallium, and Titanium complexes attributes to this transport mechanism as tumor cells express a large number of transferrin receptors on their membranes (Nejmeddine *et. al,* 1998). Redox molecules like glutathione interact with heavy metals and the reduced form of this peptide is known to interact with complex, which would activate the metal

complexes to bind to biopolymers in the hypoxic environment of tumors. Ruthenium (III) compounds can also serve as diagnostic tumor imaging agents, using the nuclides ^{97}Ru or ^{103} Ru (Srinivasa *et. al,* 1989)

Numerous ruthenium compounds have earlier been reported to possess anticancer activity, some of them are potent than cisplatin (Giraldi *et. al,* 1977, Clarke 1989, Seva *et. al,* 1984, 1989, Mestroni *et. al,* 1989, Pacor *et. al,* 1991, Keppler *et. al,* 1990). two of these compounds are trans-[indazolium bis 9indazole) tetrachlororuthenate (III), cis-[Ru II Cl_2 (dimethysulphoxide) and trans-[Ru III Cl (dimethysulphoxide) Imidazole] Na+

In vitro analysis of these ruthenium complexes show that these complexes interact with DNA and the topoisomerase II in a bi-directional manner resulting in the formation of a TopoII mediated DNA cleavage complex. The formation of such a cleavage complex is the main route for the anti cancer action of the TopoII poisons. Our laboratory synthesized and studied mechanism of ogranometallic ruthenium derivatives like RuBen (dmso), RuBen Pyr, RuBen Apy, RuBen Agu, RuBen Aba and analyzed for their anti cancer activity Among these Ruben Apy showed significant topo II poisoning and anti cancer activities. Further to understand the structural component required for topo II poisoning by Ruben Apy, we used the these molecules as lead and designed various structural analogues to identify the structural orientation required for Ruben Apy for topo II poisoning. Four compounds in which the amino pyridine group is replaced with pyridine derivatives like pyridine 3-carboxaldehyde, Pyridine 3-sulphonic, 3- hydroxy pyridine and nicotinamide derivatives. These complexes are analyzed for their inhibitory action on topo II catalytic activity and the antiproliferative activity.

Methodologies: the following methods are used in the present study. Ruthenium Organometallic Complexes:

Synthesis of the dimeric starting compound:

This complex was synthesized as previously described by (Zelonka *et. al,* 1972). Briefly, freshly synthesized 1, 3-cyclohexadiene (6 ml) was added to $RuCl_3.3H_2O$ (1.7 gm) in 100 ml of aqueous ethanol. The solution was maintained at 45 ^0C for 3 hrs to form a red precipitate which was washed in ethanol and dried in vacuum to give the dimeric complex of $[RuCl_2(C_6H_6)_2]$. This dimer was the starting compound for the synthesis of all the complexes of the 'RuBen' type.

RuBen Pyridine 3-carboxaldehyde

To the 25 mg RuBen dimer, equimolar concentrations of pyridine 3- carboxaldehyde was added and incubated to get a brick red colored precipitate, which was washed in ethanol and dried in a vacuum drier.

The structure of the complex was confirmed by infrared spectroscopy (spectra 1).

RuBen Pyridine 3- sulphonic acid

To 25 Mg of Ruben dimer equimolar concentrations of pyridine 3- sulphonic acid derivative was added and incubated to get a dark green colored precipitate, which was washed with ethanol and vaccum dried.

The structure of the complex was confirmed by infrared spectroscopy (spectra 2).

RuBen 3- hydroxy pyridine

To 25 mg of Ruben dimer equimolar concentrations of 3- hydroxy pyridine derivative was added to get a brown precipitate, which was washed with ethanol and vacuum dried.

The structure of the complex was confirmed by infrared spectroscopy (spectra 3).

RuBen nicotinamide

To 25 mg of Ruben dimmer equimolar concentrations of nicotinamide derivatives was added to get a brown colored precipitate.

The structure of the complex was confirmed by infrared spectroscopy (spectra 4).

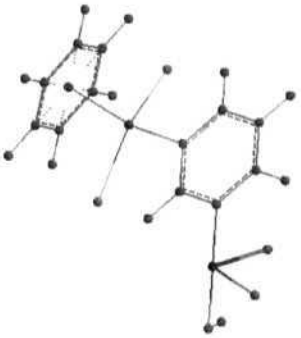
Molecular modeling of the Ruthenium derivatives

Structural conformations of the Ruthenium derivatives synthesized were determined by molecular modeling analysis. The models were generated using the PC SPARTAN molecular modeling software (wave function)

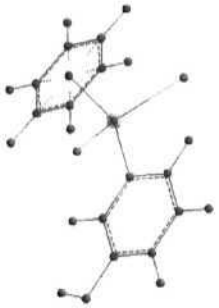
SPARTAN graphic interphase was used to generate the 3- dimensional models of the molecules, which were subjected to energy minimization. Optimized structures were used to understand the structural variation between amino pyridine and other derivatives.

Ruthenium Derivatives

Ruben Pyridine-3-Carboxaldehyde



Ruben Pyridine-3-Sulphonic Acid



Ruben-3-Hydroxy Pyridine

Ruben Nicotinamide

Methods:

- Protein estimation was done according to the colorimetric method described by Bradford (1976).
- SDS-PAGE electrophoresis was carried out according to the procedure of Laemmli (1970).
- Silver Staining of the SDS-PAGE protein gels was carried out according to the method of Blum *et. al,* (1987).
- Western Blotting was done following the procedure of Towbin et. al., (1979).

PURIFICATION OF pRYG NEGATIVELY SUPERCOILED PLASMID DNA:

The negatively supercoiled pRYG plasmid DNA was purified from the E.coli HB101 strain containing the plasmid, using the alkaline lysis procedure of Wang and Rossman (1994). The procedure described is for a llitre culture, which can be scaled up to 4 liters. An overnight culture of the plasmid containing bacteria (grown in the presence of 70 μg/ml ampicillin) was used for purification of the plasmid.

Butters used in the purification:

Lysis buffer: 50 mM glucose, 25 mM tris-HCl, pH 8.0, 10 mM EDTA and 5 mg/ml lysozyme.

Alkaline solution: 0.2 NNaOH and 1% SDS.

3M Sodium Acetate solutions

Tris buffer saturated Phenol

Sodium acetate buffer: 50 mM Tris, 100mM Sodium acetate

Tris-EDTA buffer: 10 mM tris-HCl, pH 7.5 and lmM EDTA.

Procedure:

Bacterial cell growth and harvesting:

25 ml of LB broth was inoculated with a single bacterial colony containing the plasmid. The culture was grown in a shaking incubator for 8 h at 36 °C. This culture was used for inoculating 1 liter of LB broth. The 1-liter culture was grown overnight (12 -14 h) at 37 ^{0}C in a shaker incubator. The purification procedures were carried out 4 °C.

Cells were harvested by centrifugation at 5000 rpm for 10 min. The cells were lysed with 40 ml of lysis buffer by constant stirring over a period of 15 min.

Alkaline lysis:

80 ml of freshly prepared alkaline solution was added and the constituents were mixed by swirling in a bottle. The mixture was placed on ice for 10 min. 50 ml of freshly prepared 3M-sodium acetate solution was added gently against the walls of the bottle. The bottle was placed on ice for 10 min. The precipitated proteins were removed by centrifugation at 12,000 rpm. The supernatant was clarified by filtering it through glass wool. Ice-cold isopropanol (0.7 volume) was added to the supernatant and placed on ice for 1 hour.

*Acidic **phenol-** chloroform extraction of RNA:*

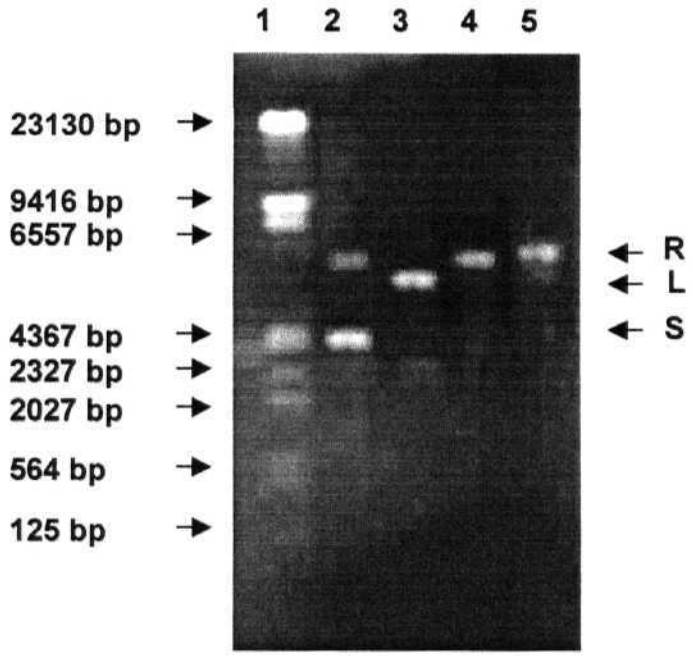
The precipitated DNA was pelleted at 12,000 rpm. The supernatant was removed and the pellet allowed for drying. This pellet was dissolved in 40ml of sodium acetate buffer solution. After 5 min on ice, equal volumes of Tris saturated phenol and chloroform are added and vortex mixed for 2 min in 50 ml tubes. The tubes were centrifuged at 12,000

rpm for 10 min. The aqueous phase was taken in an autoclaved conical flask and the phenol phase was removed.

Precipitation and dissolution of DNA:

The aqueous phase containing the DNA was treated with 0.7 volume of ice-cold isopropanol and 0.1 volume of 3 M sodium acetate (pH 4.2) and placed on ice for 20 min. The DNA was pelleted and washed twice with ice-cold ethanol (70%). The pellet was dissolved in a proper volume of Tris-EDTA buffer.

Agarose **gel** electrophoresis of pRYG plasmid DNA



lane 1 marker DNA
lane 2 supercoiled pRYG DNA
lane 3 BamH1 digest of pRYG
lane 4 Topo II α relaxed DNA
lane 5 Topo II β relaxed DNA

Purification of Topoisomerase II from rat liver:

Topoisomerase II was purified from rat liver tissue following the procedure of (Galande and Muniyappa 1996). In principle, the procedure involves the isolation of enriched nuclei for minimization of protease action. From the nuclei, topoisomerase II is isolated by polymin P precipitation of chromatin, followed by salt extraction of proteins, ammonium Sulfate precipitation and finally two rounds of gradient elution in a hydroxyapatite column. All the steps were carried out in a cold room at 4 °C.

Buffers used in the purification:

Buffer A: 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 25 mM KC1, 0.34 M sucrose and 0.1 mM PMSF.

Lysis Buffer: 5 mM potassium phosphate (pH 7.5), 100 mM NaCl, 10 mM 2-mercaptoethanol and 0.5 mM PMSF.

PR buffer: 20 mM potassium phosphate (pH 7.5), 10 mM $NaHSO_3$, 10% glycerol, 10 mM 2-mercaptoethano and 0.5 mM PMSF.

Storage buffer: 30 mM potassium phosphate (pH 7.5), 50% glycerol, 0.1 mM EDTA and 0.5 mM DTT.

Procedure:

Isolation of enriched nuclei:

400 gm liver from 2 month old rats (wistar strain) was washed twice in ice cold saline, minced thoroughly and homogenized in 2.5 liters of buffer A. The cell-free homogenate was centrifuged at 5000 rpm for 10 min. The pellet suspended in 700 ml of buffer A

containing 2.2 M sucrose and the supernatant was discarded. Enriched nuclei were obtained by ultracentrifugation of the reconstituted pellet at 28,000 rpm for 1 h in a Beckman Ti-70 rotor. The nuclear pellet was washed once at 15,000 rpm with 200 ml of buffer A containing 1 M sucrose followed by 200 ml of buffer A with 0.1% triton X-100.

Lysis of Nuclei:

The nuclear pellet was resuspended in lysis buffer and subjected to lysis in an MSE sonicator with a macroprobe for 4 times, 30 sec. each, with two min intervals.

Polymin P precipitation:

10% Polymin P (pH 7.8) was added slowly to the lysate, while stirring to a final concentration of 0.35% during a period of 15 min. The precipitate was pelleted at 6000 rpm for 10 min. The Pellet was resuspended in 200 ml of PR buffer. Proteins were extracted from the chromatin-polymin P complex with 0.55 M NaCl, while stirring for 30 min. Nucleic acids were reprecipitated by adding extra polymin P up to concentration of 0.7% while stirring for 15 min. The nucleic acid precipitate was removed by centrifugation and the supernatant was filtered through glass wool.

Ammonium Sulfate precipitation and dialysis:

The clarified supernatant was subjected to ammonium Sulfate (60%) precipitation with continuous stirring for 1 h. The precipitate was collected by centrifugation at 12,000 rpm for 20 min. The pellet was resuspended in 100 ml of PR buffer and dialyzed against 3 X 1

liters of the same buffer over a period of 15 h. A precipitate formed during the dialysis, which was removed by centrifugation at 26,000 rpm for 20 min.

Separation of low molecular proteins by Amicon filters.

The clarified supernatant obtained after ultra centrifugation was taken in Amicon filters with cut off range 100 and centrifuged at 1500 g for 30 minutes at 4°C. The retentate obtained contained the high molecular weight fractions with higher amounts of topoisomerase **II.**

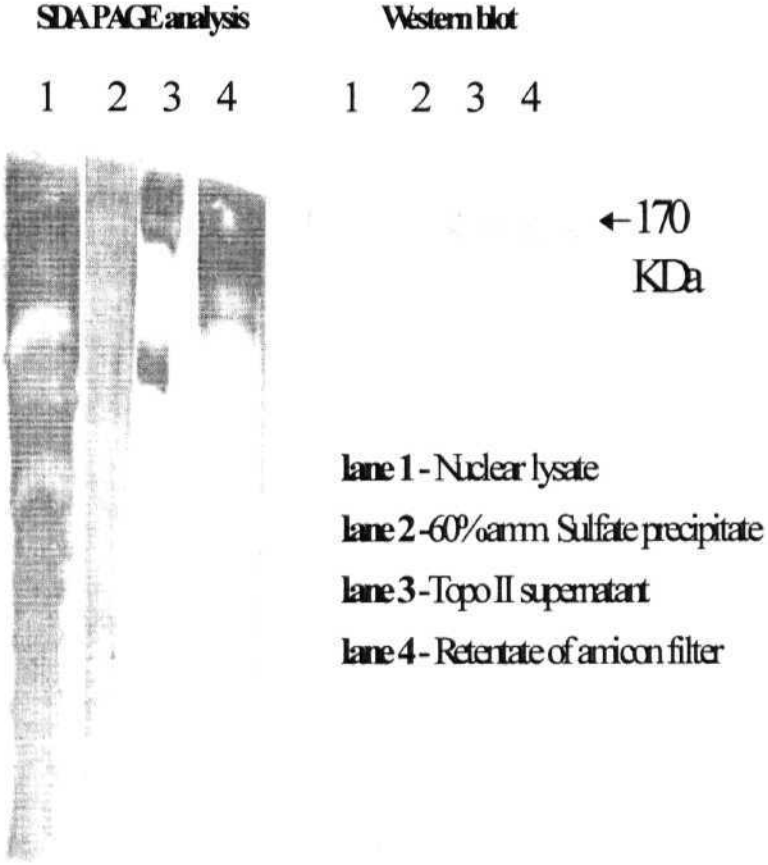
Characterization of topoisomerase II:

The purification profile of topo II is shown in a silver stained SDS-PAGE gel (10%) in The protein was confirmed by western blotting, with a monoclonal antibody against topo II a . Protein concentration was determined by the Bradford method (1976).

Enzyme activity was determined using the relaxation assay described in the 'Topoisomerase II Activity Assays' sectioa

Definition *of enzyme activity:* One unit of topo II activity is defined as the minimal amount of the enzyme required to completely relaxing 0.3 μg (0.5 nM base pairs) of negatively supercoiled pBR322 plasmid DNA in the presence of Mg^{2+} ions, in a specified period of time at 30 °C.

Purification of Topoisomerase II fromR=tli\£r



DNA Binding studies:

Thermal denaturation studies of calf thymus DNA was carried out to determine binding affinity of the metal complexes to DNA:

Calf thymus DNA (sodium salt) was dissolved in 1 mM sodium phosphate buffer containing 1 mM sodium chloride. DNA concentration was adjusted to give an absorbance of 1.0 in 1 ml at 260 nm. The metal complexes were added to DNA at concentrations, which gave drug to nucleotide ratios of 1:40, 1:20, 1:10, 1:5 and 1:1 respectively. The samples were incubated in 1 ml quartz cuvettes for 2 minutes to allow drug-DNA interaction. A Hitachi 150-20 spectrophotometer was set to give a 1 °C rise in temperature per minute with a KPC-6 thermo-programmer and SPR-7 temperature controller. Increase in absorbance at 260 nm was recorded from 40 to 90 °C. T_m was determined from the denaturation curves and the data was plotted.

Topoisomerase 11 activity assays to determine molecular mechanism of action of the metal complexes:

DNA relaxation assay:

Relaxation of the supercoiled DNA occurs in a stepwise manner. Each DNA relaxation step of topo II involves a change in the linking number of DNA by 2. Thus, in an incomplete reaction, the supercoiled DNA band (form S of DNA) disappears and a ladder of bands (each band differing in linking number by 2 from its successive band) in different stages of relaxation appear. Complete relaxation of the plasmid DNA can be visualized as a single band (form R) of DNA. This is because; all the bands in the ladder

are partially relaxed into form II DNA. In the present study, an incomplete relaxation reaction has been employed for the drug assays.

This assay was performed following the procedure of Osheroff et al. (1983). The reaction mixture (50 μL) contained relaxation buffer (50 mM Tris-HCl, pH 8.0, 120 mM KC1, 0.5 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 30 μg/mL BSA, 1 mM ATP), 0.6 μg of negatively supercoiled pRYG plasmid DNA and increasing concentrations of the RuBen drugs. The reaction was initiated by adding 2 units (~8 nmole) of topo II and incubated at 30 °C for 15 min. The reaction was stopped by adding 2μL of 10% SDS. To this, 3μL of loading dye (0.5% bromo-phenol blue, 0.5% xylene cyanol, 60% sucrose, 10 mM tris-HC1, pH 8.0) was added, and the products were separated on a 1% agarose gel in 0.5x TAE buffer (20 mM tris-acetate, 0.5 mM EDTA) at 50 V for 8 h. The gel was stained with ethidium bromide, visualized using a Photodyne UV transilluminator and photographed.

DNA Cleavage Assay:

In presence of a topo II poison, a ternary 'cleavage complex' consisting of 'cleaved DNA-drug-topo IF is formed. The formation of this complex is confirmed by treating the reaction products of the cleavage assay with SDS. This detergent treatment denatures the enzyme, thus liberating the cleaved DNA. Proteinase K treatment completely disrupts the associated enzyme with DNA. This cleaved DNA is visualized as form L (linear plasmid DNA) in an agarose gel.

The formation of cleavage complex was assayed following the procedure of Zechiedrich et al (1989). The 50 μL reaction mixture contained relaxation buffer (minus ATP), 0.6 μg

of pRYG supercoiled DNA and increasing concentrations of drugs. The reaction was initiated by adding 10 units (40 nmol) of topo II and incubated at 30 °C for 15 min. The reaction was stopped with 2 μL of 0.5 M EDTA and 2 μL of 10% SDS. The DNA bound protein was degraded by incubating the reaction mixture with 2 μL of 1 mg/ml Proteinase K at 45 °C for 1 h. The products were separated on 1% agarose gel for 8 h at 50 V in 1x TAE buffer (40 mM tris-acetate, 1 mM EDTA), stained and photographed. The linear DNA band was quantified in terms of percentage of total DNA in a UVP gel documentation system.

Anticancer Activity assay:

[^{3}H]-Thymidine incorporation by proliferating cells was the assay used for determining the *in vitro* anticancer activity of the RuBen drugs. Colo-205 (colon adeno-carcinoma), cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (A-431 cells were grown in DMEM with 10% serum. 0.2 x 10^6 cells/200 μl were distributed in triplicates in 96 well microtiter tissue culture plates. The cultures were incubated for 16 hours at 37 ^{0}C in a CO_2 incubator (Forma Scientific) maintaining 5% CO2 atmosphere. Increasing concentrations of the drugs were added to the cells in culture. The DNA intercalating topo II poison, m-AMSA, was used as a positive drug control (positive and negative controls always contained an equal amount of DMSO present in the drug treated samples). The drug treatment was stopped after 6 h by centrifugation and change of media. After this, the cultures were further incubated for 48 h. The cultures were then pulsed with 0.5 μCi of ^{3}H-thymidine and incubation was continued for 4 h to allow thymidine incorporation by the proliferating cells.. In the adhering cell cultures Colo-205

the medium was removed and the adhering cells were treated with 10 μL of trypsin-EDTA (0.25% trypsin, lmM EDTA) for 5 min at 37 °C to release the cells from the adhering surface. Trypsinization was stopped by adding 10 μL of serum to the cells. The original cultures were then added back to the wells and the cells were harvested as described above. Radioactivity was measured in a Wallac liquid scintillation counter. The mean result of three independent experiments conducted in triplicates was plotted as drug concentration versus percentage of proliferation.

Results:

The observations of the experiments performed in the presence of the ruthenium derivatives will be described as below. Various experiments revealing the topoisomerase II inhibition, DNA binding nature and antiproliferative nature of these derivatives was conducted as follows.

Topoisomerase II inhibition by ruthenium derivatives.

Relaxation assay:

The inhibition of the topoisomerase II mediated relaxation activity by the ruthenium derivatives is monitored in the relaxation assay. Increasing concentration of Ruthenium derivatives are used in this assay. The known Topoisomerase II inhibitor mAMSA used in this assay showed complete inhibition of the topoisomerase II activity at a concentration of 100μM. Various Ruthenium derivatives were tested for their topo II inhibitory activity, the results show that the pyridine 3-carboxaldehyde derivative showed complete inhibition of the relaxation activity at a concentration of 400 μM (figure 28 panel A), which is 4 times more than the standard inhibitor that is mAMSA. Pyridine 3-sulphonic acid showed partial inhibition at a concentration of 500μM (figure 28 panel B) with no further inhibition at a concentration of 600 μM. The nicotinamide derivative showed complete inhibition of relaxation activity at 400 μM concentration. (Figure 28 panel B) Where as the 3-hydroxy pyridine derivative showed partial inhibition only at 500μM concentration (figure 26 panel B).

Cleavage assay:

Topoisomerase II mediated cleavage of DNA double strand and the formation of the formation of enzyme -drug- DNA ternary complex is observed by the cleavage assay. In the presence of topoisomerase II inhibitors the enzyme interacts with the DNA and cleaves it, **but** fails to religate the double stranded break. The enzyme-inhibiting drug freezes the enzyme and the DNA in the ternary cleavage complex. The cleaved DNA in the cleavage complex is released by SDS and proteinase K treatment. The circular supercoiled plasmid DNA used in this assay is linearized and appears as the third nicked DNA band. This linear DNA is a measure of the cleavage complex formed by the topoisomerase II Inhibitors. The cleavage assay was carried out at increasing concentration of ruthenium derivatives. The results of the assay carried out in the presence of pyridine 3-carboxaldehyde showed that this derivative formed cleavage complex formation at a concentration of 300μM with a slight increase in the intensity of the nicked DNA band at a concentration of 400 and 500μM (Figure 29 panel A), the pyridine 3- sulphonic acid derivative showed cleavage complex formation at a concentration of 500μM (Figure 29 panel B), the 3- hydroxy pyridine derivative showed the nicked DNA in the cleavage assay with a drug concentration of 300μM, with an increase in the nicked DNA with increase in the drug concentration. The nicotinamide derivative showed the cleaved or nicked DNA at a concentration of 400 and 500μM. these results correlated with the drug concentrations at which the drugs showed inhibition towards the relaxation activity.

DNA binding of Ruthenium derivatives:

The DNA binding nature of the ruthenium derivatives was studied by the monitoring the melting of calf thymus DNA in the presence of ruthenium drugs. The melting temperature in the presence of drugs (at 1: 1 concentration) showed an increase. The calf thymus DNA in the absence of the drug showed a Tm value of 67 ° C where as in the presence of mAMSA, which is a DNA intercalating agent showed an increase of 8° C with a Tm value of 74 ° C. The ruthenium derivatives also showed an increase in the Tm value indicating the DNA binding nature of these drugs (figure 30). In the presence of Ruben pyridine carboxaldehyde the Tm value was found to be 71, RuBen pyridine 3-sulphonic acid showed a Tm of 73, Ruben 3- hydroxy pyridine showed a Tm of 75 and in the presence of Ruben nicotinamide the Tm was found to be 78. The change in the Tm values indicated that tiiese compounds have DNA binding ability.

Anti proliferative MTT assay:

MTT assay was carried out to check if the ruthenium derivatives show anti proliferative or cytotoxic activity. Colo- 205 cells which are stable in terms of proliferation and reproducibility of drug assays were used. The results of this assay suggest that the RuBen pyridine 3-carboxaldehyde, RuBen Pyridine 3-sulphonic acid,, RuBen 3- hydroxy pyridine and Ruben nicotinamide show inhibition almost 90-100 % inhibition of the proliferation of the Colo cancer cells at concentrations ranging from 300- 350μM (figure 31).

Molecular modeling analysis:

The modeling was done using SPARTAN software. The molecular models show that the coordinated groups are in different structural orientation. The different orientation of these groups may contribute to their interaction with the enzyme. The group pyridine 3-carboxaldehyde show an orientation which is coplanar with the organometallic bond and these complexes have shown inhibition of topo II activity at lower concentration when compared to pyridine 3- sulphonic acid, nicotinamide and 3- hydroxy pyridine in which the groups are away from the organometallic bond of Ruthenium atom (figure 32).

Discussion:

Topoisomerase II is a major nuclear enzyme that maintains the DNA topology in the complex chromosomal environment. The enzyme resolves numerous torsional problems in the DNA, which arise during replication and transcription. It also helps in catenation / decatenation, condensation/ decondensation of DNA and segregation of chromosomes during cell division. The enzyme performs these functions by nicking a segment of DNA passing second strand through a gate formed by the nicked DNA and finally rejoining the nicked segment. This DNA breaking activity of topoisomerase II results in loss of genetic integrity and therefore is the target for anticancer drugs. These Topoisomerase II drugs inhibit the catalytic activity of the enzyme by enhancing the cleavage complex formation and block the religation activity. Cancer cells, which over express topoisomerase II when treated with the inhibitors will harbour numerous Topoisomerase II, induced DNA breaks formation. The damaged DNA Stimulates repair and recombination pathways leading to sister chromatid exchange, large insertions/ deletions translocations and chromosomal aberrations. The accumulation of these aberrations triggers a series of events, which ultimately result in cell death by apoptosis and necrosis.

The present study show that ruthenium compounds can be attributed the anticancer activity because of their topoisomerase II inhibition. Earlier studies on the ruthenium compounds like the RuBen (dmso) and derivatives have implicated this molecule as a potent anticancer drug, whose molecular target is topoisomerase II. This particular molecule served as a parent molecule for the development of potent derivatives in which the topoisomerase II interacting DMSO group will be replaced by the other ligands. Further studies in this area lead to the development of derivatives of ruthenium in which

the dmso group is substituted with pyridine, 3-aminopyridine, aminobenzioc acid or aminogaunidine. These four molecules showed DNA binding capacity as well as Topoisomerase II inhibition at concentrations less than the RuBen (dmso) derivative. These derivatives showed potential anticancer activity also. Among these derivatives the amino group containing amino pyridine, amino guanidine and amino benzoic acid, showing that the amino group may be responsible for interaction with Topoisomerase II. [Vashisht Gopal et al 2002]. These studies showed that the spatial orientation shown by the single amino group containing RuBenApy made it a potent inhibitor for Topoisomerase II. The conformation and the special orientation of the interacting amino group is responsible for the cleavage complex formation. The Ruthenium atom in the complex binds to the DNA and the ligand binds to the topoisomerase II.

It was previously suggested that in aqueous solution the chlorides and the dmso groups on the RuBen dmso hydrolyze and the complex exists in an equilibrium between the aqua and the chloro ions as the Ru (C_6H_6) (H2O). Hydrolysis of the chloride leaving groups enables the interaction of the Ruthenium atom with nucleotide phosphate of the DNA double strand. Long-lived DNA/ protein interaction and eventual hydrolysis of the Ruthenium complex, which leads to the formation of the covalent cross-links. Hydrolysis of the coordinated groups is an important determinant of the macromolecular interaction. by theses complexes.

In the present study we have made derivatives of ruthenium benzene in which the amino pyridine group is replaced with substituted pyridine with the carboxaldehyde, hydroxy, suphonic acid and nicotinamide groups. The DNA binding studies reveal that the derivatives have strong DNA binding affinity. All the ruthenium derivatives showed

similar DNA binding affinity suggesting that the ruthenium atom interacts with the DNA. Their interaction with DNA may be ionic bonding with the phosphate backbone or covalent binding of nucleotide without disturbing the helix. The ruthenium derivatives do not show an intercalative mode of DNA binding.

The results of the Topoisomerase II inhibition experiments help in proposing a probable mechanism for the inhibitory activity of the ruthenium derivatives. The metal atom interacts with covalently or non covalently with DNA nucleotides and the ligands form cross links with the enzyme and prevent DNA religation leading to the formation of stable drug induced cleavage, which is a characteristic of the topoisomerase II inhibitors. Such topoisomerase II inhibitors forming the cleavage complex formation will harbor the DNA double strand breaks. The accumulation of such double strand breaks in the cells ultimately leads to the cell death by apoptosis/necrosis.

The molecular modeling analysis shows that the complexes with groups, which are oriented in plain with the organomettalic bond of the ruthenium atom, show higher topo II inhibition when compared to the complexes with groups going away from the organomettalic bond. The interaction of the metal complexes with topo II enzyme could be determined by the orientation of the groups in the complexes.

Conclusions

- The Topoisomerase II isoforms shows significant changes in rat brain tissue. The Topoisomerase II a is found to be negligible in rat brain, where as the β isoform is predominantly present. Topoisomerase II β is expressed at different levels in various regions of brain showing the highest level of expression and activity in the cerebellar region. This observation is confirmed by immunohistochemical analysis. An age dependent change is observed in the levels of Topoisomerase II P with maximum protein seen in the young rats. Among the two cell types studied The cerebellar neurons showed the highest activity when compared to the astrocytes. Topo II β activity in cerebellar neurons shown to be decreased with increasing age.
- The activities of the topoisomerase II a and β have shown variation in different tissues with the a isoform showing negligible activity in the brain tissues of young adult and old rats. In Liver and testes tissues the activity is found to be high indicating higher level 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 activity with age. The topoisomerase II β activity is found to be high in all the three tissues with variation seen in the brain tissue with age. As reported earlier the young extracts of brain have shown highest activity of topoisomerase II p. There was a slight decrease in the activity of topoisomerase II β in the adult rats when compared to young and old rats. No much change is seen in the testes extracts of

the three age groups for the topoisomerase II β activity. Topo II a shows high sensitivity towards Topo II poisons than the topoII β.

- RuBen Pyridine 3-carboxaldehyde indicate that this particular derivative inhibit the DNA relaxation activity of topoisomerase II at a concentration of 400 and 500 |iM The Ruben pyridine 3- sulphonic acid showed inhibition of the relaxation activity at a concentration of 500 μM with no increase in the inhibition at higher concentrations. The Ruben 3- hydroxy pyridine showed inhibition of the relaxation activity at a concentration of 400 μM with no further increase of inhibition seen at higher concentrations. The RuBen nicotinamide derivative showed inhibition of the relaxation activity at a concentration of 500 μM Cleavage activity was also shown by the complexes at concentrations similar to that of the concentrations at which they show inhibition of the relaxation activity. These derivatives have shown DNA binding property by changing the melting temperature from 67 to 70, 71,75,78 by the Ruben pyridine3- carboxaldhyde, RuBen pyridine 3- sulphonic acid, RuBen 3- hydroxy pyridine, Ruben nicotinamide. The derivatives have shown antiproliferative activity at concentrations of 300- 350 μM with Ruben pyridine3- carboxaldhyde, Ruben pyridine 3- sulphonic acid, Ruben 3- hydroxy pyridine, RuBen nicotinamide respectively. Analysis of Topo II poisoning by RuBenPy derivatives, show that the interaction group of Pyridine should be in plane with ruthenium atom so as to allow its interaction with the enzyme.

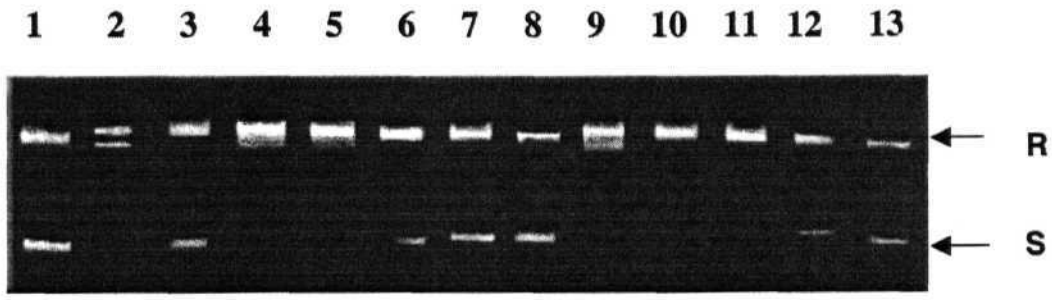
Figure 28

Relaxation assay in presence of ruthenium derivatives;

~0.6 mg of supercoiled DNA (lane1) was incubated with Topo II in absence (lane 2) or **presence** of 100μM mAMSA **(lane 3) of 100,200,300, 400 and 500** μM RuBenCHO (panel A lane 4-8), RuBen SA (panel A lane 9-13), RuBen OH (panel B lane 4-8), RuBen NA (panel B lane 9-13). The products were resolved on 1% agarose gel, ethidium bromide stained and photographed.

Figure 28

A



B

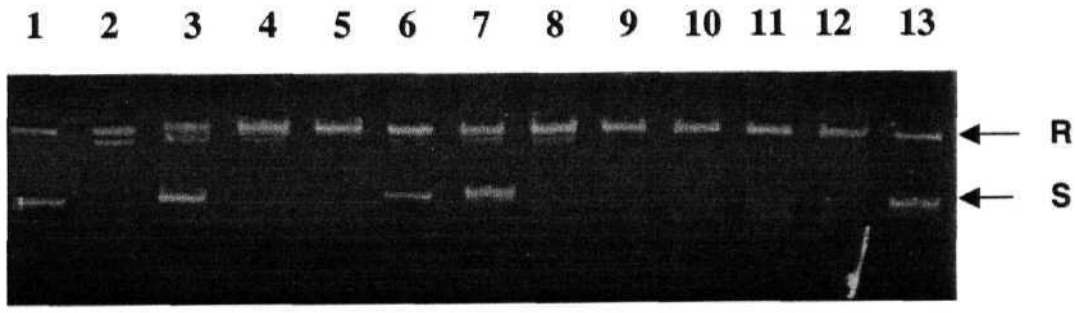


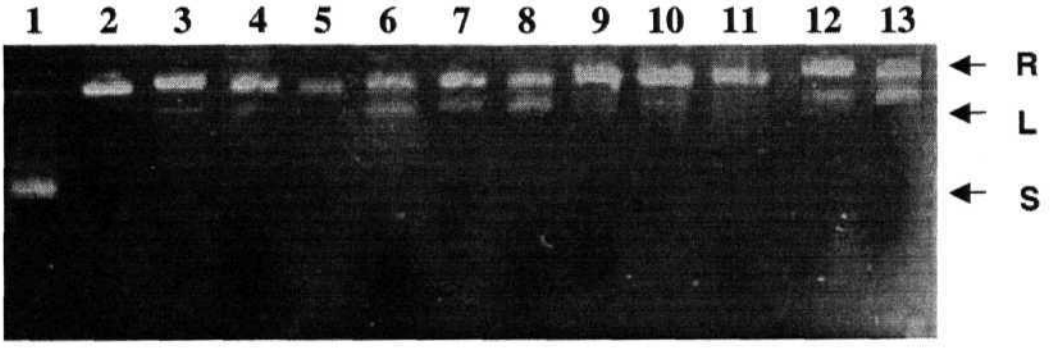
Figure 29

Cleavage assay in presence of ruthenium derivatives:

Cleavage assay was performed by incubation supercoiled DNA with topo II in absence (lane 2) or presence of 100μM mAMSA (lane 3) of 100,200,300, 400 and 500 μM RuBenCHO (panel A lane 4-8), RuBen SA (panel A lane 9-13), RuBen OH (panel B lane 4-8), RuBen NA (panel B lane 9-13). The products were resolved on 1% agarose gel, ethidium bromide stained and photographed.

Figure 29

A



B

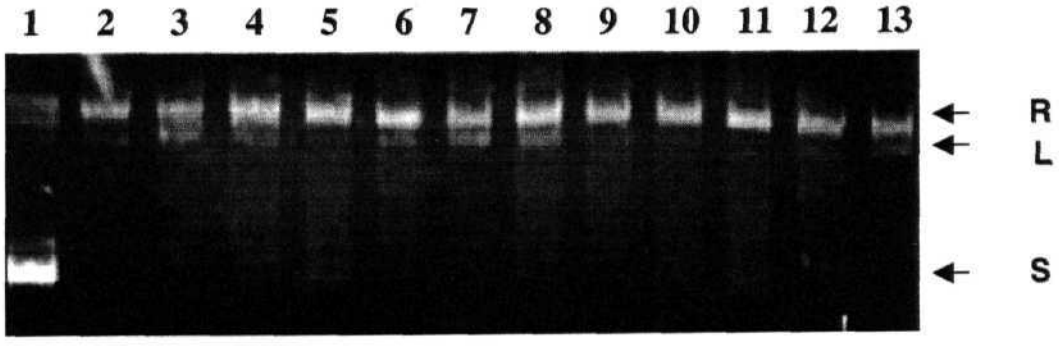


Figure 30

Tm analysis was carried out with the ruthenium derivatives and the values obtained are graphically represented in the figure. The changes in the Tm values are clearly seen with the shift in the peak.

Figure 30

Melting temperature studies

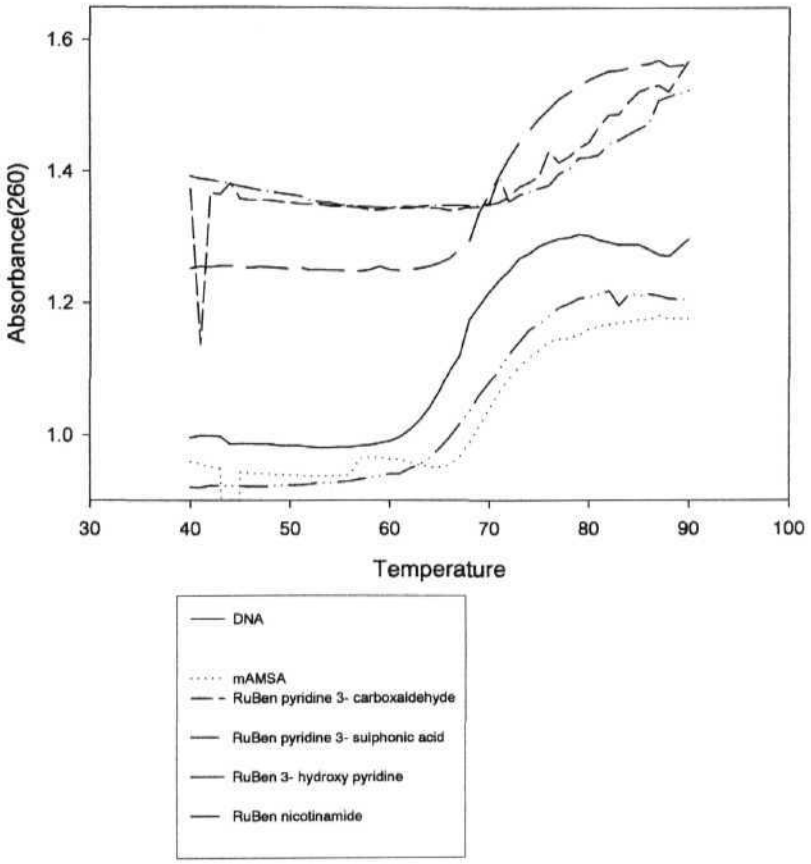


Figure 31

The anticancer activity of Ruben CHO, Ruben SA, Ruben oh and Ruben NA was examines using MTT assay on colon cancer cells. The results of the study show the antiproliferative potency of the ruthenium derivatives. The highest potency is shown by mAMSA.

Figure 31

MTT assay

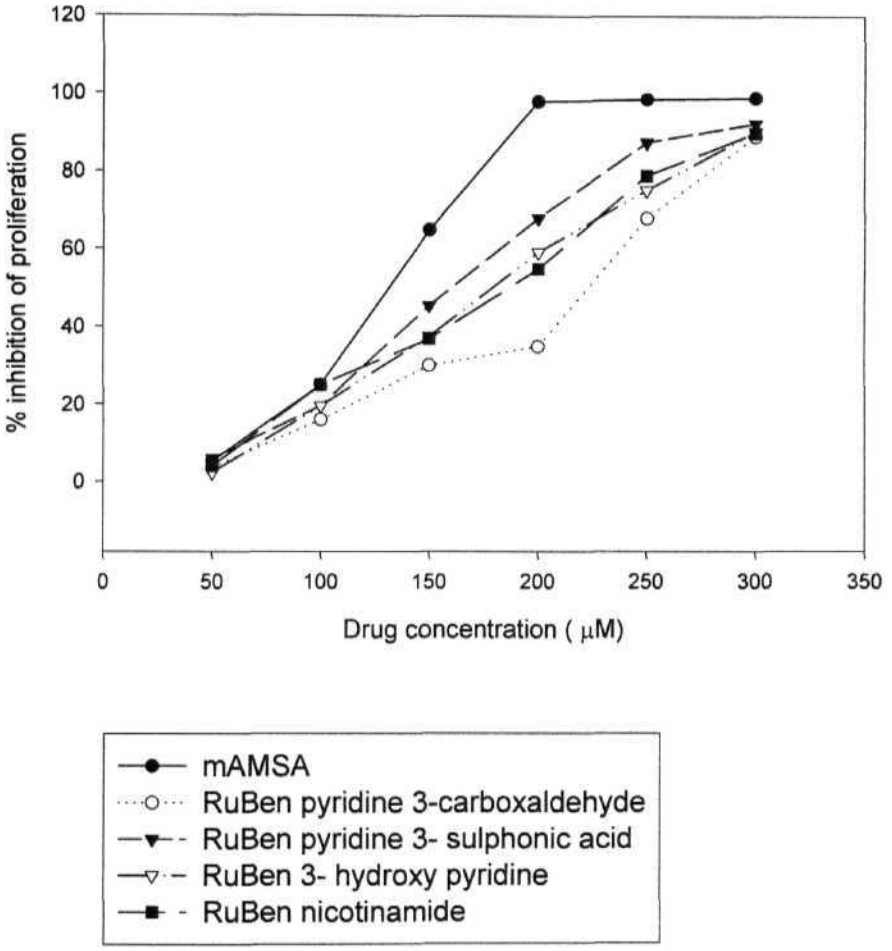


Figure 32

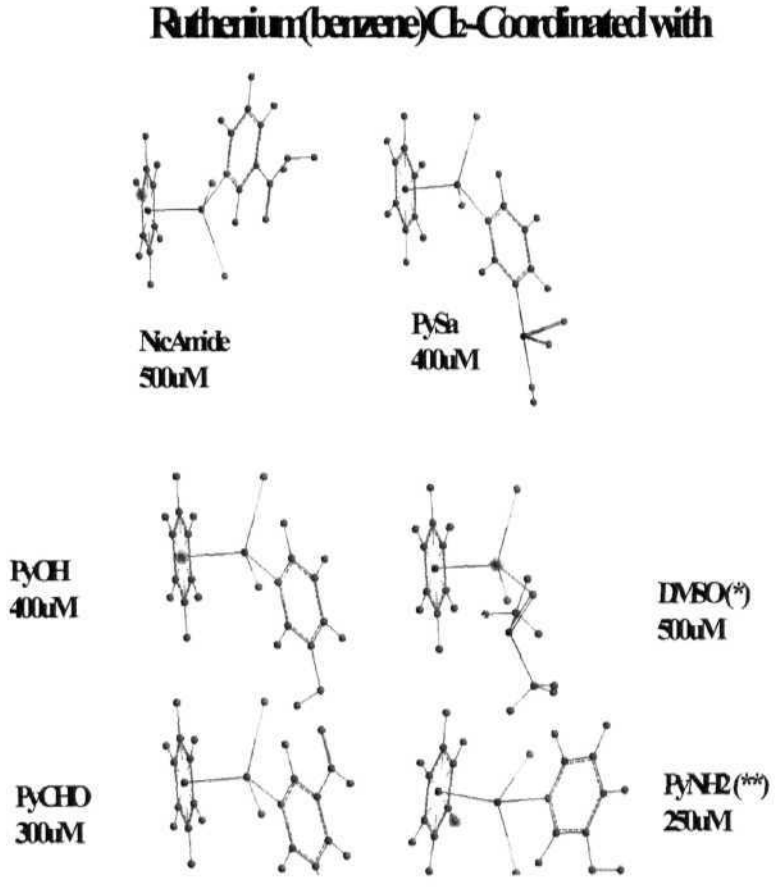

Ruthenium(benzene)Cl2-Coordinated with
NicAmide
500uM
PySa
400uM
PyOH
400uM
DMSO(*)
500uM
PyCHO
300uM
PyNH2(**)
250uM

Figure 32

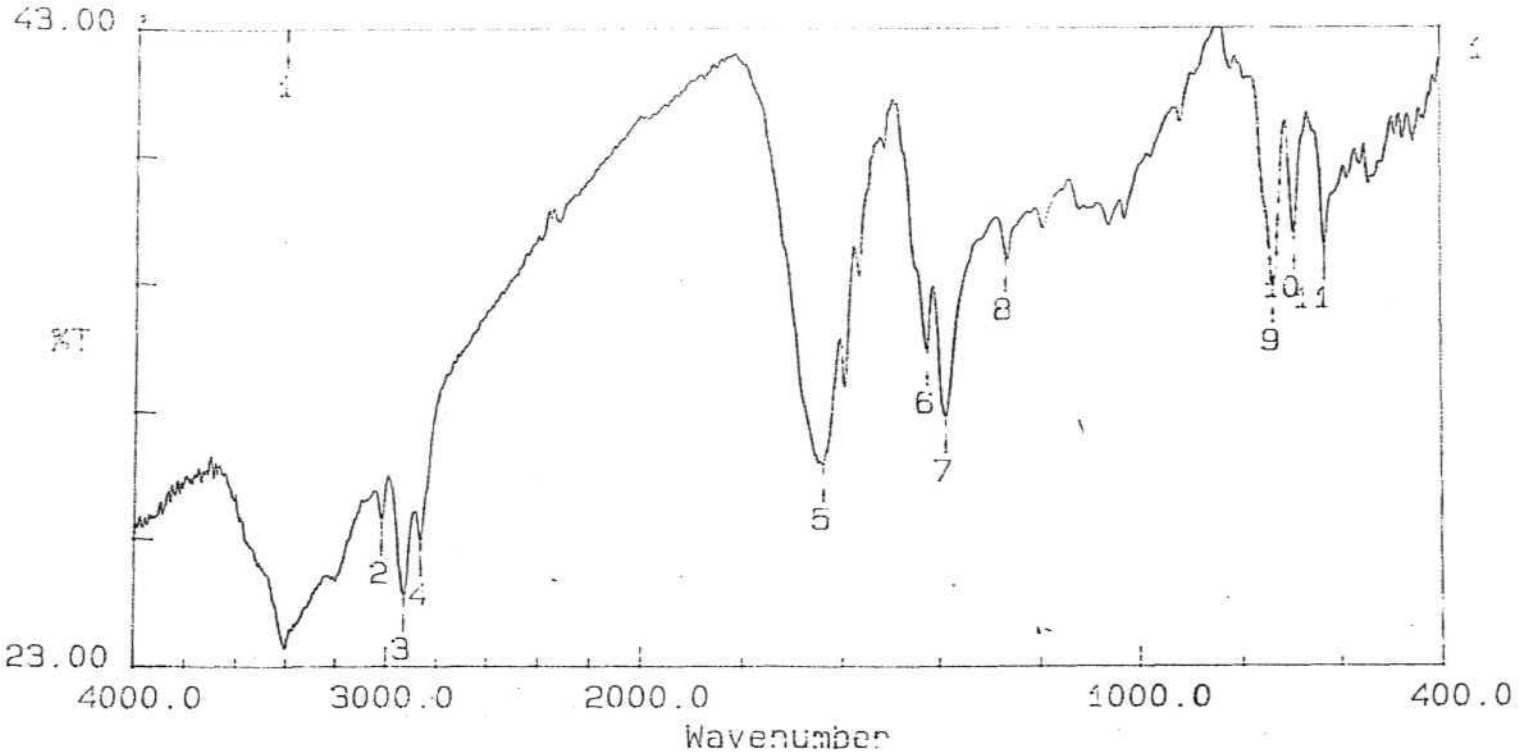
The molecular models of Ruben NA, Ruben DMSO, Ruben Apy, Ruben CHO, Ruben OH and Ruben SA show the 3- dimensional orientation of the groups around the organometallic bond of ruthenium. The angular orientation may be responsible for the complexes to intact with the enzyme and align them in to the active sit.

JASCO FT/IR-6300 02/04/19 12:21

File Name : Sample Name : 7BIOCHM/KBR

Resolution : 4 Scans : 4 Gain : 5 Apodization : CS

RUBEN OH



Condition

upper 43.00 lower 23.00 depth 2.00

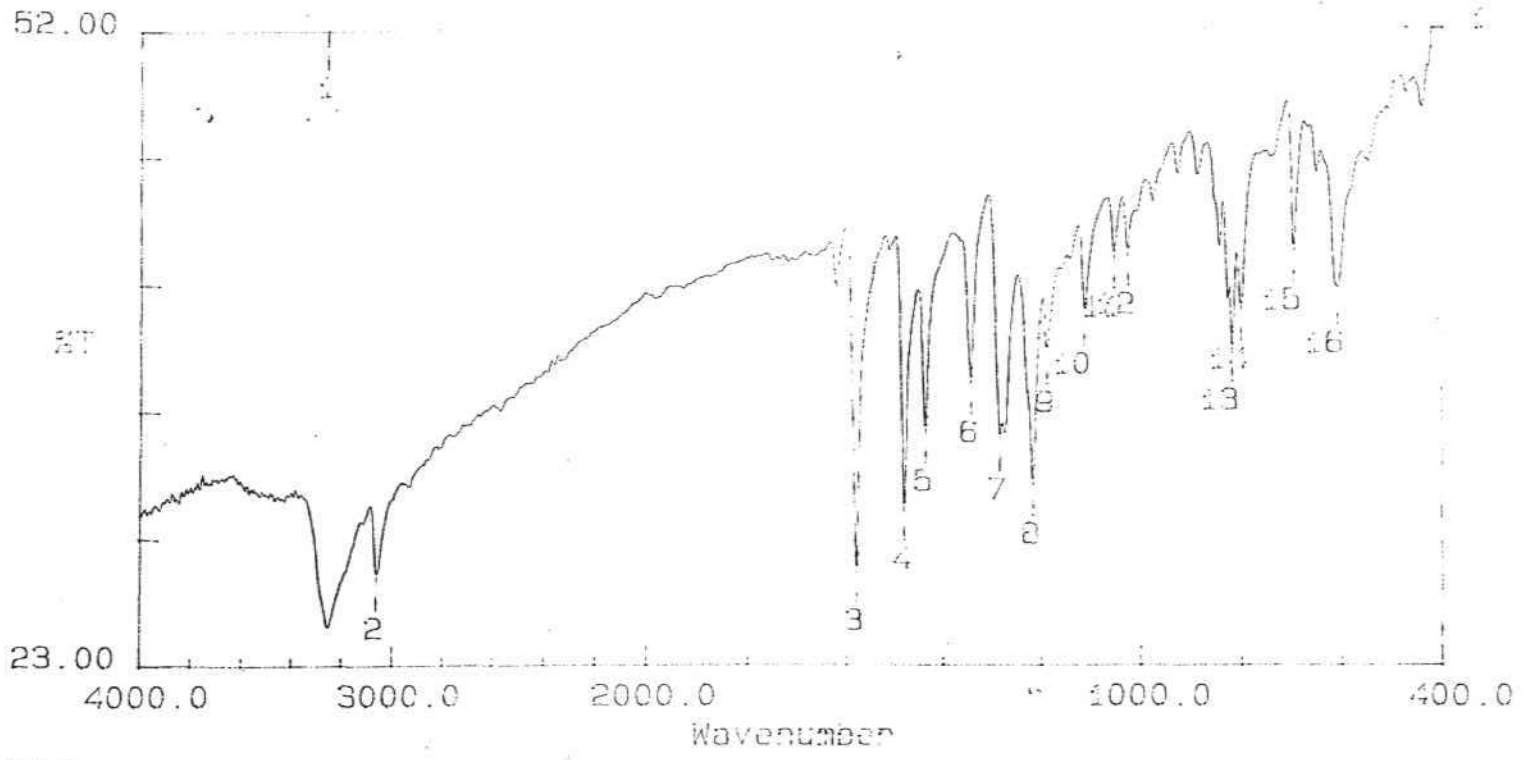
Peak table

1:	3404.67 (23.6)	2:	3016.94 (27.7)	3:	2928.21 (25.3)	4:	2860.69 (27.0)
5:	1637.71 (29.4)	6:	1423.59 (33.1)	7:	1386.94 (31.0)	8:	1269.28 (36.0)
9:	736.67 (35.0)	10:	692.51 (36.6)	11:	630.72 (36.3)		

RUBEN SA

File Name : Sample Name :

Resolution : 4 Scans : 4 Gain : 5 Apodization : 0.5



Condition

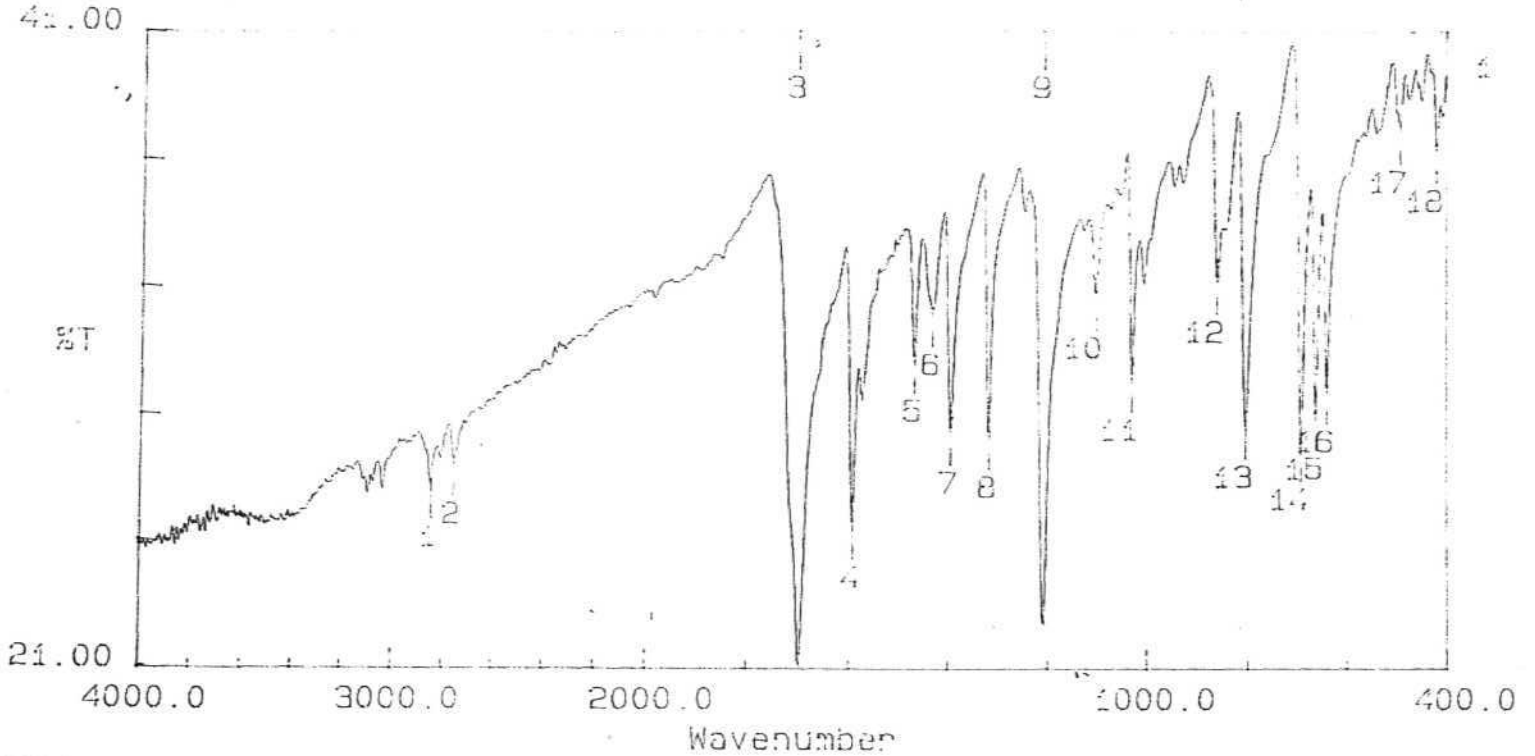
upper 52.00 lower 23.00 depth 2.00

Peak table

1:	3254.21 (24.8)	2:	3063.24 (27.3)	3:	1577.91 (27.6)	4:	1479.53 (30.5)
5:	1435.17 (34.0)	6:	1342.58 (36.2)	7:	1284.71 (33.6)	8:	1217.19 (31.6)
9:	1188.26 (37.6)	10:	1111.10 (39.3)	11:	1053.23 (42.0)	12:	1026.22 (42.1)
13:	819.82 (37.7)	14:	802.46 (39.5)	15:	696.37 (42.2)	16:	609.56 (40.3)

File Name : Sample Name : RuBen CHO

Resolution : 4 Scans : 4 Gain : 5 Apodization : CS



Condition

upper 41.00 lower 21.00 depth 2.00

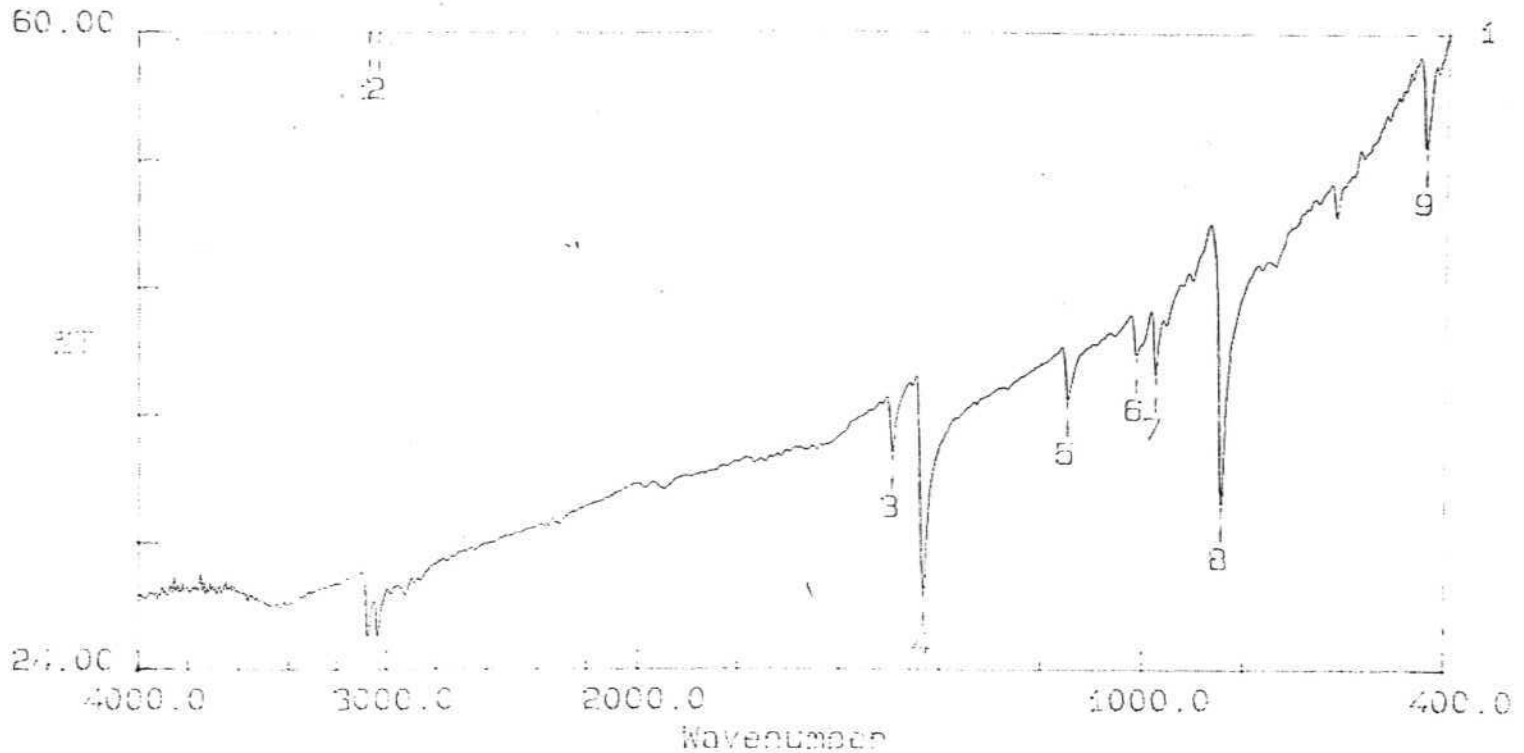
Peak table

1:	2847.19 (27.0)	2:	2754.60 (27.6)	3:	1697.51 (21.1)	4:	1591.42 (25.7)
5:	1466.03 (30.9)	6:	1429.38 (32.4)	7:	1392.73 (28.7)	8:	1315.57 (28.5)
9:	1207.55 (22.4)	10:	1103.38 (32.9)	11:	1030.08 (30.3)	12:	860.33 (33.4)
13:	804.39 (28.8)	14:	694.44 (28.1)	15:	663.57 (29.1)	16:	640.42 (30.0)
17:	493.82 (38.0)	18:	420.52 (37.4)				

[illegible] FT/IR-6300 02/04/19 11:[illegible]

File Name : Sample Name : 1-BIOCHEM RUBEN DIMER

Resolution : 4 Scans : 4 Gain : 5 Apodization : CS



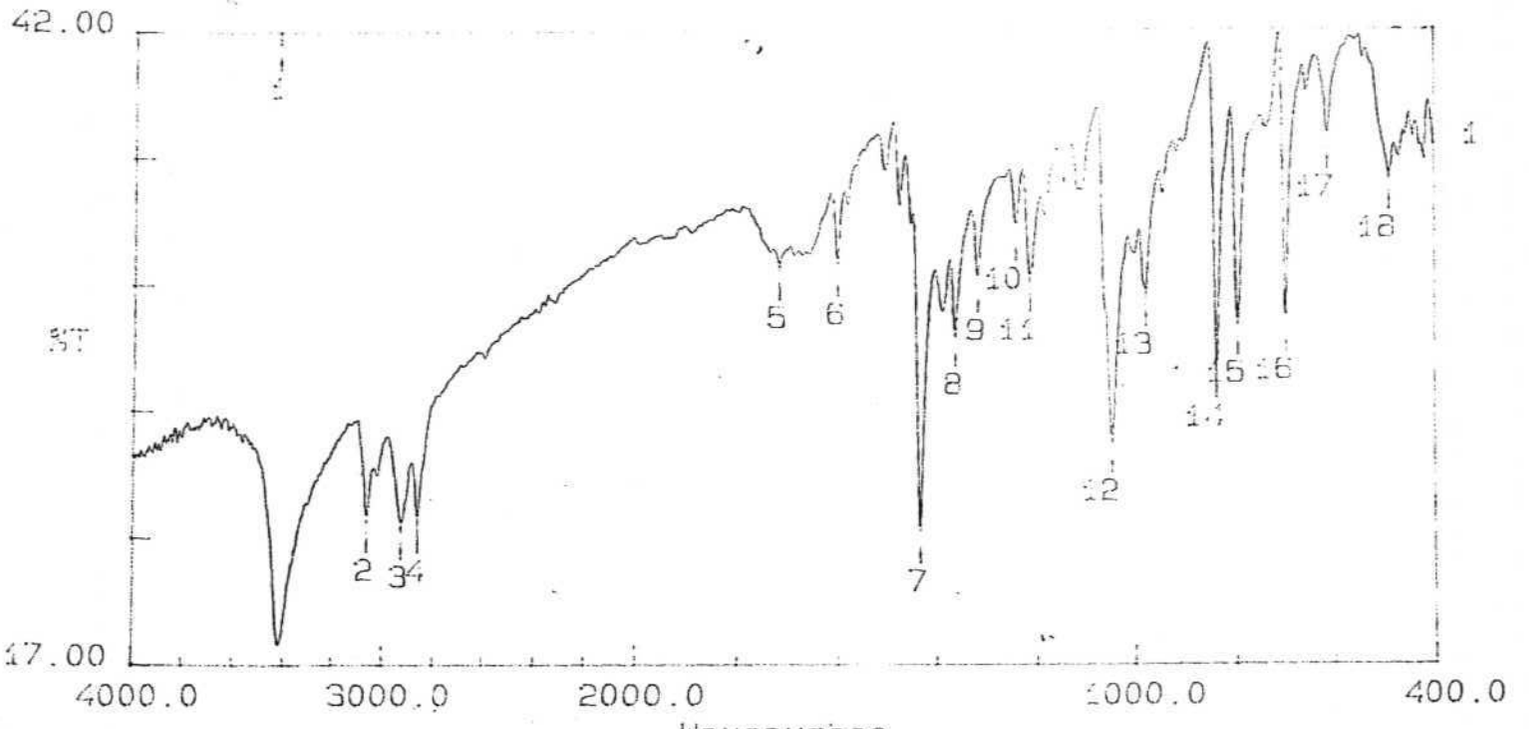
Condition

Upper 60.00 Lower 24.00 depth 2.00

Peak table

1:	3072.28 (26.0)	2.	3034.30 (26.0)	3.	1481.97 (36.4)	4.	1431.31 (28.7)
5:	1347.78 (39.1)	6.	1012.72 (42.0)	7:	974.14 (40.8)	8.	842.97 (33.6)
9:	439.24 (53.6)						

JASCO FT/IR-6300 02/04/19 12:27
File Name : Sample Name : [illegible] RUBEN NA
Resolution : 4 Scans : 4 Gain : 5 Apodization : CS



Condition
upper 42.00 lower 17.00 depth 2.00

Peak table

1:	3418.17 (17.8)	2:	3061.31 (23.0)	3:	2924.35 (22.7)	4:	2856.83 (23.0)
5:	1714.87 (33.0)	6:	1599.13 (33.1)	7:	1433.24 (22.5)	8:	1361.87 (30.3)
9:	1317.50 (32.4)	10:	1242.27 (34.5)	11:	1213.33 (32.4)	12:	1045.51 (26.1)
13:	972.00 (31.9)	14:	839.11 (29.0)	15:	796.67 (30.7)	16:	698.29 (30.8)
17:	613.42 (38.0)	18:	489.96 (36.4)				

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1. Y,N,Vashisht Gopal, **Neelima Konuru**,Anand K. Kondapi
   Topoisomerase II antagonism and anticancer activity of coordinated derivatives of $[RuCl_2(C_6H_6)(dmso)]$
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   Analysis of age dependent changes of Topoisomerase II **a** and (3 in rat brain.
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1. Ahmed Kamal, N. Laxman, G. Ramesh. **K. Neelima** and Anand K Kondapi
   Synthesis of novel non-cross linking pyrrolobenzodiazepines with remarkable DNA binding affinity and potent anti tumor activity
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Analysis of age dependent changes of Topoisomerase II α and β in rat brain

Anand K. Kondapi [a,*], Neelima Mulpuri [a], R.K. Mandraju [a], B. Sasikaran [b], K. Subba Rao [a]

[a] *Department of Biochemistry and Center for Research on Ageing and Brain, University of Hyderabad, Hyderabad 500046, India*
[b] *Department of Pathology, National Institute of Nutrition, Jamai Osmania, Hyderabad 500007, India*

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Abstract

Eukaryotic Topoisomerase II (Topo II) is present in two isoforms α and β. The α isoform is predominantly localized in proliferative tissue, while β isoform is present in all tissues. In the present study we report the activity and protein levels of Topoisomerase II α and β in rat brains of different age groups viz.: E11 (Embryo day 11), E18 (Embryo day 18), post-natal day 1, young (<10 days), adult (<6 months) and old (>2 years). Topoisomerase II β isoform is found to be the predominant form in brain tissue but Topoisomerase II α is found in embryos up to post-natal day 1. The studies to examine the regional distribution of Topoisomerase II β in brain showed highest activity in cerebellar region and that too only neuronal cell fraction. There was a significant age-dependent decline in this activity. Hence, Topoisomerase II β may have some unknown function in cerebellum and the low levels of Topoisomerase II β activity in ageing cerebellum may contribute to the genomic instability in cerebellar region of ageing brain.

Keywords: Brain; Cerebellum; Neurons; Ageing; Topoisomerase II β

1. Introduction

Topoisomerase II (Topo II) is a nuclear enzyme playing a key role in DNA replication, transcription, chromosome condensation, genetic recombination and repair (Wang, 1996). In mammals, Topo II is found to be present as 170 kDa, α and 180 kDa, β isoforms (Drake and Hofmann, 1989). Both the isoforms show structural similarity but are genetically, immunologically and biochemically distinct. They show distinct cellular localization and cell cycle expression profiles. Topo II α activity is shown to be highest during the G_2/M phase of the cell cycle (Woessner et al., 1991). On the other hand, Topo II β activity is constant throughout the cell cycle. Topo II α is distributed in the nucleoplasm, whereas Topo II β is localized in the nucleolus during interphase, and in the cytoplasm during mitosis (Chaly et al., 1996). In the embryonic stage, Topo II β in the brain is a nucleoplasmic enzyme showing higher levels of expression in the differentiating neurons (Tsutsui et al., 2001). Both the isoforms show different patterns of tissue distribution. Topo II α is shown to be higher in testes, spleen, bone marrow and liver. Topo II β is detected in high levels in differentiated tissue like brain (Jurenke and Holden, 1993; Capranico et al., 1992). However, a systematic study to examine the levels of the isoform of Topoisomerase II, α and β in different regions and cell types of brain is lacking.

We have been interested in the DNA repair mechanisms in ageing brain (Rao, 2002; Rao et al., 2000) and also Topo II as possible drug target (Gopal et al., 1999, 2002). Therefore a systematic study of the levels of Topo II α and β in different regions and in two cell types (neurons and astrocytes) in rat brain of different ages has been taken-up. We report the age-associated changes in the protein and activity levels of the Topo II β isoform in different regions of brain. The changes in the levels of Topo II β at the cellular level were also studied, viz. neurons and astroglia from whole brain, cerebellum, and cerebral cortex. The results of our experiments show that Topo II β is the only isoform that is significantly present in rat brain and that too predominantly in cerebellar neurons.

2. Experimental procedures

2.1. Materials

Topo II α and β monoclonal antibodies were obtained from Pharmingen. Goat–anti mouse IgG and FITC

* Corresponding author. Tel.: +91-40-3010-500x4571; fax: +91-40-3010-120/145.
E-mail address: akksl@uohyd.ernet.in (A.K. Kondapi).

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conjugated Goat–anti mouse IgG was obtained from Bangalore Genei, India. Nonidet, ATP were from Boehringer Manhheim. PMSF, BSA, protein A agarose, Triton X-100, ficoll, DTT, trypsin and trypsin inhibitor were from Sigma, USA, PVDF membrane was obtained from PALL Life Sciences, USA.

2.2. Preparation of tissue extracts from embryos

Tissue extracts were prepared from cerebellum of E11 (Embryo day 11), E18 (Embryo day 18) and post-natal day 1 pups by sacrificing them through decapitation and collecting the cerebellum tissue. At E11 stage there is no organ differentiation so we have taken cephalic region of the embryos as brain source. The tissue was homogenized in extraction buffer (20 mM Tris–HCl pH 7.5, 0.1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 0.1% Triton X-100, 0.5 mM KCl, 0.5 mM PMSF and 1 μg/μl pepstatin and leupeptin.). The homogenate was kept at 4 °C for 1 h and centrifuged at 100,000 × *g* for an hour in an ultracentrifuge. The supernatant containing the cytosolic and nuclear proteins was used as a source for Topo II.

2.3. Preparation of tissue extracts from whole brain

Whole tissue extracts were prepared from young (<10 days), adult (~6 months), old (>2 years) rats. The brain, liver and testes tissue were collected separately. The tissue was homogenized in extraction buffer (20 mM Tris–HCl pH 7.5, 0.1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 0.1% Triton X-100, 0.5 mM KCl, 0.5 mM PMSF and 1 μg/μl pepstatin and leupeptin.). The homogenate was kept at 4 °C for 1 h and centrifuged at 100,000 × *g* for an hour in an ultracentrifuge. The supernatant containing the cytosolic and nuclear proteins was used as a source for Topo II.

2.4. Isolation of neurons and astroglia

Neurons and astrocytes were isolated from brain tissues of rats that are collected after decapitation essentially as per the

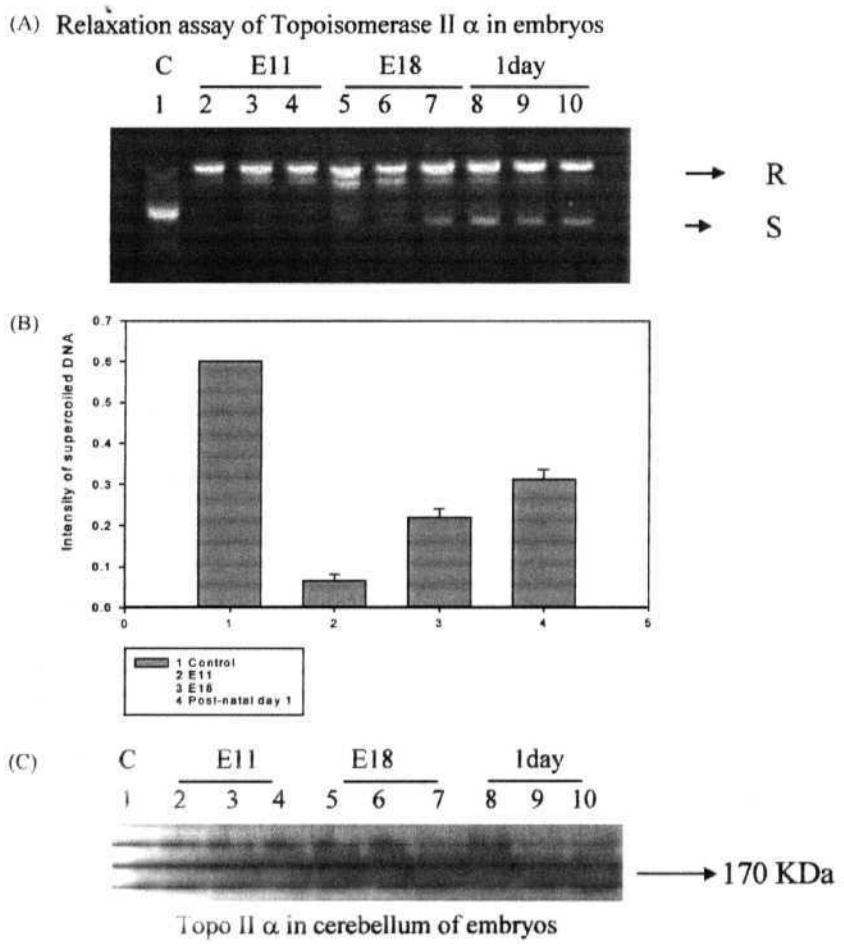

Fig. 1. Enzymatic activity and [illegible] of Topo II α in cerebellum of rat embryos: activity: Topo II α activity was measured in 100 μg protein of cerebellum extracts of E11 [illegible] E18 (lanes 5–7) and post-natal day 1 (lanes 8–10) rat pups, lane 1 shows ~0.6 μg of PRYG plasmid DNA. Activity of Topo II α in cerebellum of [illegible] E18 and post-natal day 1 shown as gel in panel A, corresponding quantified supercoiled DNA is shown in panel B. Protein: 75 μg of protein [illegible] was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against Topo II α, immunoblot of Topo II α [illegible] panel C, lane 1 shows 25 μg of Topo II α positive control.

procedure of Usha Rani et al. (1983). The brain tissue was placed in isolation medium (10 mM KH_2PO_4, 8% glucose, 5% fructose PH 6.0) and cleared of blood vessels and minced well. Young rat brains were incubated with medium; adult and old brains were incubated with medium containing 0.1% trypsin for 30 min. After incubation the trypsin medium was decanted and 0.1% trypsin inhibitor was added and left on ice for 10 min. Tissue was passed through 103, 80 and 48 μm nylon meshes and rinsed with isolation medium thrice. Before washing the tissue was minced well on the mesh using a flat glass rod. Filtrate was centrifuged at 800 × *g* for 15 min the supernatant is decanted and the pellet was resuspended in 7% ficoll medium and centrifuged at 300 × *g* for 10 min and pellet containing the neurons was suspended in 2% ficoll medium. The supernatant containing the astrocytes was diluted with 2% ficoll medium and centrifuged at 1100 × *g* for 10 min. Astrocyte pellet with was suspended in 2% ficoll isolation medium. Both the suspensions were centrifuged at 1500 × *g* for 10 min and the pellets are further washed with medium with out ficoll followed by washing with PBS. The final pellet containing the neurons and the astrocytes was suspended in required amounts of extraction buffer then vortexed and was kept at −20 °C overnight. These suspensions were sonicated and spun down at 100,000 × *g* for 1 h.

2.5. Immunoprecipitation of Topoisomerase II isoforms

Brain extracts (100 μg total protein) prepared from cerebellum of embryos (E11, E18 and 1 day old) and whole brain, cerebellum, cerebral cortex and midbrain regions of the young, adult and old age groups were taken in Eppendorf tubes for immunoprecipitation and Topo II α or β antibody (1:1000 dilution in Immunoprecipitation buffer containing 100 mM Tris–HCl pH 8, 750 mM NaCl, 2 mM EDTA, 1 mM PMSF, 0.75% Nonidet) was added to each sample. The antigen–antibody mixture was incubated at room temperature for 1 h and 25 μl of 6% protein A agarose beads were added. The beads were incubated at 4 °C for 15 min, spun down and the supernatant was removed. The protein A agarose beads were washed twice with 0.5% Triton X-100 in PBS. The beads were directly used for monitoring the relaxation activity of Topo II. The total amount of protein was constant as assessed from the Western blot analysis of β-actin.

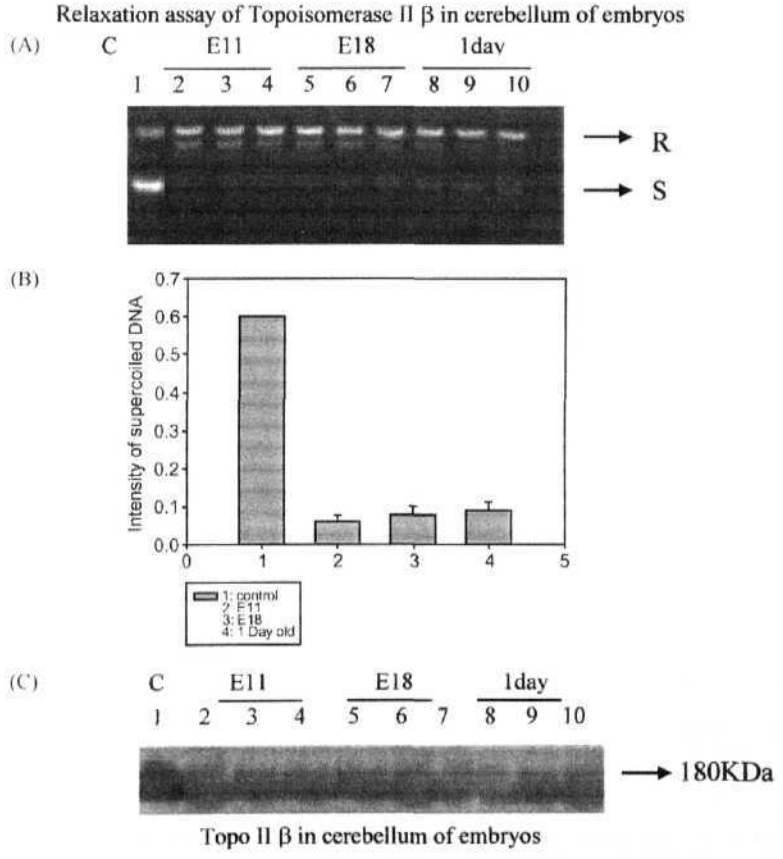

Fig. 2. Enzymatic activity and immunoblotting analysis of Topo II β in cerebellum of rat embryos: activity: Topo II β activity was measured in 100 μg protein of extract of E11 (lanes 2–4), E18 (lanes 5–7) and post-natal day 1 (lanes 8–10) rat pups, lane 1 shows ~0.6 μg of pRYG plasmid DNA Activity of Topo II β in cerebellum of E11, E18 and post-natal day 1 shown as gel in panel A, corresponding quantified supercoiled DNA is shown in panel B. Protein: immunoblot of Topo II β was shown in panel C.

2.6. DNA relaxation assay

DNA relaxation by Topo II involves the change in the linking number of DNA by 2. During relaxation the super coiled DNA band (Form I) disappears and completely relaxed plasmid DNA (form II) appears. About 0.6 μg of super coiled plasmid DNA is incubated with the immunoprecipitated Topo II captured on to the Protein A agarose beads in relaxation buffer (50 mM Tris–HCl pH 8.0, 120 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 30 μg/ml BSA, 1 mM ATP) for 30 min at 37 °C. The beads were spun down at 300 × *g* for 5 min and the supernatants were collected separately. The reaction was stopped by addition of 10% SDS and the DNA products were resolved on 1% agarose gel and stained with ethidium bromide and photographed.

2.7. Immunoblotting analysis

Seventy-five micrograms of total protein of brain tissue extracts were electrophoresed on a 7.5% SDS polyacrylamide gel and transferred on to polyvinyl diphenyl fluoride (PVDF) membrane for immunoblotting analysis. Blot was incubated with 5% non-fat dry milk powder solution in Tris-buffered saline (TBS: 10 mM Tris and 150 mM NaCl) to block the non-specific binding. The membrane was

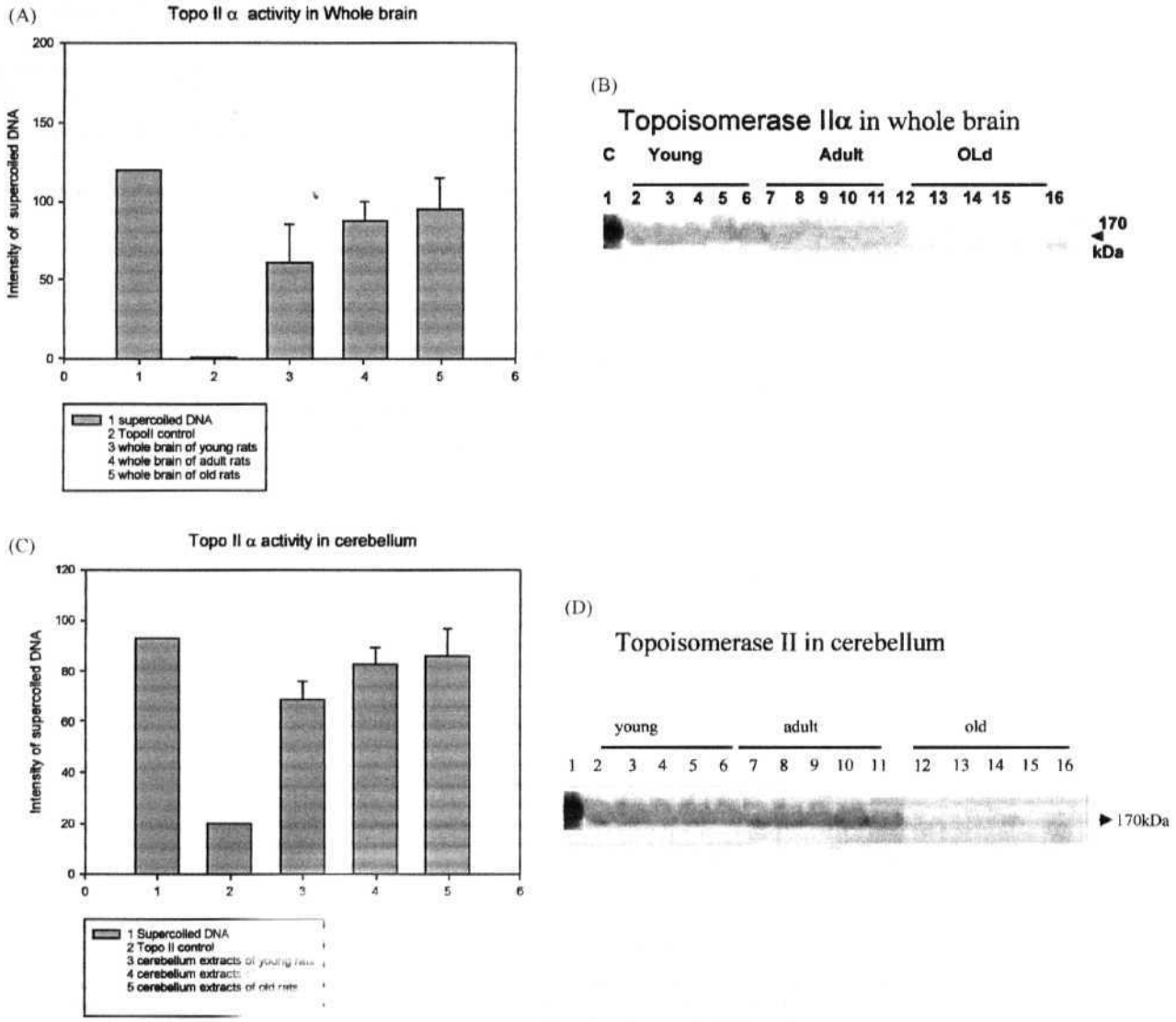

Fig. 3. Enzymatic activity and i[illegible] analysis of Topo II α in whole brain: activity: 100 μg of protein of extract was assayed for Topo II α catalyzed relaxation activity, the [illegible] measured in terms of amount of supercoiled DNA present in the gel. Quantified supercoiled DNA was plotted on *Y*-axis and sample names were [illegible] *X*-axis. Panels A, C, E and G are the results of activity of Topo II α in extracts of whole brain, cerebellum, cerebral cortex and mid brain respectively. [illegible] 75 μg of protein of extract was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against To[illegible] [illegible]lows Topo II α control with 25 μg. Panels B, D, F and H show immunoblots of Topo II α in extracts of whole brain, cerebellum, and [illegible] and mid brain respectively.

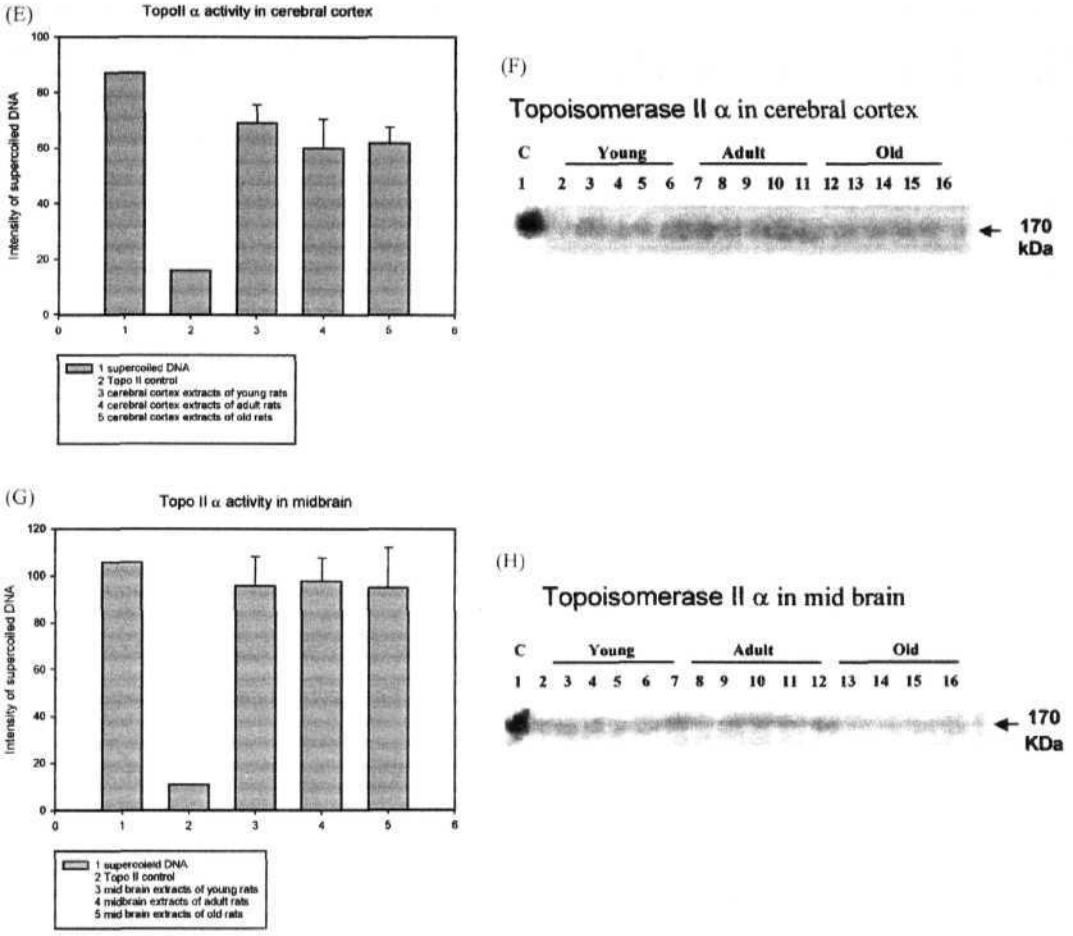

Fig. 3. (*Contineud*).

thoroughly washed using TBS containing 0.15% Tween 20 and incubated with Topo II α or β antibodies (1:1000 dilution in TBS) for 30 min at room temperature. The membrane was washed thrice and incubated with the alkaline phosphatase conjugated anti mouse IgG antibody (1:2000 dilution in TBS) for 60 min at room temperature and washed with TBS containing 0.15% Tween. The blots were developed using NBT-BCIP substrate in TBS.

2.8. Phosphorylation of Topoisomerase II

Fifty micrograms of protein from the extracts of whole brain or cerebellum was phosphorylated with 5 μCi of γ^{32}P-ATP in kinase buffer (10% glycerol 0.1 M EDTA and 50 mM $MgCl_2$). The mixture was incubated at 37 °C for 1 h. Topo II β antibody was added to the above mixture. After incubation at 37 °C for 30 min, 20 μl of protein A agarose was added. The tissue lysates were incubated at 4 °C for 15 min with intermittent shaking and centrifuged at 300 × ***g***. The sediment was washed twice with PBS and eluted with 20 μl of 5% trichloroacetic acid (TCA). Ten microliters of elute was spotted on Whatman no. 1 filter paper discs and ^{32}P was measured with a Wallac 1400 DSA liquid scintillation counter. Each experiment was carried out in triplicate and all data points represent an average of results from three experiments.

2.9. Immunofluorescence

The cerebellar regions from brains of young, adult and old rats were dissected out and placed in medium containing 10 mM potassium phosphate, 8% glucose and 5% fructose. Frozen sections ~4 microns/μm of the cerebellum from the three different age groups were taken in a cryostat and mounted on glass slides. The sections were treated with ethanol for 10 min followed by washes with double distilled water and PBS. Then the sections were treated with 0.3% Triton X-100 in PBS for 3 min. The sections were blocked with 1% BSA in PBS and incubated for 1 h at room temperature. After washing with PBS twice, the sections were then incubated with Topo II antibodies diluted in PBS containing 1% BSA for 1 h and then washed with PBS twice. The sections were then incubated in FITC conjugated IgG secondary antibody for 1 h and washed with PBS twice. The slides were then viewed using a fluorescence microscope and photographed.

3. Results

The levels of the two isoforms of Topo II were analyzed in whole brain, and also in the three regions of brain viz. cerebellum, cerebral cortex and midbrain. The levels of this enzyme were also examined in the different cell types, viz. neurons and astrocytes, that are widely used for various studies for understanding mechanisms of DNA damage and repair.

Topo II α and β present in the corresponding extract was immunoprecipitated using isoform-specific monoclonal antibody. The activity of the Topo II α and β present in immunoprecipitate was analyzed by monitoring the extent of Topo II catalyzed relaxation of supercoiled plasmid pRYG DNA. PRYG DNA plasmid contains Topo II binding and cleavage sites. The products were analyzed on 1% agarose gels and stained with ethidium bromide. Agarose gels along with quantified supercoiled DNA were shown as bar graphs in results wherever we have observed significant Topo II activity. In the results where we have observed low levels of Topo II activity, we have given data only in bar graph for quantified supercoiled DNA bands that is inversely proportional to the relaxation activity of the enzyme. Topo II α and β proteins were monitored through Western blot analysis of the Protein using isoforms-specific monoclonal antibodies.

3.1. Topoisomerase II α in cerebellum of rat embryos

Analysis of Topo II α in cerebellum extracts of embryos shows high activity in E11 and E18 embryos but in post-natal day 1 pups shows moderate activity (Fig. 1A). This observation was correlated well with corresponding protein levels on the immunoblot analysis (Fig. 1C). This analysis indicates that Topo II α level is high in proliferating tissue like developing rat embryos brain.

3.2. Topoisomerase II β in cerebellum of rat embryos

Our analysis on Topo II β in cerebellum extracts of embryos shows same activity in all three age groups (Fig. 2A). There are no detectable changes in Topo II β activity

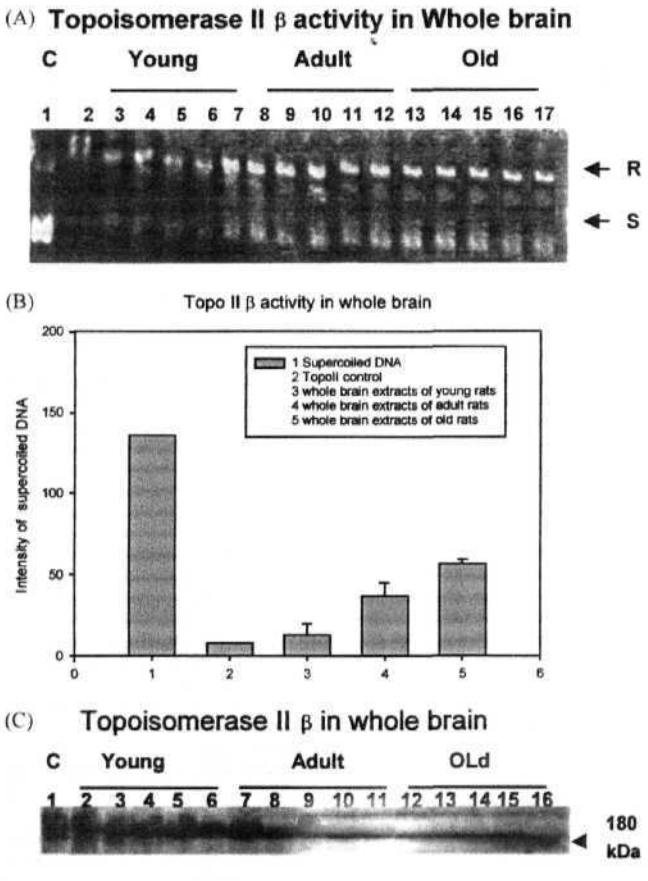

Fig. 4. Enzymatic activity and protein levels of Topo II β in whole brain: ACTIVITY: Topo II β activity is measured in 100 μg protein of extract of young (lanes 3–7), adult (lanes [illegible] (lanes 13–17) rats. Lane 1 shows ~0.6 μg of pRYG plasmid [illegible] lane 2 shows DNA with 2 units of Topo II from whole brain [illegible] regions of young, adult and old rats. Activity of Topo II β in whole brain was shown as gel in panel A, corresponding quantified [illegible] DNA is shown in panel B. Protien: immunoblots of Topo II β [illegible] whole brain was shown in panel C.

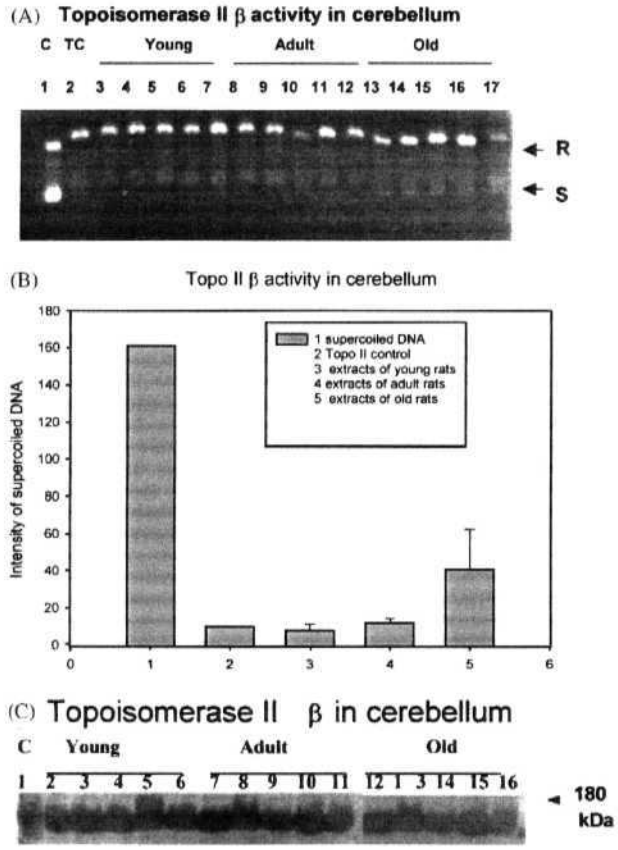

Fig. 5. Enzymatic activity and protein levels of Topo II β in cerebellum: activity: Topo II β Activity is measured in 100 μg protein of extract of young (lanes 3–7), adult (lanes 8–12) and old (lanes 13–17) rats. Lane 1 shows ~0.6 μg of pRYG plasmid DNA and lane 2 shows DNA with 2 units of Topo II from cerebellum. Activity of Topo II β in cerebellum was shown as gel in panel A, corresponding quantified supercoiled DNA is shown in panel B. Protien: immunoblots of Topo II β in extracts of cerebellum was shown in panel C.

between three age groups. This result closely supported by immunoblot analysis of Topo II β level in embryos (Fig. 2C).

3.3. Topoisomerase II α in whole brain and three regions of rat brain

Analysis of Topo II α in whole brain extracts showed very low activity of this enzyme in young rat brain and negligible activity in adult and old rat brain (Fig. 3A), this observation is correlated well with corresponding protein levels on the immunoblot analysis in Fig. 3B.

Topo II α activity was also examined in the three regions of brain viz. cerebellum (Fig. 3C), cerebral cortex (Fig. 3E) and mid brain (Fig. 3G), the results showed negligible activity of Topo II α in these three regions and at all the ages studied. To know protein levels of Topo II α, we have carried out immunoblot analysis of Topo II α in extracts of cerebellum (Fig. 3D), cerebral cortex (Fig. 3F) and midbrain (Fig. 3H), the results of the these experiments confirm that Topo II α activity as well as protein level remains low in these three regions at all ages.

3.4. Topoisomerase II β in rat whole brain and three regions of brain

Since the above studies indicated that Topo II α activity remained low or even undetectable in brain at all the ages studied, we have taken-up to study the activity and protein levels of Topo II β in whole brain and three regions of brain in young, adult and old rats.

The results are shown in Fig. 4, the results show a significant activity of Topo II β in whole brain extract (Fig. 4A and B). The "Young" rat brain showed high activity of this isoform, while moderate activity is seen in "Adult" brain

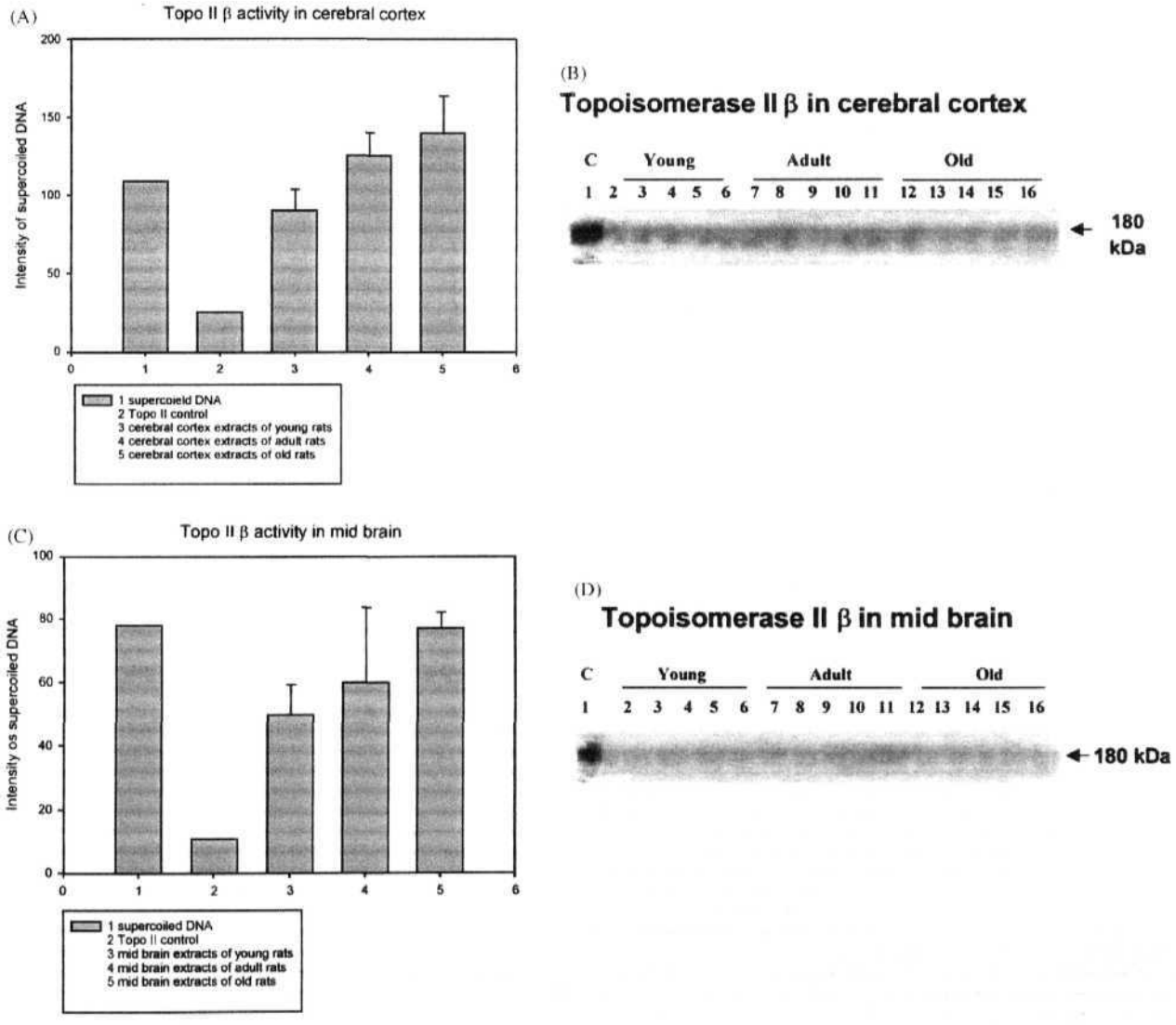

Fig. 6. Panels A and C show the activity of Topo II β in extracts of cerebral cortex and midbrain in bar diagram. Protein: immunoblots of Topo II β in extracts of cerebral cortex and midbrain were shown in panels B and D, the blots labeled appropriately, lane 1 shows Topo II β control with 25 μg.

and least activity was detected in 'Old' brain (Fig. 4A and B). The analysis of protein levels of Topo II β as shown in Fig. 4C, correlate well with the observations seen in the enzymatic activity suggesting that high enzymatic activity and protein of Topo II β is observed in young brain and these levels deceased with increasing age. Further, we notice that the migration of Topo II β protein in "Old" rat brain is slightly ahead of "Young" and "Adult" suggesting the possibility of slight variation in molecular form of Topo II β in ageing brain.

The results concerning the Topo II β activity in cerebellum of brain and at three different ages are shown in Fig. 5A and B and the protein levels are shown in Fig. 5C. The Topo II β activity was highest in "Young" as well as "Adult", while the activity is decreased in "Old" rat cerebellum. Western blot analysis of protein levels of Topo II β in rat cerebellum as shown in Fig. 3C suggest that Topo II β protein remains high in "Young" and in "Adult" rat cerebellar extracts, while protein levels decrease in "Old" rats. These observations closely correlated the results of enzymatic activity (Fig. 5A and B) Further, we notice the migration of Topo II β in "Old" rat cerebellum is slightly faster than that of Topo II β in "Young" and "Adult" rat cerebellum (similar to the observation in whole brain) suggesting the molecular form of Topo II β in "Old" rat cerebellum may a slightly altered one.

On the other hand, activity and protein of Topo II β are negligible in cerebral cortex and mid brain (Fig. 6A–D) at all the ages studied. Similar analysis of Topo II β specifically in hippocampus region also showed negative results (data not shown). These results thus point out the interesting aspect of Topo II β being found only in cerebellar region.

3.5. Phosphorylation of Topoisomerase II β in whole brain and cerebellum

Cellular Topo II β activity is known to be regulated by its phosphorylation and the phosphorylated form being the active form (Isaacs et al., 1998). To understand whether the Topo II β activity in brain also maintained through its phosphorylation, Topo II β phosphorylation was analyzed in whole brain and cerebellum extracts. The results in Fig. 7 show that whole brain and cerebellum region have the maximum level of phosphorylated Topo II β and there was a decrease in this level in an age-dependent manner (Fig. 7).

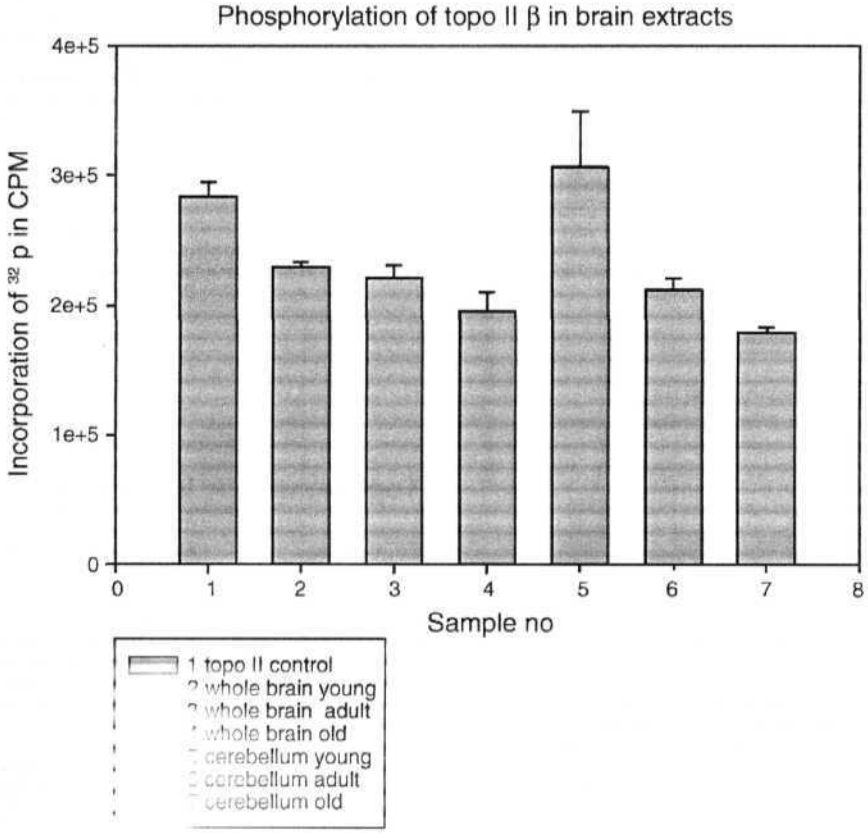

Fig. 7. Phosphorylation of Topo II [illegible] extracts. Topo II β from 50 μg total protein of whole brain (lane 2–4), and cerebellum of young, adult and old rats was phosphorylated with [illegible] in presence of kinase buffer. Twenty-five micrograms of Topo II (sample no. 1) was used as a control. The phosphorylated enzyme captured on to the protein A agarose beads was TCA eluted and spotted on to Whatman filter papers and the radioactivity was counted using a Wallac scintil[illegible] sample nos. 2–4 are Topo II β of whole brain extract of young, adult and old rats respectively, sample nos. 5–7 are of cerebellum extract [illegible] adult and old rats respectively.

3.6. Levels of Topoisomerase II β in different cell types of brain

Since Topo II β is found only in the cerebellar region of brain, we have examined whether Topo II β has any exclusive localization in a particular cell type. Neuronal and astroglial cell fraction were prepared from whole brain, cerebellum and cerebral cortex and topo II β activity in the extracts of these cell fractions were examined. The results are shown in Fig. 8. Only neuronal extracts from whole brain and cerebellum showed activity of Topo II β. The activity of the cerebellar neuronal extracts (Fig. 8A and B) was higher when compared to whole brain (Fig. 8A and B) and in contrast Topo II β activity was negligible in cerebral cortex neurons (Fig. 8C and D). Analysis of age-dependent changes in Topo II β activity in whole brain and cerebellar neurons showed that the enzymatic activity decreased with increasing age. Similar analysis of astrocytes showed negligible amounts of Topo II β (Fig. 8E).

3.7. Immunohistochemical analysis of Topoisomerase II α and β

To confirm the above findings we have performed the immunohistochemical analysis of Topo II β in brain sections and neurons. The analysis of Topo II β was carried out in the sections obtained from cerebellum (young, adult and old) (Fig. 9, panel A) cerebral cortex (young), midbrain (young) (data not shown). Immunofluorescence of Topo II β was high in cerebellum sections, while Topo II β was negligible in sections of cerebral cortex and mid brain. Further, immunofluorescence of Topo II β showed a decrease with increasing age, highest amount of fluorescence is seen in the young rat cerebellar section (Fig. 9, panel A), in contrast the Topo II α analyzed in the cerebellum sections of the three

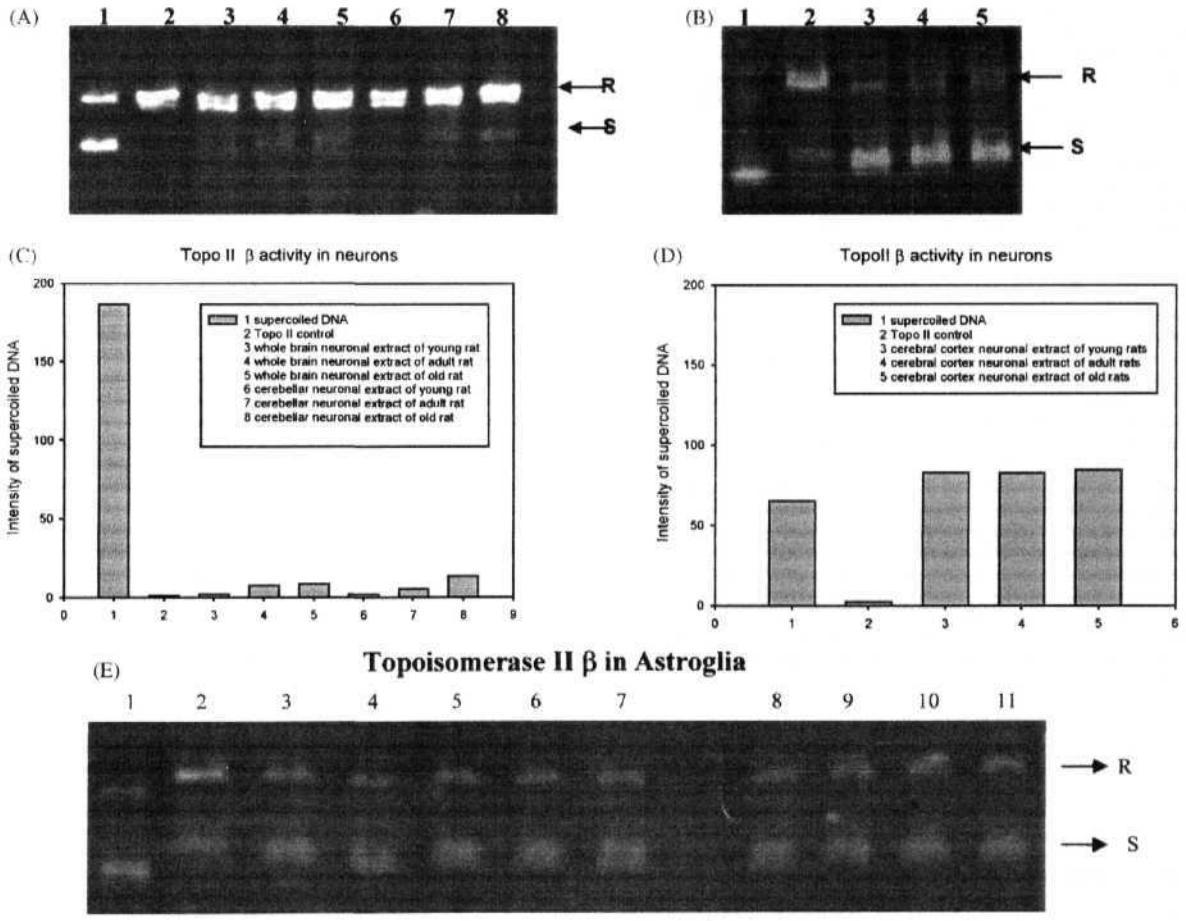

Fig. 8. Relaxation activity of Topo II β from neuronal cells: 100 μg protein of neuronal cell extracts was assays for Topo II β activity and data presented in form of relaxation of supercoiled DNA as analyzed by 1% agarose gel electrophoresis followed by ethidium bromide straining and photography, in addition a bar diagram is appended to show the quantified supercoiled DNA. Panel A, lanes 3–5 show activity of Topo II β in neuronal extracts of whole brain of young, adult and old respectively, while lanes 6–8 show the activity of Topo II β in neuronal extracts of cerebellum of young, adult and old respectively, quantified supercoiled DNA in gel is given in panel B and panel C, lanes 3–5 show activity in neuronal extract of cerebral cortex of young, adult and old rats, quantified supercoiled DNA in gel is given in panel D. Panel E shows activity in astroglial extracts of whole brain (3–5), cerebellum (6–8), and cerebral cortex (9–11) of young, adult and old rats. In all gels, lanes 1 show the DNA alone, lane 2 show DNA with purified Topo II β.

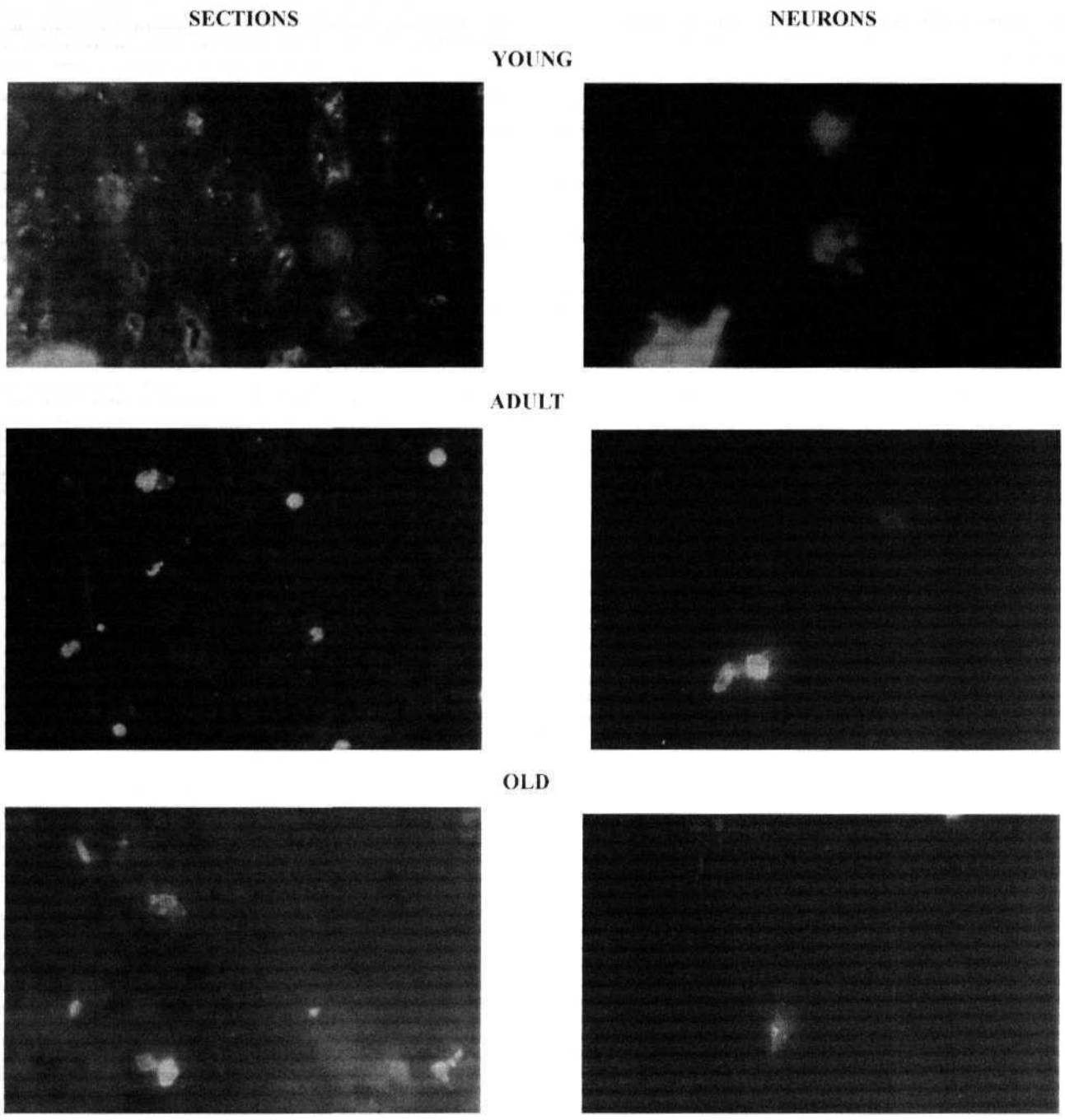

Fig. 9. Immunoflourescence of cerebellar section and cerebellar neurons: cerebellar sections of young (A), adult (B) and old (C) rats were dissected out and frozen in liquid nitrogen. Sections were taken by cryostat, they were fixed with acetone and probed with Topoisomerase II β monoclonal and stained with FITC conjugated anti mouse IgG and fluorescence images were seen in fluorescence microscope and photographed. The cerebral neurons from young (D), adult (E) and old (F) were isolated and cytospinned. The monolayer was fixed with ethanol and probed and stained as explained above.

age groups showed very low amounts of immunofluorescence (data not shown). Analysis of Topo II β immunofluorescence in cerebellar neurons at young, adult and old rats showed that the young rat cerebellar neurons possess highest amount of Topo II β immunofluorescence, while lowest being in the old rats. These observations correlate well with the above studies on the activity and protein levels of Topo II isoforms in three regions.

4. Discussion

The above studies indicated that Topo II β is predominant in brain, while Topo II α is predominant in embryos (up to post-natal day 1) and remains low in aged groups. Further higher activity and protein levels of the topo II β isoform was seen only in cerebellar region, that is having laminar arrangements of cells. The studies of Tsutsui et al. (1993,

2001) on localization of Topo II isoforms in developing rat brain also showed that developing rat cerebellar region possess high levels of Topo II β during the early post-natal period suggesting the importance of Topo II β in rat cerebellum in its development and growth.

The results of the age-dependent changes of Topo II α and β suggested that α isoform remains low and unaltered with age, while the activity of β isoform is higher in young, moderate in adult and low in old rat cerebellar region suggesting an age-dependent decline in Topo II β protein and activity. The enzymatic activity may be regulated by phosphorylation as phosphorylated form of Topo II decreases in an age-dependent manner. Furthermore, we have noticed in Western blot data of Topo II β in whole brain and cerebellum (Fig. 2C and F) that Topo II β migration in whole brain and cerebellum of old rat is slightly faster than that in young ones indicating that the change in migration could be due to different phosphorylation status in addition to protein levels. The above results suggest that Topo II β activity in rat brain may be regulated through its phosphorylation.

The increased topo II β mRNA levels seen by Tsutsui et al. (2001) in the cerebellar granule cells that are differentiating to granular layer during the first two post-natal weeks of the rat cerebellar development suggest that the topo II β is essential for some functions in cerebellum during its development and growth. The present results show that this essential function of Topo II β is depleting during ageing. Higher excision nuclease activity, which is required in excision repair, is detected in cerebellum (Brook, 1998) and it is to be seen whether Topo II β is associated with a specific DNA repair activity in cerebellum.

The present results also suggest that only neurons possess significant Topo II β activity. Further, Topo II β activity in neurons decreased in an age-dependent manner. Studies of Woessner et al. (1991) showed that in developing rat neurons Topo II β is present is Purkinjee cells and granule cells during development suggesting that Topo II β is expressed in cerebellar neurons during cerebellar development and growth. These studies indicate that Topo II β may be required for cerebellar neurons for certain recombination

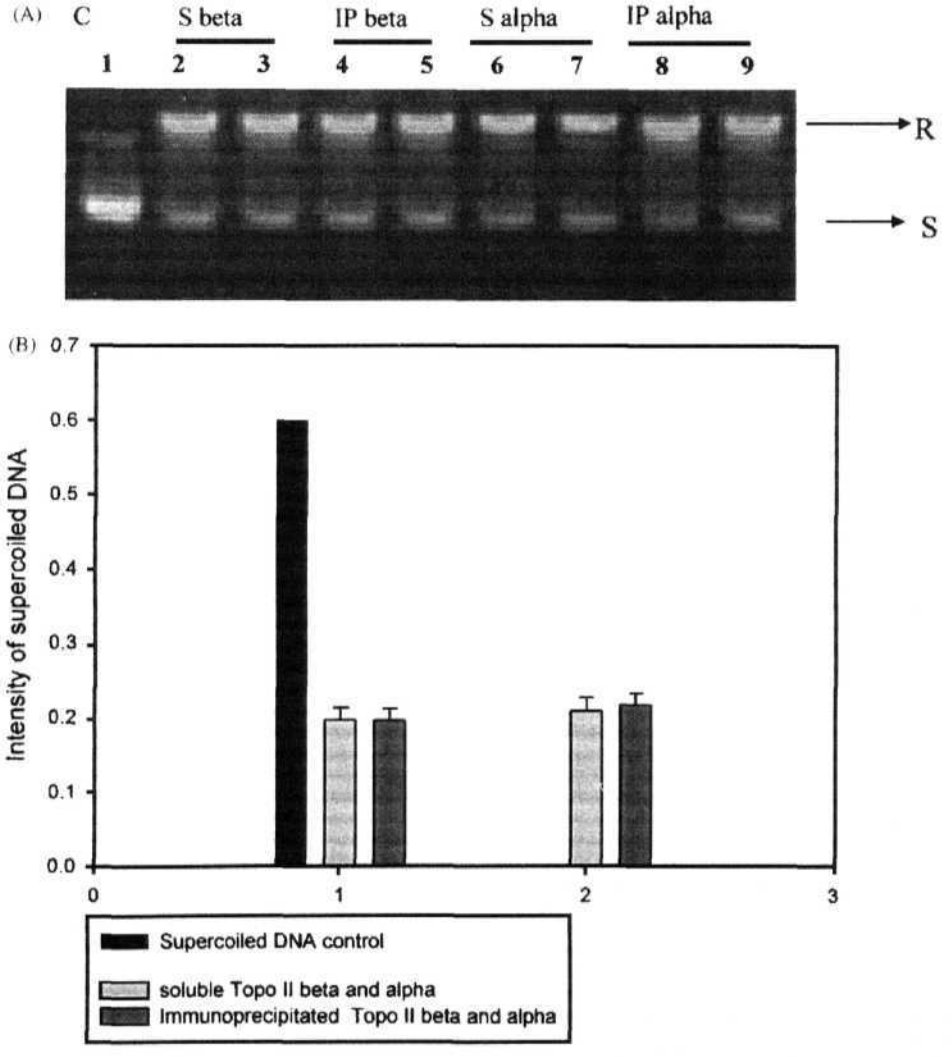

Fig. 10. Comparison of enzymatic activity of soluble and immunoprecipitated Topo II α and β.

and repair activity of differentiated cerebellar cells during their development and growth. Since Topoisomerases are reported to be involved in promiscuous recombination, and cellular response to radiation damage of DNA (Asami et al., 2002; Kohji et al., 1998; Ishii and Ikushima, 2002; Pastor and Cortes, 2002; Franchitto et al., 2000; Wu et al., 1999), the above results on the decreased activity of Topo II β in cerebellum of aged rat may be correlated as one of the factors contributing towards decrease in repair activity in aged brain tissue. Topo II β catalytic activity may be required for resolving certain topological restrictions formed during DNA rearrangements that take place in DNA repair and recombination in cerebellum.

5. Supporting data

Comparison of enzymatic activity of soluble and immunoprecipitated Topo II α and β are shown in Fig. 10. Tissue extracts were prepared from cerebellum of young (1 day old) rat pups. For soluble Topo II, tissue extracts were precipitated with 60% ammonium sulphate and dialyzed then treated with 0.2% heparin (nucłease inhibitor). Panel A, lanes 2 and 3 show soluble Topo II β, lanes 4 and 5 show immunoprecipitated Topo II β, lanes 6 and 7 show soluble Topo II α and lanes 8 and 9 show immunoprecipitated Topo II α. Lane 1 shows ~0.6 μg of pRYG plasmid DNA and quantified supercoiled DNA are shown in panel B.

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Topoisomerase II antagonism and anticancer activity of coordinated derivatives of $[RuCl_2(C_6H_6)(dmso)]$

Y.N. Vashisht Gopal, Neelima Konuru, and Anand K. Kondapi*

Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India

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Abstract

Topoisomerase II poisoning and anticancer activity by the organometallic compound $[RuCl_2(C_6H_6)(dmso)]$ was shown by us in an earlier study [Biochemistry 38 (1999) 4382]. Since high concentrations of this complex were required to achieve either effects, we have synthesized four derivatives of this complex in which the dimethyl sulphoxide group on the ruthenium atom was replaced with pyridine, 3-aminopyridine, *p*-aminobenzoic acid, and aminoguanidine. Three of these molecules showed enhanced potency of topoisomerase II poisoning and consequently also showed higher anticancer activity in breast and colon carcinoma cells in vitro. Detailed analysis of the molecular action of these compounds on topoisomerase II activity was carried out using the classical relaxation and cleavage activity of the enzyme, which revealed that the compounds poison topoisomerase II by freezing the enzyme and enzyme-cleaved DNA in a ternary "cleavage complex". The cleavage complex is implicated in the anti-neoplastic activity of these compounds. DNA interaction studies showed that these compounds interact with DNA in much the same way as $[RuCl_2(C_6H_6)(dmso)]$, by external binding of the DNA helix. This is unlike most other topoisomerase II poisons, which predominantly interact with DNA through intercalation with the double helix.

Keywords: Topoisomerase II; Cleavage complex; Ruthenium compounds; Anticancer activity; DNA external binding

The accidental discovery of the cytostatic properties of cisplatin in 1969 [1] sparked off an extensive search for other platinum and non-platinum metals containing inorganic and organometallic antitumor agents. Numerous complexes were synthesized and tested for antitumor effects. Although very few of them actually matched the cytostatic efficacy of cisplatin, some of the non-platinum compounds were active against tumor types which are unresponsive to cisplatin and other existing anticancer drugs, e.g., gastrointestinal carcinomas insensitive to cisplatin and other chemotherapeutic treatment are responsive to treatment with antitumor titanium compounds, a noteworthy and promising aspect for the development of non-platinum anticancer metal complexes. Some of the metals and their complexes which showed promising anticancer activity are spirogermanium, a germanium complex [2], gallium nitrate [3,4], titanium metal complexes like titanocene dichloride and budotitane [5,6], ruthenium complexes like *trans*-indazolium[bisindazole]tetrachloro ruthenate [7], and imidazolium *trans*-imidazolediemethylsulfoxidetetrachlororuthenate (NAMI-A) complex [8]. Each of these molecules has been shown to possess distinct molecular interactions with DNA, RNA, and proteins, which define their anticancer activities. For example, the gallium salts interfere with ribonucleotide reductase activity and inhibit DNA nucleotide synthesis [9]. Titanocene complexes and the ruthenium complex NAMI have been shown to interfere with type IV collagenolytic activity, which corresponds to a pronounced increase of extracellular matrix components in tumor parenchyma [10]. This is known to hinder metastasis formation and blood flow to the tumors [11,12]. Earlier in vitro studies showed that cyclopentadiene and carbadecacarboranyl complexes of iron [13–15], salicylaldoxime and carborane complexes of cobalt [16,17], and the ruthenium

* Corresponding author. Fax: +91-40-3010-120 or 145.
E-mail address: akksl@uohyd.ernet.in (A.K. Kondapi).

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complexes $[RuCl_2(C_6H_6)dmso]$ (RuBen(dmso))[1] and *trans*-indazolium[bisindazole]tetrachlororuthenate [18, 19] interfere with the mechanistic activity of topoisomerase II. Ruthenium complexes utilize multiple biological mechanisms for transport and macromolecular binding and coordinate with a wide range of biological macromolecules, which is a promising aspect for the development of complexes that interact with specific biological molecules to bring about targeted anticancer activity. In most cases, DNA binding by ruthenium complexes has been associated with anticancer activity. Many ruthenium complexes appear to be transported in blood mostly by transferrin (80%) and to a lesser extent by albumin [20,21]. Tumor localization of ruthenium, gallium, and titanium complexes has been attributed to this transport mechanism because tumor cells express a large number of transferrin receptors on their membranes [22]. Also redox molecules like glutathione interact with heavy metals and the reduced form of this peptide (GSH) is known to reduce metal complexes (e.g., $Ru^{III} \rightarrow Ru^{II}$), which would activate the metal complexes to bind biopolymers in the hypoxic environment of tumors [23,24].

Detailed in vitro analysis of $[RuCl_2(C_6H_6)dmso]$ (referred here as RuBen(dmso)) showed that this complex interacts with DNA and topoisomerase II (topo II) in a bi-directional manner, resulting in the formation of a topo II-mediated DNA cleavage complex [19]. The formation of such a cleavage complex is the main route for the anticancer action of topo II poisons. RuBen(dmso), however, was required in a high concentration for DNA cleavage complex formation, which is undesirable for in vivo applications. In the present study, we have synthesized four analogs of this complex by replacing the dmso group with other biologically active groups—pyridine, 3-aminopyridine, *p*-aminobenzoic acid, and aminoguanidine. Three of these complexes exhibited higher topo II poisoning compared to RuBen(dmso). The proliferative response of a breast cancer cell line and a colon cancer cell line was analyzed in presence of the new ruthenium complexes. The data show that these complexes have significant anti-proliferative activity on the two fast dividing cancer cell lines, suggesting that these anti-topo II complexes are promising anticancer agents.

[1] *Abbreviations used:* ATP, adenosine triphosphate; DMSO, dimethyl sulphoxide; RuBen(dmso), (η⁶-benzene) dichloro sulfinyl bis(methane)-*O*-ruthenium (II)]; RuBen, [(η⁶-benzene) (pyridine)-*N*-ruthenium (II)]; RuBenAPy, [(η⁶-benzene) (3-aminopyridine)-N_1-ruthenium (II)]; RuBenABa, [(η⁶-benzene) (*p*-aminobenzoic)-*O*-ruthenium (II)]; RuBenAGu, [(η⁶-benzene) (aminoguanidine)-N_1-ruthenium (II)]; topo II, topoisomerase II.

Experimental procedures

Materials. Topo II was purified from rat liver following the procedure of Galande et al. [25]. The enzyme concentration was determined using Bradford colorimetric assay [26] and unit enzyme activity was defined as the amount of enzyme that completely relaxes 0.3 μg of supercoiled DNA in standard relaxation assay conditions described in the experimental section. The negatively supercoiled pBR322 plasmid DNA was purified as described by Wang and Rossman [27]. RPMI 1640 medium, m-AMSA, and calf thymus DNA were from Sigma Chemical. The chemicals used for synthesis of the ruthenium compounds were from Aldrich, fetal calf serum and antibiotics were from Gibco-BRL, PEI (polyethyleneimine) cellulose-F TLC sheets were from Merck, Proteinase K and ATP were from Boehringer–Mannheim, [γ^{32}-P]ATP and ^{3}H-labeled thymidine were supplied by BRIT, India. Other chemicals and biochemicals were of analytical grade.

Synthesis of the ruthenium compounds

RuBen(dmso). This compound was synthesized as previously described by Zelonka et al. [28]. Briefly, freshly synthesized 1,3-cyclohexadiene (6 ml) was added to $RuCl_3 \cdot 3H_2O$ (1.7 g) in 100 ml of aqueous ethanol. The solution was maintained at 45 °C for 3 h to form a red precipitate which was washed in ethanol and dried in vacuum to give the dimeric complex of $[RuCl_2(C_6H_6)_2]$. To this dimer, DMSO was added to form the monomeric DMSO complex $[RuCl_2(C_6H_6)dmso]$ (RuBen (dmso)) which was vacuum dried to give a bright red precipitate. The compound was characterized by NMR spectroscopy.

RuBenPyr. One hundred milligrams of the above dimer was stirred in 10 ml of pyridine for 48 h to give an orange-red solid. This was filtered and washed in methanol followed by diethyl ether. The compound was characterized by NMR spectroscopy.

RuBenAPy. Five millimoles of the dimer was suspended in dry absolute ethanol and 10 mmol of 3-aminopyridine was added. The mixture was refluxed for 12 h in dry nitrogen atmosphere. A dark green product formed which was filtered and washed in ethyl alcohol:diethyl ether (70:30) mixture. The compound was characterized by NMR spectroscopy.

RuBenABa. To 5 mmol of the dimer in dry ethanol, *p*-aminobenzoic acid was added. This was refluxed for 6 h in dry nitrogen atmosphere to give a dark purplish-pink precipitate. This precipitate was dried in diethyl ether. The compound was characterized by IR spectroscopy.

RuBenAGu. Ten millimoles of aminoguanidine was added to 5 mmol of the dimer in dry alcohol and refluxed for 6 h. The solution was cooled and allowed to

stand at room temperature for 24 h. A dark blue precipitate formed, which was washed and dried in diethyl ether. The compound was characterized by NMR spectroscopy. The chemical structures of these compounds are shown in Fig. 1.

Relaxation assay

This assay was performed following the procedure of Osheroff et al. [29]. The reaction mixture (20 μL) contained relaxation buffer (50 mM Tris–HCl, pH 8.0), 120 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 30 μg/mL BSA, 1 mM ATP), 0.6 μg of negatively supercoiled pBR322 plasmid DNA (~75% supercoiled) and increasing concentrations of the ruthenium complexes. The reaction was initiated by adding two units (~8 nmol) of topo II and incubated at 30 °C for 15 min. The reaction was stopped by adding 2 μL of 10% SDS. Three microliters of loading dye (0.5% bromophenol blue, 0.5% xylene cyanol, 60% sucrose, 10 mM Tris–HCl, pH 8.0) was added, and the products were separated on a 1% agarose gel in 0.5× TAE buffer (20 mM Tris–acetate, 0.5 mM EDTA) at 50 V for 8 h. The gel was stained with ethidium bromide, visualized in a Photodyne UV transilluminator and photographed.

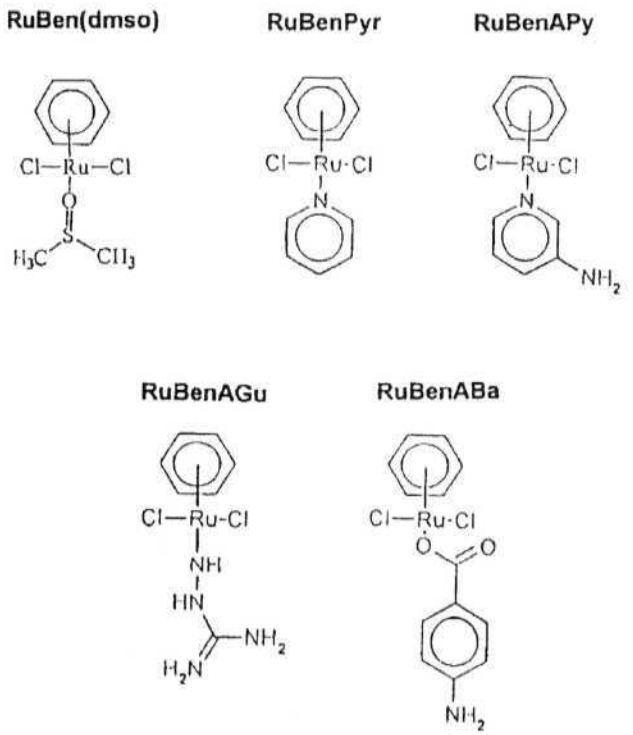

Fig. 1. Chemical structures of the RuBen compounds—RuBen(dmso), RuBenPyr, RuBenAPy, RuBenAGu, and RuBenABa. In these organometallic compounds, the π-electron cloud of the benzene ring forms an organometallic bond with the ruthenium atom, shown as a bond between the center of the aromatic ring and the metal atom.

ATPase assay

This assay is a modified procedure of Osheroff et al. [29]. The 20 μL reaction mixture contained relaxation buffer (the 1 mM ATP component contained 0.025 μCi [γ^{32}-P]ATP), 0.6 μg of pBR322 DNA and increasing concentrations of the ruthenium complexes. The reaction was initiated with two units of topo II and incubated at 30 °C for 15 min. The reaction was stopped with 2 μL of 0.5 M EDTA. The reaction mixture was spotted on PEI cellulose-F TLC sheets and the sheets were subjected to thin layer chromatography in 1 M lithium chloride solution. Under these conditions, γ^{32}-P_i migrates first followed by ADP and [γ^{32}-P]ATP. After resolution, the bands were monitored under reflected UV light at 366 nm in a Photodyne transilluminator. The illuminated bands of ATP, ADP, and P_i (inorganic phosphate) were cut out of the sheet and counted for ^{32}P in a liquid scintillation counter.

Cleavage assay

The formation of cleavage complex was assayed following the procedure of Zechiedrich et al. [30]. The 20 μL reaction mixture contained relaxation buffer (minus ATP), 0.6 μg of pBR322 supercoiled DNA and increasing concentrations of the ruthenium complexes. The reaction was initiated by adding 10 units (40 nmol) of topo II and incubated at 30 °C for 15 min. The reaction was stopped with 2 μL of 0.5 M EDTA and 2 μL of 10% SDS. The DNA bound protein was degraded by incubating the reaction mixture with 2 μL of 1 mg/mL Proteinase K at 45 °C for 1 h. The products were separated on 1% agarose gel for 8 h at 50 V in 1× TAE buffer (40 mM Tris–acetate, 1 mM EDTA), stained and photographed. The linear DNA band was quantified as percentage of total DNA in a UVP gel documentation system.

DNA thermal denaturation assay

This assay was carried out following the procedure of Gopal et al. [19]. Calf thymus DNA (sodium salt) was dissolved in 1 mM sodium phosphate buffer containing 1 mM sodium chloride. DNA concentration was adjusted to give an absorbance of 1.0 in 1 ml at 260 nm. The ruthenium compounds were added to DNA at concentrations which gave drug to nucleotide ratios of 1:40, 1:20, 1:10, 1:5, 1:2, and 1:1, respectively. The samples were incubated in 1 ml quartz cuvettes for 2 min to allow drug–DNA interaction. A Hitachi 150-20 spectrophotometer was set to give a 1 °C rise in temperature per minute with a KPC-6 thermo-programmer and SPR-7 temperature controller. Increase in absorbance at 260 nm was recorded from 40 to 90 °C. T_m was determined from the denaturation curves. Curve width

of the individual melting curves was calculated by the procedure of Kelly et al. [31]. Curve width is the temperature range between which 10 and 90% of the absorbance increase occurs. The data were plotted and analyzed.

Circular dichroism studies for the analysis of drug–DNA interaction

The ruthenium compounds and pBR322 DNA corresponding to 350 μM drug and 0.6 μg DNA (the maximum concentration of drug and DNA used in the relaxation assay) were incubated in the relaxation buffer for 5 min to allow drug–DNA interaction. The DNA intercalator, m-AMSA, corresponding to 100 μM in the topo II assay was included as a control. CD spectra of the samples were measured between 240 and 300 nm wavelength in a Jasco J-715 spectropolarimeter. Molar ellipticity [θ] was calculated from the CD spectra using the formula

$$[\theta] = M_r * \psi / 100 Lc,$$

where [θ] is molar ellipticity, M_r is mean residual weight, ψ is CD value in millidegrees, L is path length of the cuvette in centimeters and c is the concentration of the sample (DNA) in g/ml.

Anti-proliferation activity

This was analyzed through [^{3}H]thymidine incorporation assays using two human cancer cell lines—Colo-205 (colon adenocarcinoma) and ZR-75-1 (breast carcinoma). The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Then 0.2×10^6 cells/200 μL were distributed in triplicates in 96 well microtiter tissue culture plates. The cultures were incubated for 16 h at 37 °C in a CO_2 incubator (Forma Scientific) maintaining 5% CO_2. Increasing concentrations of the ruthenium complexes were added to the cells. m-AMSA was included as a control. All controls always contained an equal amount of DMSO present in the drug treated samples. The drug treatment was stopped after 6 h by centrifugation and change of media. The cells were further incubated for 48 h. The cultures were then pulsed with 0.5 μCi of [^{3}H]thymidine and incubation was continued for 4 h to allow thymidine incorporation by cells. After this incubation, the culture medium was removed and the adhering cells were treated with 20 μL of trypsin–EDTA (0.25% trypsin, 1 mM EDTA) for 5 min at 37 °C to release the cells from the adhering surface. Trypsinization was stopped by adding 20 μL of serum to the cells. The cells were harvested on glass microfiber strips using a Skatron automated cell harvester. Radioactivity was measured in a Wallac liquid scintillation counter.

DNA polymerase assay by Klenow extension

The inhibitory activity of ruthenium derivatives for DNA polymerase was assayed by using the Klenow extension labeling assay. Plasmid pRYG DNA (0.6 μg) was digested with restriction enzyme *Hin*dIII and incubated with ruthenium derivatives at respective concentrations at which they completely inhibit Topoisomerase II activity. The 3′ ends of the restriction digested plasmid were labeled with [α^{32}-P]ATP in the presence of one unit of Klenow enzyme. The reaction was done at room temperature for 15 min and was stopped by heating the samples to 70 °C in a water bath for 5 min. The DNA was TCA precipitated and spotted on Whatmann filter paper and the radioactivity was measured by placing the filters in scintillation fluid and counted in Wallac counter.

Results

Topoisomerase II poisoning by the ruthenium compounds

Inhibition of topo II mediated relaxation of supercoiled DNA was investigated through the relaxation assay. It was observed that RuBenPyr does not affect the DNA relaxation activity of topo II over the concentration range employed while RuBenAPy completely inhibits topo II activity at a concentration of 200 μM. RuBenAGu and RuBenABa show complete inhibition at 250 and 350 μM concentration. The complexes show dose dependency in the inhibition of topo II mediated DNA relaxation activity (Fig. 2).

Drugs that poison the mechanistic action of topo II also interfere with the DNA-stimulated ATPase activity of the enzyme. Hydrolysis of ATP to ADP and P_i can be UV-visualized on a TLC sheet as individual migrating bands, which are cut out of the sheet and scintillation counted to get a direct measure of the ATPase activity of topo II. The results of the ATPase assay indicate that the three ruthenium drugs inhibit the DNA dependent ATPase activity of topo II, while RuBenPyr does not appreciably do so (Fig. 3). Inhibition of ATPase activity by the three drugs was concomitant with that of the relaxation activity.

The topo II mediated cleavage of DNA in presence of the ruthenium drugs was monitored through the cleavage assay. In presence of a topo II poison, the enzyme interacts with DNA and cleaves it but fails to religate it. The drug (topo II poison) then freezes the enzyme and DNA in a ternary cleavage complex of topo II–drug–DNA. After ending the reaction, the cleaved DNA from the ternary complex is released by SDS and proteinase K treatment. Since a closed circular plasmid DNA is used in this assay, cleavage of this DNA results in its linearization. The resulting linear DNA is a direct

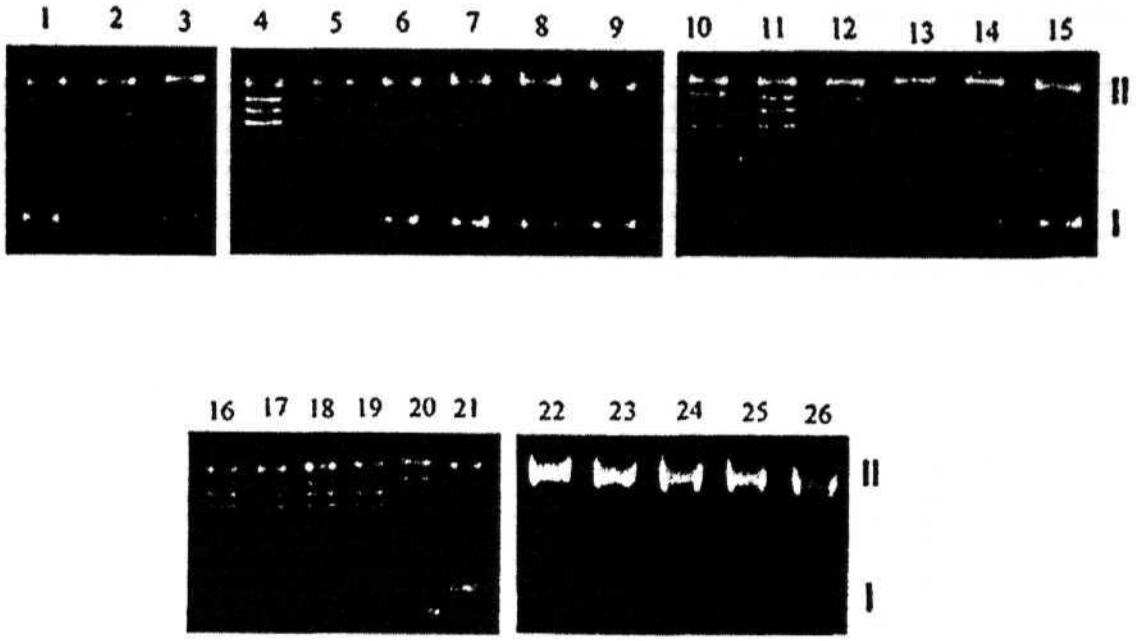

Fig. 2. Inhibition of the topo II catalyzed relaxation of supercoiled DNA. Supercoiled pBR322 DNA (lane 1) was incubated with topo II in the absence (lane 2) or presence of 75 μM m-AMSA (lane 3), 100, 150, 200, 250, 300, and 350 μM of RuBenAPy (lanes 4–9), RuBenAGu (lanes 10–15), RuBenABa (lanes 16–21), and 100, 200, 300, 400, and 500 μM of RuBenPyr (lanes 22–26). The positions of supercoiled (form 1) DNA and relaxed (nicked circular or form 2) DNA are indicated by I and II.

quantifiable measure of cleavage complex formation by topo II poisons. The cleavage assay carried out in presence of increasing concentrations of the drugs showed that RuBenAPy, RuBenABa, and RuBenAGu induced linearization of the plasmid DNA, but RuBenPyr could not do so (Fig. 4A). Density quantification of linearization shows that RuBenApy has the highest potency of cleavage complex formation followed by RuBenABa and RuBenAGu (Fig. 4B). The topo II poison m-AMSA had a slightly higher potency of cleavage complex formation compared to RuBenAPy. These results correlate with the relaxation and ATPase inhibition activities of the three ruthenium complexes. The aqua solubilized complexes retained the anti-topo II activity for up to 72 h after solubilization, after which the activity decreased significantly. It is known that ruthenium complexes covalently bound to DNA could generate strand breaks in the DNA by generation of free radicals and other oxidation products [42]. To understand whether the ruthenium complexes under study can generate double or single strand breaks, the cleavage assay was carried out in the absence of topo II, and samples incubated at 37 °C for a period of 12 h. The result showed that the ruthenium complexes at 300 μM concentration did not induce linear DNA formation and also did not increase the percentage of the nicked circular DNA (data not shown). This suggests that the Ru in these complexes may not form covalent interactions with DNA and induce DNA oxidation. This may also be the case in vivo too, especially in the hypoxic environment of tumor cells. But since topo II-drug cleavage complexes are long-lived and the ruthenium complexes could form other long-lived protein–DNA cross-links, Ru may eventually form covalent N-glycosidic bonds with purines.

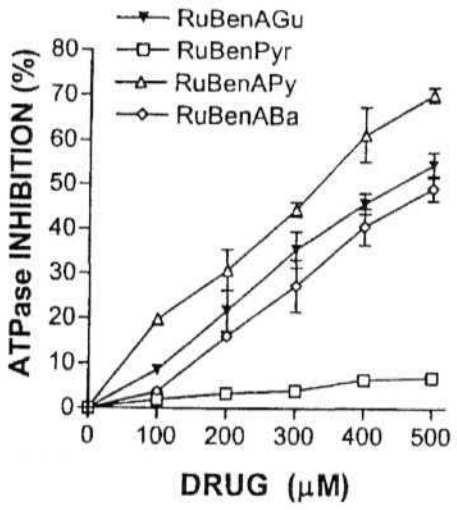

Fig. 3. Inhibition of the ATPase activity of topo II by RuBenPyr (□), RuBenAPy (△), RuBenAGu (▼), and RuBenABa (◇). ATP hydrolysis in the presence of increasing concentrations of the drugs are presented as mean of three experiments. Data are plotted as percent inhibition of ATP hydrolysis versus concentration of drug in μM.

DNA interaction by the ruthenium drugs

Analysis of DNA interaction by these complexes was made through DNA thermal denaturation studies. Drug

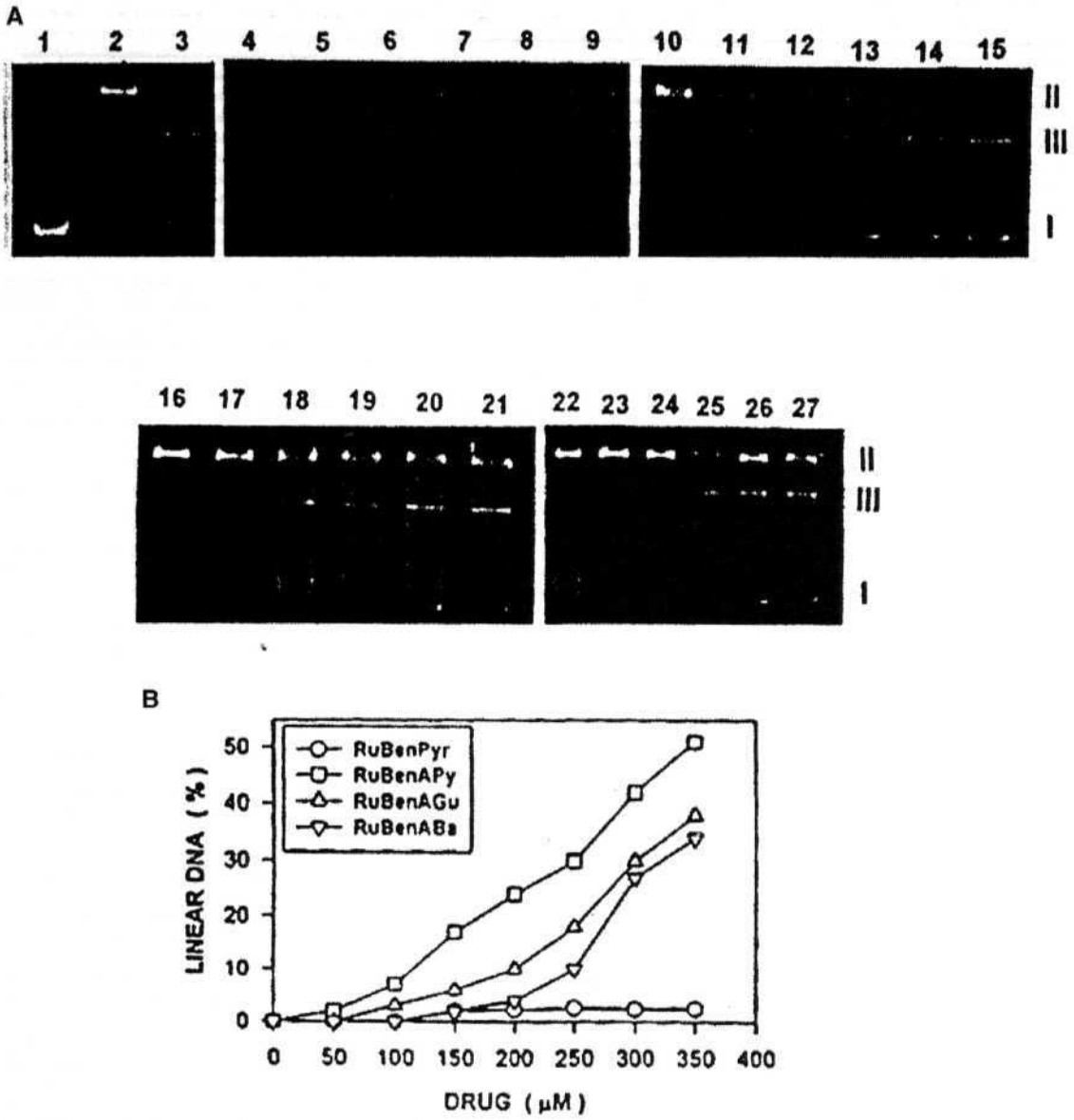

Fig. 4. (A) Cleavage assay was performed by incubating supercoiled pBR322 DNA (lane 1) with topo II (lane 2) in the presence of 100 μM m-AMSA (lane 3), 100, 150, 200, 250, 300, and 350 μM RuBenPyr (lanes 4–9) or RuBenAPy (lanes 10–15) or RuBenAGu (lanes 16–21) or RuBenABa (lanes 22–27). The positions of supercoiled, nicked circular, and linear (form 3) DNA are indicated by I, II, and III. The formation of the cleavage complex is seen as the appearance of the linear DNA (III). (B) Quantification of the linear DNA gives a direct indication of cleavage complex formation. As seen in the figure, RuBenPyr does not show cleavage complex formation, while RuBenAPy promotes >50% cleavage of the pBR322 DNA. RuBenAGu and RuBenABa also show significant cleavage complex formation.

titration was carried out which showed a DNA binding saturation at 1:1 or higher drug : nucleotide base ratio indicating that the drug molecules bind to single nucleotide bases. T_m values were plotted on a logarithmic scale of the drug to nucleotide ratio against temperature. The data clearly indicate [illegible] DNA binding affinity for the ruthenium compl[illegible] 5A). Curve width analysis of the thermal d[illegible] curves was carried out to determine the mod[illegible] interaction by the ruthenium drugs. Fig. 5B [illegible] that ruthenium drug titration with DNA does not induce any significant change in the curve width of the thermal denaturation curves. In the case of m-AMSA, however, a large increase in the curve width is noticed. This indicates that the ruthenium drugs interact with DNA without disturbing the DNA helical conformation, which is typical of molecules that interact externally with the DNA helix, possibly by ionic interactions with the phosphate backbone or nucleotide bases [31]. Verification of such an interaction was made by CD spectral analysis of DNA in presence of these molecules, which reveals that the ruthenium drugs do not induce a significant

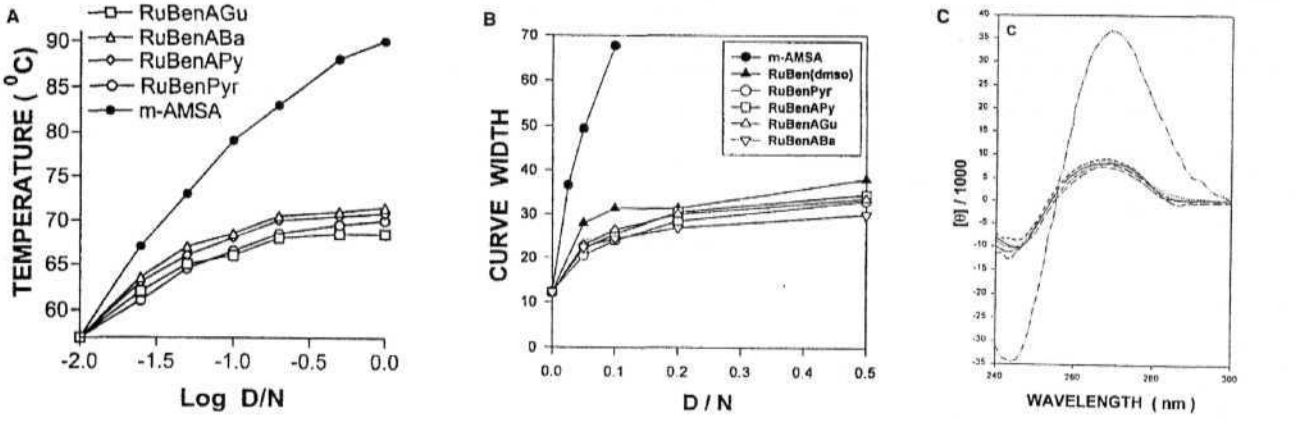

Fig. 5. (A) In the absence of any drug, the melting temperature curve of calf thymus DNA showed a T_m of 57 °C. In general, it was observed that at a drug to base nucleotide ratio 1:1, the ruthenium complexes raised the T_m of calf thymus DNA between 68.5 and 70.5. The DNA intercalator, m-AMSA, however, induces a significant increase in T_m (92 °C) at the similar concentration. Log D/N (drug/nucleotide) was plotted against the T_m for the ruthenium complexes, which showed that while all the ruthenium complexes were able to protect thermal denaturation to the same extent, m-AMSA showed a much higher protection. (B) D/N (drug/nucleotide) ratio was plotted against curve width of the T_m curves. Four curves for drug to DNA nucleotide ratios of 1:20, 1:10, 1:5, and 1:2 in case of the respective ruthenium drugs and three curves in case of m-AMSA were taken for curve width analysis. The data show a characteristic increase in curve width for m-AMSA (•), while the ruthenium drugs do not greatly affect the curve width of the melting temperature curves, suggesting that they do not intercalate with DNA. (C) The circular dichroism spectra of pBR322 DNA (——) in the presence of RuBenAGu (- - -), RuBenPyr (- - - -), RuBenAPy (· · ·), and RuBenABa (— · —) show that these drugs induce minute changes in the molar ellipticity of DNA, while m-AMSA (— · · —) shows a very prominent change at a concentration of ~3.5 times less than that of the ruthenium drugs. This large increase in the CD signal indicates a conformational change induced in the DNA due to intercalation by m-AMSA.

conformational change in the DNA. In contrast, a steep increase in the CD signal of DNA is noticed in presence of m-AMSA, which is a characteristic feature of DNA intercalating molecules (Fig. 5C).

Anti-proliferative action of the ruthenium compounds

The [^{3}H]thymidine incorporation assays on the two cancer cell lines show that the anti-proliferation activity and the anti-topo II activity of the three ruthenium complexes show a similar trend. RuBenAPy shows the highest potency of anti-proliferative action followed by RuBenAGu and RuBenABa, similar to their potency on topo II inhibition. But RuBenPyr also has a significant action on the proliferative response of the cancer cells, which does not correlate with its negligible action on topo II activity (Fig. 6). The DNA intercalating drug, m-AMSA, however, shows the highest anti- proliferative activity. The ZR-75-1 cells are more resistant to the anti-proliferation activity of these complexes compared to the colo-205 cells to an extent of 5–10% (Figs. 6A and B).

Discussion

Topoisomerase II is a major nuclear enzyme that maintains DNA topology in the complex chromosomal milieu [32,33]. The enzyme resolves numerous torsional problems in DNA, which arise during replication and transcription [34]. It also helps in catenation/decatenation, condensation/decondensation of DNA, and segregation of chromosomes during cell division [35,36]. The enzyme performs these functions by nicking a segment of duplex DNA, passing a second segment through a gate formed by the nicked DNA and finally rejoining the nicked segment [37]. This DNA breaking action of topo II has widespread implications resulting in loss of genetic integrity and is therefore a target for anti-cancer drugs. These topo II drugs are popularly known as topo II poisons because, rather than inhibiting the catalytic activity of the enzyme, they promote one part of the reaction mechanism, namely DNA cleavage, and block the second part, which is religation of the cleaved DNA [38]. Cancer cells generally over-express topo II, and these cells treated with the topo II poisons will harbor numerous topo II induced DNA double strand breaks which will become permanent double strand fractures following traversal by replication or transcription complexes [39]. This DNA damage will stimulate repair and recombination pathways, leading to sister chromatid exchange, large insertions/deletions, translocations and chromosomal aberrations. When these genetic aberrations accumulate at high concentrations, they trigger a series of events, which will ultimately culminate in cell death by apoptosis or necrosis [40,41].

Our earlier study with RuBen(dmso) introduced this molecule as a potential anticancer drug whose molecular target is topo II [19]. Even though high concentrations of this drug are required for topo II poisoning and anticancer activity, it nevertheless served as a lead molecule for the development of potent derivatives, in which

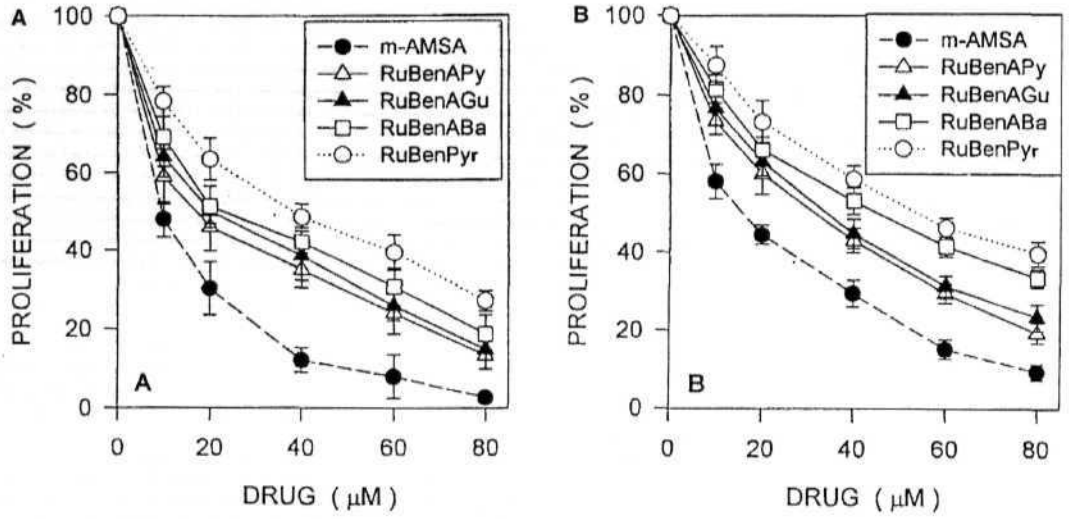

Fig. 6. In vitro anti-proliferation activity of the ruthenium compounds was tested on two fast growing carcinomas: (A) Colo-205 and (B) ZR-75-1. The cells were incubated with increasing concentrations of the ruthenium drugs and proliferation was measured by [^{3}H]thymidine incorporation, as described in Experimental procedures. Cell proliferation measured in the presence of 10, 20, 40, 60, and 80 μM ruthenium complexes is shown in the figure. m-AMSA has the most potent anti-proliferative action, followed by RuBenAPy, RuBenAGu, RuBenABa, RuBen(dmso), and RuBenPyr. Compared to the ZR-75-1 cells, the colo-205 cells are slightly more sensitive to the action of the drugs. The data presented are means of three independent experiments carried out in triplicates.

the topo II interacting 'dmso' group was replaced with pyridine, 3-aminopyridine, aminobenzoic acid, or aminoguanidine. All the four molecules bind to DNA with a similar affinity and three of them poison topo II activity, indicating a similar mechanism of molecular action.

The results of the relaxation assay, ATPase assay and the cleavage assay indicate that RuBenAPy, RuBenAGu, and RuBenABa poison topo II by cleavage complex formation. RuBenAPy is the most potent followed by RuBenAGu and RuBenABa. Surprisingly, RuBenPyr, which has a structure and DNA binding affinity similar to RuBenAPy, does not poison topo II. This large difference between the two similar molecules could be due to the amino group on the pyridine ring of RuBenAPy, which may solely interact with topo II. Similarly, in RuBenAGu and RuBenABa, the amino groups may be responsible for topo II interaction leading to effective poisoning of the enzyme. The higher potency of RuBenAGu could be due to multiple interactions with the enzyme by the three amino groups of the aminoguanidine ligand, as compared to that of a single amino group in RuBenABa. The drug conformation during topo II interaction may also be an important determinant for topo II poisoning. This is because RuBenApy has a single amino group, but is the most potent topo II poison among the RuBen drugs. The conformation and spatial orientation of the amino pyridine ligand in this drug may be appropriate for effectively freezing topo II in the cleavage complex. These studies reveal that the three poison drugs interact bidirectionally with DNA and topo II, similar to RuBen(dmso). The ruthenium atom binds to DNA and the ligand (aminopyridine, aminobenzoic acid, or aminoguanidine) interacts with topo II.

It was suggested that in aqueous solutions, the chlorides and the dmso group on RuBen(dmso) hydrolyze and the complex may likely exist in equilibria between aqua and chloro ions as $[Ru^{II}(C_6H_6)(H_2O)_3]^{2+}$ [28,42]. But the topo II assays with the complexes having different coordinated groups show that each has a different potency of topo II poisoning, which is retained in their aqueous solutions for a period of at least 72 h. Though hydrolysis of the coordinated group would eventually occur, the topo II inhibition data suggest that it may occur over a period of time rather than immediately. Hydrolysis of the chloride leaving groups may be an immediate reaction, which may enable interaction of the ruthenium atom with nucleotide phosphates of the DNA double strands. Covalent DNA cross-linking may not occur initially since the DNA interaction studies suggest a largely electrostatic binding. Long-lived DNA/protein interactions and eventual hydrolysis of the ruthenium complexes may, however, lead to covalent cross-linkages. Hydrolysis of the coordinated groups may also be an important determinant of the macromolecular interaction by these complexes. A recent work by Morris et al. [43] shows that similar complexes of the type $[(\eta^6\text{-arene})Ru^{II}(en)X]^+$ do not poison the activity of both topoisomerases, I and II up to concentrations of 50 μM, which may be true for most DNA binding metal complexes. Typically high concentrations of metal complexes in the range of 200 μM to 1 mM have been shown to poison the activity of topo II [13,14,16,19].

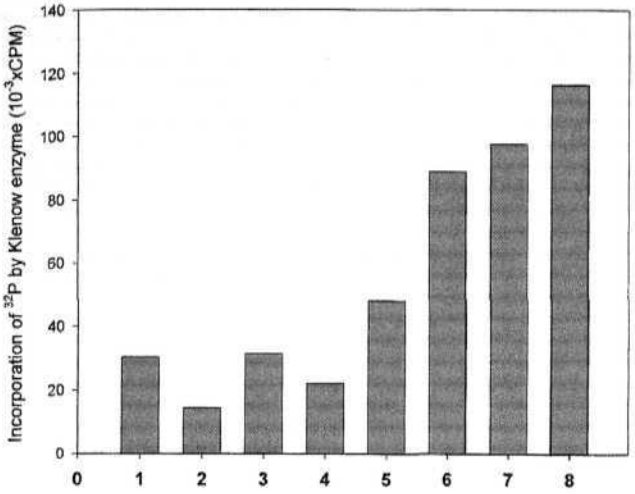

Fig. 7. Effect of ruthenium compounds on Klenow extension was studied by using *Hin*dIII digest of pBR322 DNA. The 3′ ends of the DNA are extension labeled using [α^{32}P]ATP by Klenow enzyme as described in the methodology. Samples 4–8 are first incubated with 500 μM Ruben(dmso) (4), 500 μM RubenPy (5), 200 μM RubenAPy (6), 250 μM RubenABa (7), and 350 μM RubenAGu (8), and the labeled DNA was analyzed. The experiment was also conducted in absence of compound (1) and in presence of 2′, 3′, dideoxy ATP (2) and DMSO (3). Each data point represents an average of two determinations.

How much of this activity actually translates into anticancer action needs to be further evaluated.

The possibility of inhibition of DNA polymerase activity by these compounds was tested using [α^{32}-P]ATP. The data given in Fig. 7 show Ruben(dmso) marginally inhibit, polymerase while RubenAGu, RubenABa, RubenAPy, and RubenPyr are shown to enhance polymerase action rather than inhibition, suggesting that these compounds may not interfere with DNA polymerase activity. We have verified DNA polymerase activity using [^{3}H]TTP with Klenow enzyme; these results confirm the above observations (data not shown). The reason for increase in activity in DNA polymerase activity is not clear to us.

The anti-proliferation assay on the two cell lines shows that the ruthenium compounds are effective anticancer agents and merit detailed analysis. RuBenPyr does not poison topo II but still shows significant antiproliferation activity, indicating that this drug as well as the other ruthenium compounds would interact with other cellular constituents as well. Though the present work details topoisomerase II antagonism, there would be other in vivo targets for these ruthenium complexes, which may or may not be important in cancer. This brings up the question of tissue and organ toxicity, which needs to be addressed for therapeutic purposes. In the present study, m-AMSA was found to be more effective than the ruthenium compounds in topo II poisoning and anticancer activity, but it is a DNA damaging agent and a possible mutagen, which limits its therapeutic potential, whereas the ruthenium compounds under study interact externally with DNA without destabilizing the DNA helix. More detailed studies involving animal models would be helpful in understanding the molecular targets vis-à-vis therapeutic efficacy and generalized toxicity.

Acknowledgments

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