List of publications:

1. Y.N.Vashish Gopal, Neelima Konuru, Anand K. Kondapi
   Topoisomerase II antagonism and anticancer activity of coordinated derivatives of
   \([\text{RuCl}_2(\text{C}_6\text{H}_6)(\text{dms})]\)
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   Analysis of age dependent changes of Topoisomerase II \(\alpha\) and \(\beta\) in rat brain.

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   Dimers with efficient DNA binding ability and potent anti tumor activity.
Analysis of age dependent changes of Topoisomerase II $\alpha$ and $\beta$ in rat brain


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Abstract

Eukaryotic Topoisomerase II (Topo II) is present in two isoforms $\alpha$ and $\beta$. The $\alpha$ isoform is predominantly localized in proliferative tissue, while $\beta$ isoform is present in all tissues. In the present study we report the activity and protein levels of Topoisomerase II $\alpha$ and $\beta$ 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 $\beta$ isoform is found to be the predominant form in brain tissue but Topoisomerase II $\alpha$ is found in embryos up to post-natal day 1. The studies to examine the regional distribution of Topoisomerase II $\beta$ 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 $\beta$ may have some unknown function in cerebellum and the low levels of Topoisomerase II $\beta$ activity in ageing cerebellum may contribute to the genomic instability in cerebellar region of ageing brain.

Keywords: Brain; Cerebellum; Neurons; Ageing; Topoisomerase II $\beta$

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, $\alpha$ and 180 kDa, $\beta$ 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 $\alpha$ activity is shown to be highest during the G2/M phase of the cell cycle (Woessner et al., 1991). On the other hand, Topo II $\beta$ activity is constant throughout the cell cycle. Topo II $\alpha$ is distributed in the nucleoplasm, whereas Topo II $\beta$ is localized in the nucleolus during interphase, and in the cytoplasm during mitosis (Chailly et al., 1996). In the embryonic stage, Topo II $\beta$ 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 $\alpha$ is shown to be higher in testes, spleen, bone marrow and liver. Topo II $\beta$ is detected in high levels in differentiated tissue like brain (Jurekke and Holden, 1993; Capranico et al., 1992). However, a systematic study to examine the levels of the isoform of Topoisomerase II, $\alpha$ and $\beta$ 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 $\alpha$ and $\beta$ 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 $\beta$ isoform in different regions of brain. The changes in the levels of Topo II $\beta$ 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 $\beta$ 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 $\alpha$ and $\beta$ monoclonal antibodies were obtained from Pharmingen. Goat-anti mouse IgG and FITC...
conjugated Goat-anti mouse IgG was obtained from Bangalore Genei, India. Nonidet, ATP were from Boehringer Mannheim. PMSF, BSA, protein A agarose, Triton X-100, ficoll, DTT, trypsin and trypsin inhibitor were from Sigma, USA. PVDF membrane was obtained from FALL 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 MgCl2, 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 MgCl2, 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

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Fig. 1. Enzymatic activity and expression 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 (lanes 1-4), 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 α in cerebellum of E11 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 of each sample were separated on 7.5% SDS PAGE and Western transferred and probed with mononclonal Abs against Topo II α, immunoblot of Topo II α in 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 KH₂PO₄, 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% Nonioid) 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.

![Relaxation assay of Topoisomerase II β in cerebellum of embryos](image)

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 supercoiled DNA band (Form I) disappears and completely relaxed plasmid DNA (form II) appears. About 0.6 μg of supercoiled 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 MgCl₂, 30 μg/ml BSA, 1 mM ATP) for 30 min at 37 °C. The beads were spun down at 300 x 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 onto 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

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Fig. 3. Enzymatic activity and immunoblotting 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 activity is measured in terms of amount of supercoiled DNA present in the gel. Quantified supercoiled DNA was plotted on Y-axis and sample names were shown on 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. While 75 μg of protein of extract was separated on 7.5% SDS PAGE and Western transferred and probed with monoclonal Abs against Topo II α shows 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 cerebral cortex and mid brain respectively.
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 γ32P-ATP in kinase buffer (10% glycerol 0.1 M EDTA and 50 mM MgCl2). 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 3000 x 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 32P 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 isofom-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

![Figure 1A](image1)

![Figure 1B](image2)

![Figure 1C](image3)

![Figure 2A](image4)

![Figure 2B](image5)

![Figure 2C](image6)
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 ac-
tivity of Topo II α in these three regions and at all the ages
studied. To know protein levels of Topo II α, we have car-
ried out immunoblot analysis of Topo II α in extracts of
cerebellum (Fig. 3D), cerebral cortex (Fig. 3F) and midbrain
(Fig. 3H), the results of 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 signif-
ant activity of Topo II β in whole brain extract (Fig. 4A
and B). The “Young” rat brain showed high activity of this
isofrom, while moderate activity is seen in “Adult” brain

![Figure 6](image_url)

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).
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 staining 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.](image-url)
Fig. 9. Immunofluorescence 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 cyospinned. 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 of 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,
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 in Purkinje 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.

![Graph](image)

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 Topoisomerasases 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 immuno-
precipitated 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 (nuclease 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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References


Topoisomerase II antagonism and anticancer activity of coordinated derivatives of [RuCl₂(C₆H₆)(dmso)]

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Abstract

Topoisomerase II poisoning and anticancer activity by the organometallic compound [RuCl₂(C₆H₆)(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 aminoquinidine. 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 [RuCl₂(C₆H₆)(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. © 2002 Elsevier Science (USA). All rights reserved.

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-indazolylbis(indazolyl)tetrachlororuthenate [7], and imidazolium trans-imidazolylmethylsulfoxidetetrachlororuthenate (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 carbacarboranyl complexes of iron [13-15], salicylaldehyde and carbonic complexes of cobalt [16,17], and the ruthenium

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complexes [RuCl₂(C₅H₈)dmso] (RuBen(dmso)) and trans-indazolium[bis(indazole)tetraclororuthenate [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 mainly by transferrin (60%) 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., Ru³⁺ → Ru²⁺), which would activate the metal complexes to bind biopolymers in the hypoxic environment of tumors [23,24].

Detailed in vitro analysis of [RuCl₂(C₅H₈)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.

### 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 (polyethylenimine) cellulose-F TLC sheets were from Merck, Protease K and ATP were from Boehringer–Mannheim, [γ²³⁵-P]ATP and ³H-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 RuCl₂ · 3H₂O (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 [RuCl₂(C₅H₈)₂]₂. To this dimer, DMSO was added to form the monomeric DMSO complex [RuCl₂(C₅H₈)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 MgCl₂, 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 cyanole, 60% sucrose, 100 mM Tris-HCl, pH 8.0) was added, and the products were separated on a 1% agarose gel in 0.5 x 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.

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 [γ³²-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, γ³²-P migrates first followed by ADP and [γ³²-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, (inorganic phosphate) were cut out of the sheet and counted for ³²P 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 x 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. Tm was determined from the denaturation curves. Curve width

Fig. 1. Chemical structures of the RuBen compounds. RuBen(dms), 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.
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

[θ] = M_ε * ψ / 100Lc,

where [θ] is molar ellipticity, M_ε 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 μg/ml.

Anti-proliferation activity

This was analyzed through [3H]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 x 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 CO2 incubator (Forma Scientific) maintaining 5% CO2. 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 [3H]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 scintillation 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 HindIII 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-32P]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 Whatman filter paper and the radioactivity was measured by placing the filters in scintillation fluid and counted in a 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 so do (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
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
titration was carried out which showed a DNA binding saturation at 1:1 or higher drug to 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 indicated a high DNA binding affinity for the ruthenium complexes. Curve analysis of the thermal denaturation curves was carried out to determine the mode of DNA interaction by the ruthenium drugs. Fig. 5 shows 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
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 T²H-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 rejoicing 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
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 cleavage complex. These studies reveal that the three RuBen 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 [RuCl^III(C_6H_5CH_2O)]^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 macro-molecular interaction by these complexes. A recent work by Morris et al. [43] shows that similar complexes of the type [η^6-arene]RuCl^III(η^5)(en)^2 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. 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 [3H]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.
Fig. 7. Effect of ruthenium compounds on Klenow extension was studied by using HindIII digest of pBR322 DNA. The 3' ends of the DNA are extension labeled using [$\gamma$-32P]ATP by Klenow enzyme as described in the methodology. Samples 4–8 are first incubated with 500 M Ruthen(dmso) (4), 500 M RubenPy (5), 200 M RubenAPy (6), 250 M RubenAGu (7), and 350 M 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 [$\gamma$-32P]ATP. The data given in Fig. 7 show Rubent(dmso) marginally inhibit polymerase while RubenAGu, RubenPy, 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 [3H]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 anti-proliferation 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-AMS 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.

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