Design and Synthesis of Anticancer Cobalt Complexes : Molecular mechanism of Action on Topoisomerase II

A Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

BY

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Declaration

I hereby declare that the work presented in this thesis entitled ***"Design and Synthesis of Anticancer Cobalt Complexes : Molecular mechanism of Action on Topoisomerase II"*** is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of Dr. K. Anand Kumar. I further declared that to the best of my knowledge this work has not been submitted before for the award of degree or diploma from any University or Institution.

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Certificate

This is to certify that this thesis entitled ***"Design and Synthesis of Anticancer Cobalt Complexes: Molecular mechanism of Action on Topoisomerase II"*** submitted by **Mr. D. Jayaraju** for the degree of **Doctor of Philosophy** to the University of Hyderabad is based on the studies carried out by him under my supervision. This work has not been submitted before for the award of degree or diploma from any University or Institution.

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ABBREVIATIONS

A_{260}, A_{280}	absorbance at 260, 280 nm respectively
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
CoASI	cobalt (III) *N*-aminosalicylaldimine
CoAPSI	cobalt (III) *N*-phenylaminosalicylaldimine
CoDNPSI	cobalt (III) (2-4, dinitro)*N*-phenylaminosalicylaldimine
CoPSI	cobalt (III) *N*-phenylsalicylaldimine
CoSAL	cobalt (III) salicylaldoxime
CoSSC	cobalt (II) salicylalsemicarbazone
CoTSSC	cobalt (II) salicylalthiosemicarbazone
CuSAL	copper (II) salicylaldoxime
DNA	doxyribo nuclic acid
DTT	dithiotheritol
EDTA	ethylene diamine tetraaceate
EtBr	ethidium bromide
h	hours
kDa	kilodaltons
m-AMSA	*N*-[4-(9-acridinylamino)-3-methoxy-phenyl]methanesulfonamide
min	minutes
mM	millimolar
M	molecular weight
SDS	sodium dodecyl sulfate
TCA	tri chloroacetic acid
TBAP	tetra butyl ammonium perchlorate

T_m	melting temperature
tris	tris (hydroxy methyl) aminomethane
UV	ultraviolet

CONTENTS

CHAPTER I

INTRODUCTION

Cancer

Cancer is estimated to cause 23% of the person-years of the premature loss of life. Each year there are 1.25 million diagnoses and 1 million deaths from various types of cancer. Four major cancers (lung, colon-rectum, breast and prostate) account for 55% of deaths. Smoking accounts for 90% of lung cancer and about 1/3 of all cancer deaths. The death rate is a continuing reminder of the tremendous task of finding more effective treatment.

From the last 20 years percentage of survival rate has been increased because of effective treatment of various cancers by using different potential treatment methods like chemotherapy, radiotherapy, surgery and gene therapy. Presently, chemotherapy is widely used for cancer treatment.

Topoisomerases as a target for chemotherapy

One of the targets for the cancer chemotherapy are topoisomerases, a class of enzymes in the nucleus of all living cells, which alter the topological states of DNA via the breakage-reunion of one or both DNA strands. DNA topology is critically important in transcription, replication and chromosome structure. Cells die when topoisomerases are poisoned.

Enzymology of topoisomerases

Two major types of topoisomerases are recognized in eukaryotes They are classified based on their mechanism of action.

DNA topoisomerase I:

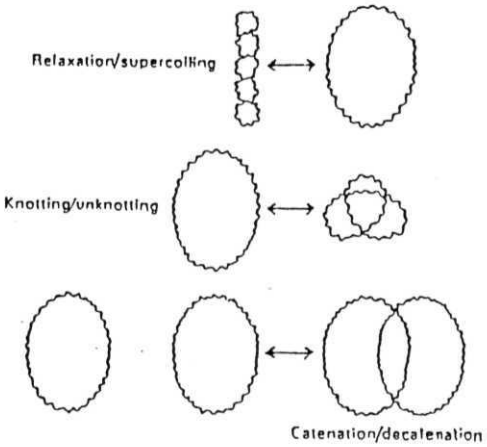
DNA topoisomerase I is a 100 k Da. monomeric protein encoded by a single copy gene located on chromosome 20q 12-13.2 (Liu & Miller, 1988; D'Arpa *et al.*, 1988). **The** enzyme is capable of relaxing both positive and negative supercoiled DNA via single-strand breakage-reunion cycle without requiring an energy cofactor ATP.

2. DNA Topoisomerase II:

DNA Topoisomerase II is a 170 k Da homodimeric protein encoded by a single copy gene located on chromosome 17q 21-22 (Miller *et al.*, 1981). The enzyme catalyzes a number of ATP-depended DNA topoisomerization reactions like, relaxation/supercoiling, catenation/decatenation and knotting/unknotting via a double-strand-passing mechanism (Liu *et al.*, 1980)

Figure. 1

Reactions Catalysed by Topoisomerase II



Structure and function of topoisomerase II:

Berger *et al* (1996) reported the three-dimensional structure of the functional yeast type II topoisomerase. Based on their observations the following structural alignments were found to be present in topoisomerase II.

The monomer

The enzyme is a homodimeric protein and the crystal structure of the 92Kda monomer was reported at a resolution of 2.7 ^{0}A which has the shape of a flattened crescent with over all dimensions of 120 x 85 x 55 ^{0} A. The polypeptide chain of the monomer folds into two sub-fragments, denoted as B' and A'. The B' sub-fragment consists of two α/β domains. The A' sub-fragment has snugly docked parts. The N-terminal part of A' contains two distinct domains and a connecting region. The first domain is a DNA binding domain containing tyrosine at position 783, the residue that becomes covalently attached to the 5' ends of the cleaved DNA strands lies in this region and the second domain is an α-β structure. The C-terminal part of A' is largely α-helical and helps in dimer contact at the bottom.

The dimer

The two crescent-shaped monomers form a pair to make a heart shaped dimer with a large central hole. The overall dimension of the dimeric protein is 120 x 120 x 55 ^{0}A and the hole is 55 °A wide at its base, 25 °A wide at the top, and 60 ^{0}A long. The primary dimer contact between the sub-units occur at the A'-A' interface at the bottom and forms a V shaped A'-A' dimer and in turn it receives a B'-B' dimer at its open end. This protein arch caps the hole between the two A' domains.

DNA binding domain

The domains in the first part of A' create a semicircular groove of 20-25 ^{0}A, which funnel down to the tunnel leading to the active site of DNA cleavage. Electrostatic potential distribution for the entire A'-A' dimer shows that this region has a strong positive potential.

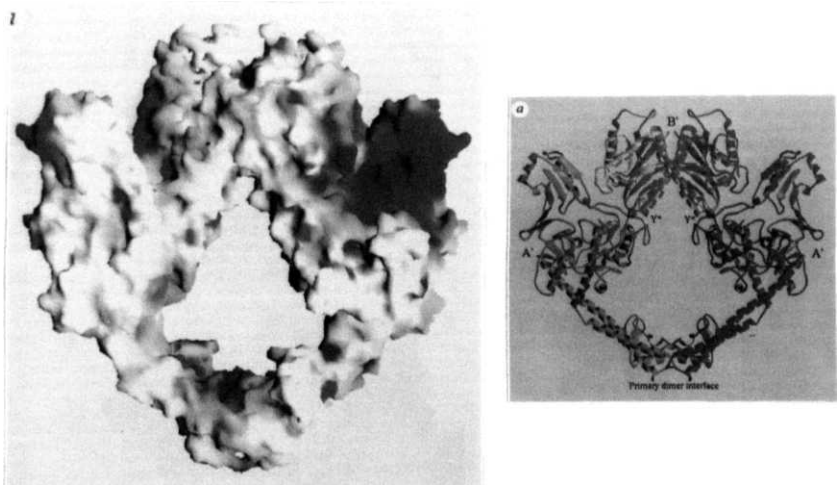
The ATPase domain

The ATPase-domain of the dimer lies on top of the B' sub-fragments, it has a large notch at the dimer interface. This notch closes off into a hole through conformational changes of its C-terminal peptides upon ATP binding.

Figure.2

The structure **of topoisomerase II**

(A model proposed by Berger et al., 1996)



The catalytic cycle of Topoisomerase II

Topoisomerase II catalyzes changes in DNA topology which requires a high-energy cofactor (ATP) and Mg^{+2}. The catalytic cycle is extremely complex and precise mechanistic details of how DNA strands are transported through an enzyme-bound DNA gate was explained by Berger *et al* (1996) using X-ray crystallographic studies. The catalytic cycle of topoisomerase II involves DNA cleavage, transport and religation, coupled to ATP binding and hydrolysis.

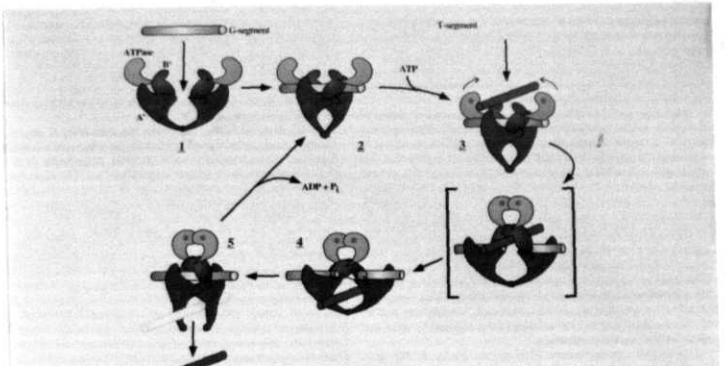
In the unliganded state the enzyme is an open conformation with the A' subfragments spread apart (panel 1 Fig 3). Upon binding of the DNA segment to be cleaved it undergoes a conformational change to bring the A' subfragments together and the active-site tyrosine's into their 'attack' positions (panel 2 Fig 3). The B' subfragments in association with this unliganded ATPase domains may assist this change.

Binding of ATP induces dimerization of the ATPase domains (panel 3 Fig 3) and initiates a conformational cascade - cleaving of DNA double strands and covalent attachment of enzyme to the 5' nicked ends of the DNA through phosphotyrosyl linkage (panel 4 Fig3). As the ATPase domains dimerize, they capture a weakly held second duplex pushing into the interior of the enzyme and through the DNA gate. Steric repulsion by the capture of second DNA segment might aid in separating the cleaved strands apart. Then ATP hydrolysis takes place and the cleaved strand is religated.

Figure 3

Mechanism of action of topoisomerase II catalytic activity

(A model proposed by Berger et al., 1996)



Function of Topoisomerase II

Segregation of chromosomes during mitosis and meiosis

This is the major function of topoisomerase II during cell cycle. A number of studies (Uemura & Yanagida. 1984; Uemura *et al,* 1987) have demonstrated that topoisomerase II is required for chromosome segregation at mitosis and meiosis, during which the enzyme is thought to separate the intertwined daughter strands created during DNA replication.

Chromatin condensation

Topoisomerase II helps in condensing the DNA into chromatin and further condensing this chromatin into mitotic chromosomes. It is also required for the decondensation of chromosomes (Uemura *et al,* 1987).

DNA replication and transcription

DNA metabolic process such as replication and transcription generates supercoiles, catenanes and knots. These are removed by topoisomerase II via a double-strand-passing mechanism.

Recombination

Mutations in the topoisomerase II gene greatly increases the recombination levels in the rDNA cluster (Christman *et al,* 1988) but the involvement of topoisomerase II in illegitimate recombination is not yet clear (Ikeda, 1990).

As chromosomal scaffold protein

Several evidences support that topoisomerase II plays a structural role in chromosomes (Gasser *et al,* 1989; Roberge & Gasser, 1992). It is a major non-histone protein present in nuclear scaffold (Earnshaw & Heck, 1985; Gasser *et al,* 1986)

Regulation of topoisomerase II functions

It has been shown that phosphorylation plays a key role both in determining specific activity and in regulating mitotic functions of topoisomerase II. Casein Kinase II, Protein kinase C, Protein kinase I are the kinases involved in topoisomerase II phosphorylation though it is not yet clear about their involvement in structure and function of topoisomerase II.

Cell cycle regulation of phosphorylation

The phosphorylation status of topoisomerase II is regulated with respect to both cellular-growth state and cell-cycle position. The level of topoisomerase II phosphorylation is higher in mitotic cells than in G1 cells, although the sites of phosphorylation are broadly similar during all cell-cycle phases. The majority of reports suggest that casein kinase II predominantly phosphorylates topoisomerase II during the cell cycle.

Topoisomerase II as target for chemotherapeutic agents

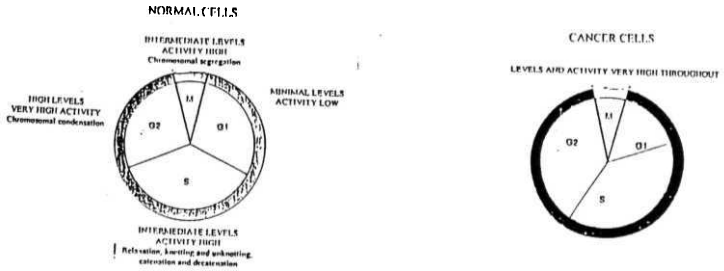
Because of its essential cellular function, topoisomerase II is a potential cellular target for number of anticancer drugs because.

Levels of topoisomerase II helps in targeting

It has been shown that the levels of mammalian topoisomerase II are generally sensitive to the growth state of cells. Topoisomerase II levels are high in rapidly proliferating cells, but decrease when cells are arrested or induced to differentiate. In general, when cells are in late G_1 or early Go state, topoisomerase II levels and activity are very low. On the other hand, stimulation of quiescent cells leads to rapid increase in topoisomerase II levels and activity (Heck *et al.*, 1988; Hsiang *et al.*, 1988). While topoisomerase II is tightly regulated by growth conditions in normal cells, cancerous cells maintain constantly high levels irrespective of growth conditions (Wang *et al.*, 1989).

Figure. 4

Topoisomerase II in cell cycle



Oncogenes near topoisomerase II genes help in targeting

The gene mapping studies for topoisomerase II gene reveal that the α and *fl* topoisomerase II genes are located in the vicinity of some of the cancer associated oncogenes. It has been shown that topoisomerase II is co-amplified along with these genes in oncogene associated tumors (Keith, 1992). Antibodies directed at the product of the oncogenes may help in targeting topoisomerase II inhibitors to cancerous cells.

The topoisomerase II a gene is very close to erbB2, the widely discussed breast cancer gene on chromosome 17q21 which is stimulated by estrogen. Estrogen stimulated human breast cancer cells also induce topoisomerase II expression (Epstein, 1989). A lung adenocarcinoma cell line, Calu3 possesses co-amplified erbB2 and topoisomerase II α (Keith, 1992) indicating this relationship may be broader than just for breast cancer. This relationship may be useful for designing of topoisomerase II targeting drugs.

Topoisomerase II inhibitors

A number of topoisomerase II inhibitors have been identified and developed. Based on their molecular action, these inhibitors can be divided into four groups. A major group are composed of DNA intercalating molecules that interfere with DNA strand passage and religation activities of topoisomerase II., e.g, m-AMSA, ellipticine, daunorubicin and adriamycin (Nelson *et al,* 1984; Tewey *et al,* 1984; Yang *et al.,* 1987). The epipodophyllotoxins (etopoiside and teniposide) fall under the non-intercalating group (Chen *et al,* 1984; Rose *et al.,* 1990). Both these groups of molecules stabilize enzyme-linked DNA intermediates "Cleavage complexes" and are widely used in treatment of human cancers and microbial infections (Liu, 1989; Schneider *et al.*, 1990, Hooper *et al.*, 1991; Reece *et al.*, 1991).

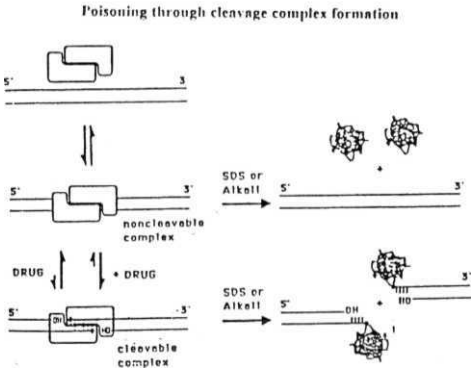
The third group constitutes the coumarine derivatives such as novobiocin and coumermycin which affect the activity of topoisomerase II by inhibiting ATP hydrolysis reaction that is required for movement of molecular clamp in its catalytic cycle (Gellert *et al.*, 1978; Sugino *et al.*, 1978). Molecules such as amonafide and RO 15-0216, quinolone derivatives and 2-nitroimidazole (Robinson *et al.,* 1990; Sorenson *et al.*, 1992) are known to stimulate topoisomerase II-mediated DNA cleavage, also fall under this category.

The fourth group of molecules have the property of inhibiting the catalytic function of topoisomerase II without aiding the formation of "Cleavage complexes". Anthracyclin derivatives, aclarubicin, bis(2,6-dioxopiperazine) and fostriecin belong to this group (Jensen *et al,* 1990).

Mechanism of action of topoisomerase II poisons

The major class of topoisomerase II poisons such as intercalating agents, eg., doxorubicin, mitoxantrone, m-AMSA, as well as non- intercalating epipodophyllotoxins, etoposide and teniposide, stabilize a reaction intermediate, termed the 'cleavable complex' consisting of a topoisomerase II protomer bound covalently to each 5′ ends of the broken DNA molecule. These drugs act at least at two points in the topoisomerase II catalytic cycle. Both the pre- and post-strand passage equilibria are disturbed as a result of a decrease in the enzyme's apparent first order constant for religation (Figure. 5). The cleavage complex formation in presence of topoisomerase II poisons can be detected by the appearance of linear DNA upon its treatment with sodium dodecylsulphate (SDS) or alkali.

Figure 5



SCOPE OF PRESENT WORK

Development of metal complexes for anticancer use was initiated from the time cisplatin, a complex of platinum was identified as an effective anti-tumor agent (Rosenberg *et al.*, 1969). The anti-tumor complexes of heavy metals like platinum, titanium and osmium show high cytotoxicity because these complexes are unnatural from the biological viewpoint and the body has no effective mechanisms for their elimination (petering *et al.*, 1980). It would be useful to develop potent anticancer metal complexes of biologically essential trace metals, because the cells have their own mechanism to eliminate the metals and their compounds when they are present in excess (Petering, 1980). Many anticancer metal complexes show anticancer activity due to their ability to interact with cellular targets. One of the possible mechanisms regarding the anticancer activity of metal complexes is that the central metal atom binds DNA nucleotide bases through covalent linkages and the ligands around it may interact with cellular targets (Kopf-Maier, 1987; Kopf-Maier *et al.*, 1989). The development of such anticancer complexes is useful in directing against specific targets.

Several leading anticancer agents eg., m-AMSA, adriamycin, daunorubicin, ellipticin, etopoiside and mitoxantrone mediate their anticancer activity by enhancing the ability of

topoisomerase II to generate double- and single-stranded breaks in the DNA (corbett et al., 1993; Froelich-Ammon et al., 1995, Chen et al., 1994; Pommier et al., 1996). The presence of elevated levels of covalent topoisomerase II-cleaved DNA complexes provokes mutagenic and cell death pathways (Nitiss et al., 1996; Ferguson et al., 1993). Thus, chemical agents which are effective in promoting enhanced topoisomerase II-

mediated DNA cleavage are referred to as topoisomerase II "poisons" because of their ability to convert this enzyme in to a potent cellular toxin. Information concerning the actual mechanisms through which these drugs exert this effect is limited; however, the ability of these compounds to interact with both DNA and topoisomerase II appear to be essential for nucleic acid cleavage. The relationship between the potent anticancer activity and enhanced topoisomerase Il-mediated DNA cleavage have been the focus of intense interest to develop more effective topoisomerase II poisons.

The present study was undertaken with the following objectives:

1. Do the known anticancer metal complexes affect the activity of topoisomerase II? if so, how?

2. Synthesis of anticancer metal complexes using the biological trace metal cobalt and analyze how they affect topoisomerase II catalytic activity.

3. Design and synthesis of more potent derivatives of the anticancer cobalt complexes which show higher topoisomerase II inhibition; and to further elucidate the molecular mechanism of action of these derivatives on topoisomerase II activity.

CHAPTER II

General methods

A) Plasmid pBR322 supercoiled DNA isolation

Transformation

A single colony of E. coli strain DH5α was inoculated in to a 100 ml liquid broth (LB) medium containing 1 g tryptone, 1 g NaCl and 0.5 g yeast extract. The culture was incubated overnight at 37 °C with vigorous shaking (200 revolutions/min). 0.5 ml of the overnight culture was inoculated in to a fresh 100 ml LB medium and further incubated for 3-4 h at 200 revolutions/min.

The cells were harvested by centrifugation at 4000 rpm for 10 min at 4 °C. The cell pellet was resuspended in 20 ml of 10 mM $CaCl_2$ and kept on ice for 10 min. Again the cell pellet was collected by centrifugation and resuspended in 2 ml of 10 mM $CaCl_2$. These cells are referred to as competent cells.

200 μl of competent E.coli cells were transferred into a 1.5 ml micro-centrifuge tube containing 10-20 ng of plasmid (pBR322). The tube was kept on ice for 30 min and then transferred to a water bath set at 45 °C for 90 sec. Immediately after, the tube was kept on ice. To this 800 μL of SOC medium (100 ml of LB medium containing 10mM Glucose and 10 mM $MgSO_4$) was added and incubated for 45 min at 37 °C. 200 μl of the transformed cells were poured onto a SOC agar (SOC medium contain 1.5% agar) plate containing appropriate antibiotic (40 μg/ml ampicillin or 10 μg/ml tetracyclin). The plate was kept in an incubator set to 37 °C for 17-20 h.

Purification of supercoiled plasmid DNA

2-3 pBR322 transformed E. coli colonies were inoculated in to a 100 ml of LB medium containing 40 μg/ml ampicillin and incubated overnight at 37 °C. 1 ml of this overnight culture was inoculated into 1 L LB medium containing 40 μg/ml ampicillin and further incubation was continued further for 17-20 h.

The cells were harvested by centrifugation at 4000 rpm for 15 min at 4 °C. The cells were lysed in 40 ml lysis buffer (50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 and 10 mg/ml Lysozyme) at room temperature for 10 min. To this, 80 ml of freshly prepared alkaline solution (0.2 N NaOH and 1% SDS) was added and mixed. The solution was kept on ice for 10 min. The proteins were precipitated by adding 50 ml of saturated ammonium acetate, mixed well and kept on ice for 10 min.

The precipitate was_removed by centrifugation at 10 000 rpm for 15 min at 4 °C and (poured the supernatant through glass wool. The crude DNA was precipitated by adding 0.7 volume of ice cold isopropanol and the solution was kept on ice for 20 min. The crude DNA pellet was collected at 12 000 rpm for 15 min at 4 °C. The supernatant was discarded and pellet was dried. The DNA pellet was dissolved in 25 ml of acid extraction buffer (0.75 M NaCl, 0.3 M sodium acetate pH 4.2 and 10 mM EDTA). To this, equal volumes of water saturated phenol was added and mixed well to remove both RNA and DNA binding proteins. The aqueous and the phenol layers were separated by centrifugation at 10 000 rpm for 15 min at 4 °C. The aqueous layer was removed without disturbing the interphase. The phenol layer was extracted once again with acid extraction buffer.

To the phenol layer 7.5 ml of reverse extraction buffer (1.5 M Tris base and 5 mM EDTA pH 8.0) and 7.5 ml of chloroform were added. The aqueous layer was collected and once again extracted with equal volumes of chloroform. The DNA was precipitated by adding 0.1 volumes of 3 M sodium acetate and 0.7 volumes of isopropanol and the DNA pellet was collected as before. The DNA pellet was washed with 70% ethanol and dissolved in 0.5 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA pH 8.0)

B) Purification and characterization of topoisomerase II

Pure nuclei isolation

All procedures were carried out at 4 °C. 500 g of liver was collected from 60 day old rats (Wistar strain) and homogenized in 3 L of homogenization buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 25 mM KC1, 0.34 M sucrose and 0.1 mM PMSF, Buffer A) by applying 10-15 strokes at 3000 rpm. The mixture was centrifuged at 5000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in 900 ml of Buffer A containing 2.2 M sucrose. Nuclei were collected by centrifugation at 26 OOOrpm for 1 h in a Beckman SW 26 rotor and supernatant was discarded. The pellet was washed with 300 ml of Buffer A containing 1 M sucrose, followed by 250 ml of Buffer A containing 0.1% Triton X-100. The nuclei pellet was dissolved in TGM buffer (10 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM DTT and 40% glycerol) and stored frozen at -20 °C.

Topoisomerase II purification

The nuclear pellet was centrifuged, the supernatant was discarded and the pellet was resuspended in lysis buffer (5 mM potasium phosphate pH 7.5, 100 mM NaCl, 10 mM 2-mercaptoethanol and 0.5 mM PMSF). The nuclei were lysed using a Branson sonicator with a macroprobe for 30 s, with 1 min intervals over a period of 5 min. The progress of lysis was monitored by phase contrast microscopy. After lysis was completed, freshly prepared 10% polymin P (pH 7.8) was slowly added to the mixture over a period of 15 min, while stirring to a final concentration of 0.35%. The resultant precipitate was collected by centrifugation at 6000 rpm for 10 min. The pellet was resuspended in 250 ml of PR buffer (20 mM potasium phosphate pH 7.5, 10 mM $NaHSO_3$, 10% glycerol, 10 mM 2-mercaptoethanol and 0.5 mM PMSF). Proteins were extracted from the chromatin-Polymin P pellet by 0.55 M NaCl, with continuous stirring over a period of 30 min. Following this, the nucleic acids were reprecipitated with the addition of Polymin P to a concentration of 0.7%. The suspension was stirred again for 15 min and the pellet was removed by centrifugation as described above. The supernatant was filtered through glass wool, to which solid ammonium sulfate was added to a final concentration of 60% with continuous stirring. After 1 h, the resultant precipitate was collected by centrifugation at 10 000 rpm for 20 min. The pellet was resuspended in 100 ml of PR buffer and dialysed against 5 x 1 L of PR buffer over a period of 15 h. The precipitate formed during dialysis was removed by centrifugation at 26 000 rpm for 20 min in Beckman SW 28 rotor.

Chromatography on Hydroxyapatite

The clarified dialysed supernatant was loaded onto a Biogel-hydroxyapatite (Biogel-HTP) column (2 x 10 cm) which had been equilibrated with a buffer containing 200 mM

potassium phosphate in PR buffer. The column was washed with the equilibration buffer until there was no A_{280} absorbing material. The bound proteins were eluted with a linear gradient of potassium phosphate (200 mM- 700 mM) in PR buffer. The fractions that contained topoisomerase II activity were pooled and dialysed against PR buffer. The dialysed fractions were re-chromatographed on a second Biogel-HTP column and the procedure was repeated as described above. The fractions containing topoisomerase II activity were combined and dialysed against PR buffer. The dialysed fractions were concentrated by using a Centricon- 10 microconcentrator.

Gel-filtration chromatography

The protein from chromatography on Biogel-HTP was layered on sephadex G 100 gel-filtration column (2 x 100 cm) which was previously equilibrated with PR buffer. Fractions of 0.5 ml were collected and analyzed for topoisomerase II activity. The fractions containing topoisomerase II activity were pooled, dialysed against PR buffer and concentrated using Centricon- 10 microconcentrator. The final enzyme preparation was dialysed against storage buffer (30 mM potasium phosphate pH 7.5, 50% glycerol, 0.1 mM EDTA, 0.5 mM DTT) and stored at -20 °C

Characterization of topoisomerase II

I. SDS-PAGE analysis

Protein concentration was measured by Bradford method. 100 ng of protein was electrophoresed on 12% SDS-PAGE gel and silver stained according to Laemmli (1970) method (Figure 6 lanes 2 & 3).

Figure 6

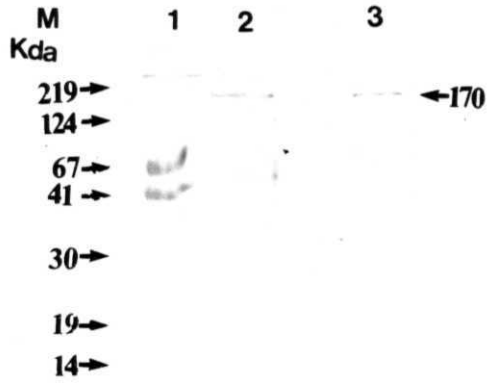


Figure 6. SDS-PAGE analysis of topoisomerase II fractions

Lane 1. Molecular weight markers

Lane 2. Bio-gel HTP fraction

Lane 3. Gel filtration (G-100) fraction

2. Immunoblottting analysis (Western blotting)

Immunoblotting was performed by the method of Towbin *et al.*, (1979). Protein from SDS-PAGE gel was transformed to 0.2 μm nitrocellulose membrane using trans blot apparatus (Biorad, USA). Protein transfer was performed at 45 mA for 3 h in 25 mM Tris-HCI (pH 8.8), 192 mM glycine and 20% methanol buffer. After blotting, the membrane was washed with TBS (10 mM tris-HCI pH 7.5 and 150 mM NaCl) and non-specific binding sites were blocked with 3% (w/v) BSA for 2 h.

Then the immunoblot was incubated overnight at 4 °C in a polyclonal antibody directed against topoisomerase II. Following three washes with TBS, the blot was incubated with secondary antibody (goat anti-rabbit conjugated with alkaline phosphatase) for 1 h. After washing three times with TBS, immunoreactive topoisomerase II was visualised using 5-bromo - 4 - chloro - 3 - indoylphosphate/nitrobluetetrazoliumchloride (BCIP/NBT) chromogen (Figure 7).

3. Enzyme Catalyzed Relaxation Assay

The reaction mixture of 20 μl contained 50 mM Tris-HCI 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 and 0.6 μg of negatively supercoiled pBR322 plasmid DNA and with increasing concentrations of drugs. The reaction was initiated by addition of topoisomerase II to the concentration of 8 nM and incubated at 30 °C for 20 min. The reaction was stopped by addition of SDS to a final concentration of 0.5%. To this reaction mixture, 3 μl of loading dye (60% sucrose ,0.5% bromophenol blue, 0.5% xylene cyanol and 10 mM tris-HCI pH 8.0) was added. Products were separated on 1% agarose gel in 0.5x tris-acetate buffer (40 mM tris acetate pH 8.3

Figure 7

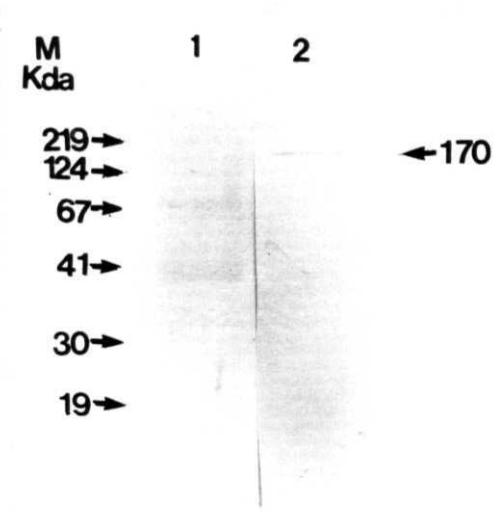


Figure 7. Western blot analysis of topoisomerase II

Lane 1. Molecular weight markers

Lane 2. 170 kDa band corresponds to Topoisomerase II.

and 2 mM EDTA) at 50 V for 16 h. The gel was stained in ethidium bromide (10 μg/ml) and visualised under uv and photographed (Figure 8 panel A).

4. Cleavage Assay

The formation of cleavage complex was assayed by the procedure of Brigette *et al* (1996) . The reaction mixture contained essentially the same buffer used in the relaxation assay with increasing concentrations of drugs. The reaction was initiated by addition of 0.6 μg pBR322 DNA and 40 nM of topo II and incubated at 30 ^{0}C for 15 min. The reaction was stopped by adding SDS to a final concentration of 0.5% and 2 μl of 250 mM EDTA. The DNA bound protein was digested by incubating the reaction mixture with 2 μl of 1 mg/ml solution of Proteinase K at 45 ^{0}C for 1 h. 3 μl of loading dye was added and the products were separated on a 1% agarose gel, stained in ethidium bromide and photographed under uv. The linear DNA band was quantified as percentage of total DNA in a UVP gel documentation system (Figure 8 panel B).

C) ATPase assay

a) Spectrophotometric assay

In the spectrophotometric assay, rapid conversion of ADP to ATP by pyruvate kinase and phosphoenolpyruvate, a reaction coupled to NADH oxidation, was used to measure the rate of ATP hydrolysis following the procedure described by Morrical *et al* (1985). Under steady-state conditions, the rate of ATP hydrolysis is directly proportional to the rate of the absorbance decrease observed at 340 nm. The assay was performed in a Shimadzu UV-650A spectrophotometer. The reaction mixture of 1 ml containing reaction buffer (20

Figure 8

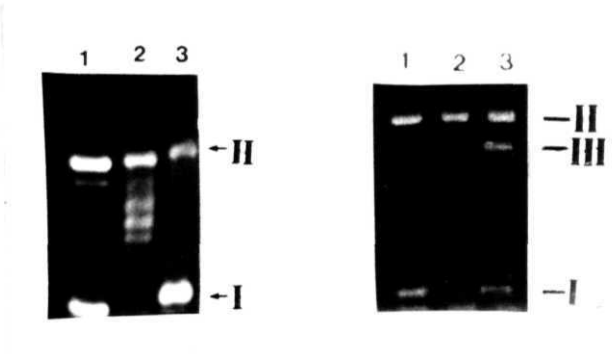


Figure 8. Characterization of Topoisomerase II. (A) Topoisomerase II catalysed relaxation activity. Supercoiled pBR322 DNA (lane 1) was incubated with topoisomerase II in the absence (lane 2) or presence of 50, μM m-AMSA (lane 3). (B) Cleavage reaction was conducted by incubating pBR322 DNA (lane 1) with topoisomerase II (lane 2) in presence of 50 μM m-AMSA (lane 3)

mM Tris-HCl pH 7.5, 0.1 mg NADH, 100 μM DTT, 1 mM ATP, 2 mM phosphoenol pyruvate , 4 mM $MgCl_2$) 12.5 units pyruvate kinase and 12.5 units lactate dehydrogenase was incubated at 37 °C for 5 min. The incubation was continued with further addition of 0.3 μg of DNA with increasing concentrations of drugs and 8 nM topo II for 30 min and absorbance was recorded at 340 nm.

***b) Thin layer chromatography** (TLC) assay*

In the TLC method, reaction mixture of 20 μl containing the enzyme catalysed relaxation assay buffer with 1 mM [γ^{32}P ATP] (0.025 Ci/mMol), 8 nM of topo II and 0.6 μg of pBR322 DNA with increasing concentrations of drugs was incubated at 30 °C for 15 min. The reaction was stopped by adding 2 μl of 250 mM EDTA. The reaction mixture was spotted on TLC plastic sheets coated with Polyethyleneimine (DC-Plastikfolien PEI-Cellulose F, Merck) and chromatographed in freshly made 1 Mlithium hloride. The bands were monitored with reflecting uv at 366 nm in a Photodyne gel documentation system. In lithium chloride solution, $^{32}P_i$ migrates first, followed by ADP and ATP. The areas corresponding to reaction products were cut out of the chromatogram and counted in a Wallac scintillation counter using a toluene based scintillation fluid (N&T, Osheroff *et al.*, 1983).

D) Immunoprecipitation assay

The cleavage reaction was conducted with cobalt drugs with the concentrations at which they form maximum 'Cleavage complex'. After 15 min, topo II in the cleavage complex and in free form was immunoprecipitated with 20 μl of 1x relaxation buffer containing

0.04 units of anti-topo II antibody. The mixture was rocked for 1 h at 4 °C before addition of 50 µl of protein-A agarose. After 1 h incubation, samples were washed five times with lx relaxation buffer and the immunocomplexes were eluted from the protein-A agarose by adding 50 µL of 4 *N* HNO3. The sample volumes were made up to 5 ml and analyzed for parts per million of cobalt metal by Atomic Absorption Spectroscopy by a Hitachi AAS650 F spectrometer.

E) DNA- Drug Binding Studies

(a) temperature melting studies

Calf thymus DNA (sodium salt) was dissolved in 1 mM sodium phosphate buffer containing 1 mM sodium chloride. The concentration of DNA was adjusted such that 1 ml of DNA gives an absorbance of ~1.0 (150 µM). This DNA was used in melting temperature studies in the presence of drugs. The concentration of metal complexes was adjusted such that drug to DNA nucleotide ratios of 1:20, 1:10, 1:5, 1:2 and 1:1 respectively were maintained in 1 ml of phosphate buffer. The samples were incubated in 1 ml quartz cuvettes for 2 min to allow drug-DNA binding. The cuvettes were placed in a Hitachi 150-20 spectrophotometer, and the instrument was set to give a 1 °C rise in temperature per min, and the increase in absorbance was recorded. The absorbance was recorded from 40 °C to 90 °C. T_m was determined from these absorbance values and the data was plotted.

(b) Circular dichroic spectral studies

Circular dichroic spectra of pBR322 DNA (20 μg) was monitored in presence of 20 mM of cobalt and copper drugs in a Jasco J-715 spectropolarimeter. The DNA and drug concentrations respectively corresponded to 0.6 μg of DNA and the concentration of drugs at which complete inhibition of enzyme activity was achieved as per the topo II relaxation assay. 2.5 mM of m-AMSA corresponding to 60 μM (as used in the relaxation assays) was included as a positive control. The spectra were measured in a quartz cuvette of 1 cm path length. The data was presented graphically as molar ellipticity ([9] X 10^{-3} deg-cm^{-2}/dmole) versus wavelength (nm).

CHAPTER III

Action of known anticancer metal complexes on topoisomerase II

Section A

Action of the Antitumor Drug

cis-Dichlorodiammineplatinum(II) *on topoisomerase II activity.*

Introduction

The action of cisplatin (cis-diamino, dichloro platinate (II) (cis DDP)) a known anticancer metal complex of platinum, which is widely used in cancer chemotherapy and in combinational therapy along with etoposide, a topoisomerase II poison was studied. It is widely accepted that DNA is the primary target for the anticancer activity of this complex.

Investigations on the mechanism of action of cisplatin revealed that its primary target is DNA (Rosenberg, 1985) and it forms >90% of 1-2 intrastrand cross-links through covalent binding to the N7 Nitrogen of adjacent purine bases. The structural analysis suggests that in 1 -2 intrastrand cross-link, DNA undergoes unwinding and bends towards majorgroove (Deboeah *et al.,* 1995). It is proposed that these cross-links are also responsible for inhibition of DNA and RNA polymerase activity. The anticancer activity of cisplatin is shown to be high in cells deficient in DNA repair (Plooy *et al.,* 1994). In repair efficient cells it is shown that 1-2 and 1-3 intra and interstrand cross-links are repaired by the nucleotide excision repair (NER). The cells that lack NER (eg; Xeroderma pigmantosum) show high sensitivity to cisplatin. An understanding of the action of cisplatin on one of the enzymes involved in DNA replication and repair, in this case topoisomerase ET, would help in gaining an insight into mechanism of action on other intracellular targets which may directly or indirectly be involved in its anti-proliferative activity and cytotoxicity.

Materials and methods:

Bio-chemicals, Enzyme and DNA:

Topoisomerase II was purified from rat testis by following the procedure of S. Galande and K. Muniyappa (1995). The concentration was determined by colorimetric assay of Bradford (1985). The negatively supercoiled pBR322 DNA was prepared as described by Wang and Rossman (1994). M-AMSA, etoposide and calf thymus DNA were from sigma. NADH, pyruvate kinase, lactate dehydrogenase and phosphoenol pyruvate were from Boehringer Mannheim. Other chemicals and bio-chemicals used were of standard grade

Synthesis of cisplatin and transplatin:

The synthesis of cisplatin and transplatin was performed as per the procedure of Kauffinan and Cowan (1954) with minor modifications.

1. Preparation of Hydrazine dihydrochloride (N_2H_2. 2HC1):

The procedure is similar to that reported by Bernard *et al*. Concentrated hydrochloric acid, (14.6 ml) was added dropwise to cold hydrazine monohydrate (6.5 ml) under constant stirring. The solution was heated to evaporate water. The solution was brought back to room temperature and concentrated HC1 (16.3 ml) was again added under stirring in cold and the precipitate formed was filtered and washed with methanol. Its melting point was recorded.

2. Preparation of $K_2[PtCl_6]$:

Potassium chloride (0.15 gms) dissolved in 2 ml of water was slowly added under stirring to a solution of hexachloroplatinic acid H_2PtCl_6. 6 H_2O (0.5 gm) in 5 ml of water. To this, methanol (7 ml) was added and the mixture was allowed to cool for 15 min in an ice bath.

The yellow salt was filtered and washed with methanol and ether. The solid was dried in air.

3. Preparation of $K_2[PtCl_4]$:

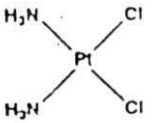
To a solution of potassium hexachloroplatinate (TV) (100 mg) in 1 ml of water, $N_2H_2.2HCl$ (0.01 mg) was added in small quantities. The mixture under stirring was heated up to 65 °C for about 2 h. The temperature was then raised to 90 °C to ensure completion of the reaction. A little excess of the $K_2[PtCl_6]$ was taken initially to prevent the complete reduction to metallic platinum. The mixture was then filtered to remove unreacted $K_2[PtCl_6]$ and washed with ice cold water.

$$2K_2[PtCl_6] + N_2H_2.2HCl \rightarrow 2K_2[PtCl_4] + N_2 + 6\ HCl$$

4. Preparation of cisplatin:

0.02 gms of NH_4Cl was dissolved in one half of the above filterate. Aqueous ammonia (3 M) was added to this until the pH reaches a value of 7. The solution was refrigerated for about 48 h. Yellow solid separated out was recrystallised from 0.1 N HC1. The recrystallised yellow crystals were washed with ice cold water and dried in air (structure I).

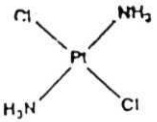
$$2K_2[PtCl_4] + 2NH_3 \xrightarrow{NH\ Cl} \text{cis } [Pt(NH_3)_2Cl_2] + N_2 + 2\ KC1$$



Structure I

5. Preparation of transplatin:

About 0.01 ml of concentrated ammonia was added to the second half of the filtrate obtained in the preparation of $K_2[PtCl_4]$. The solution was evaporated to a volume of 0.5 ml under continuous stirring. 0.6 ml of 6 N HCl was added to this and evaporation was continued to a volume of 1 ml. The mixture was then cooled and the deposited yellow powder was filtered using a sintered glass funnel (structure II).



Structure II

ir spectra:

Cisplatin: Doublet peaks appear at 4 and 5 positions which conforms chlorides in cis-conformation (Spectrum I)

Transplatin: The above peaks disappear in the spectra which conforms chlorides in trans-conformation (Spectrum II)

Cisplatin inhibits relaxation activity** of **Topoisomerase II:

Cisplatin inhibits topoisomerase II catalyzed relaxation of supercoiled DNA. The relaxation assay was conducted at increasing concentrations of cisplatin. The results show that cisplatin inhibits topoisomerase II catalyzed relaxation of supercoiled DNA at 250 μM (Figure 9 lane 11). The experiment was also carried out in the presence of increasing concentration of transplatin (trans-diamino, dichloro platinate (II) (trans-DDP)) but in this case, it did not affect the topoisomerase II catalyzed relaxation of supercoiled DNA

*Cisplatin does **not form** cleavage complex:*

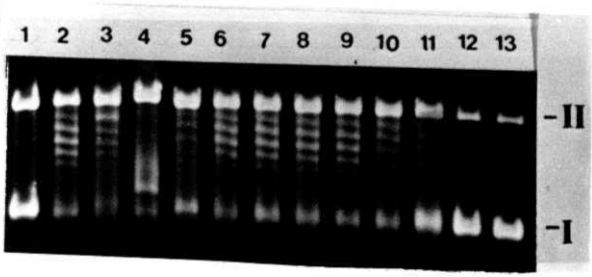
Based on their mechanism of action, topoisomerase II inhibitors are broadly classified in to two distinct classes. One class inhibits topoisomerase II activity through formation of drug-induced protein-DNA-drug cross-links called "cleavage complex". Such an inhibitor is termed as topoisomerase II poison. Where as other class of inhibitors cannot induce "Cleavage Complex", but inhibit the catalytic activity of topoisomerase II e.g., DNA stimulated ATPase activity are referred to as topoisomerase II inhibitors.

To test if cisplatin can form drug-induced cleavage complex consisting of protein-DNA-drug complex which can be seen through the appearance of linear DNA (Form III), a product formed upon treatment of cleavage complex with SDS and Proteinase K, the cleavage assay was carried out at increasing concentrations of cisplatin and it was

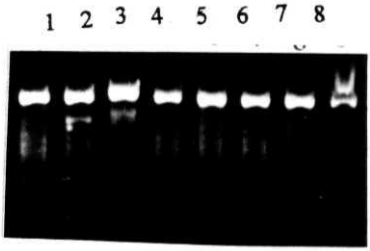
Figure 9: Effect of cisplatin and transplatin on topoisomerase II catalyzed DNA relaxation activity. (A) Supercoiled pBR322 DNA (lane 1) was incubated with topoisomerase II in the absence (lane 2) or presence of 50, 100 μM m-AMSA (lane 3-4) and 12.5, 25, 50, 75, 100, 150, 200, 250 & 300 μM cisplatin (lanes -13). (B) Supercoiled pBR322 DNA was incubated with topoisomerase II in the absence (lane 1) or presence of 25, 50, 100, 150, 200, 250 & 300 μM transplatin (lanes 2 -8) The positions of supercoiled (form 1) and nicked circular (form 2) DNA are indicated by I and II

Figure 9

A



B



observed that no linear DNA was observed even at 300 μM (Fig 10). This suggests that cisplatin inhibits topoisomerase II activity without formation of drug-induced cleavage complex.

Cisplatin affects DNA stimulated ATPase acrtivity of topoisomerase II:

The alternative mechanism through which topoisomerase II activity is inhibited may be through action on its DNA-stimulated ATPase activity. Hence the activity of cisplatin and transplatin on the DNA-stimulated ATPase activity of topoisomerase II was examined. The ATPase assay was conducted at increasing concentration of cisplatin and transplatin. The amount of NADH reduced will be directly proportional to the amount of ATP hydrolyzed in the sample. The results show that cisplatin inhibit DNA stimulated ATPase activity of topoisomerase II at 300 μM, while transplatin shows a marginal inhibition (Figure 11). This activity of cisplatin is comparable to that of its inhibition activity of topoisomerase II catalyzed relaxation of DNA. The inhibition of DNA-stimulated ATPase activity of topoisomerase II may take place when DNA bound cisplatin inhibits the binding of topoisomerase II to DNA or it may be due to interaction with a domain in topoisomerase II that is involved in ATP binding to the enzyme.

Protection of drug induced DNA cleavage of topoisomerase II by cisplatin:

Lippard *et al.*, (1981) showed that cisplatin inhibits BamH1 cleavage of DNA To test whether cisplatin can also inhibit the drug-induced DNA double strand breaks formed by the enzyme, we have conducted etoposide (Fig. 12A) and m-AMSA (Fig. 12B) induced cleavage assay in presence of increasing concentrations of cisplatin. The results show that

Figure 10

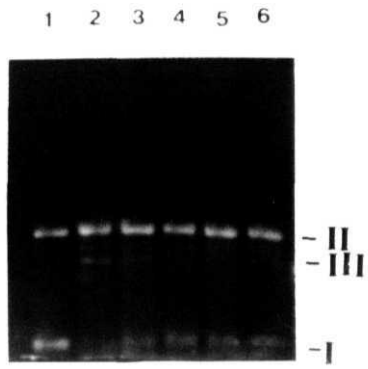


Figure 10: Cleavage reaction was conducted by incubating pBR322 DNA (lane 1) with topoisomerase II (lane 2) in presence of and 50, 100, 200 & 300μM of cisplatin (lanes 3-6). The positions of supercoiled, nicked circular and linear (form 3) DNA are indicated by **I, II and III**

Figure 11

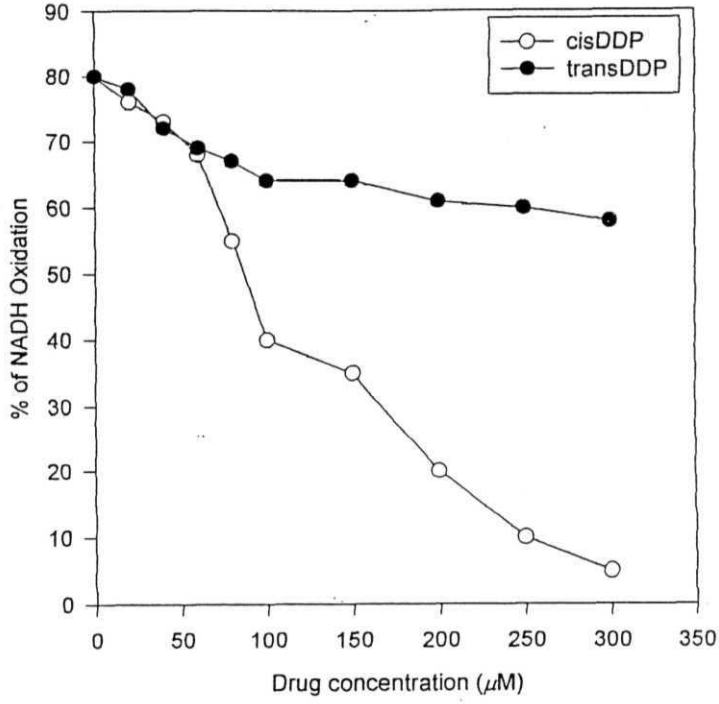


Figure 11: Inhibition of ATPase activity of topoisomerase II by cisplatin and transplatin. ATP hydrolysis in presence of increasing concentration of the drugs are presented as mean of three experiments. The plot shows the percentage of NADH oxidation versus concentration of drug in μM.

Figure 12

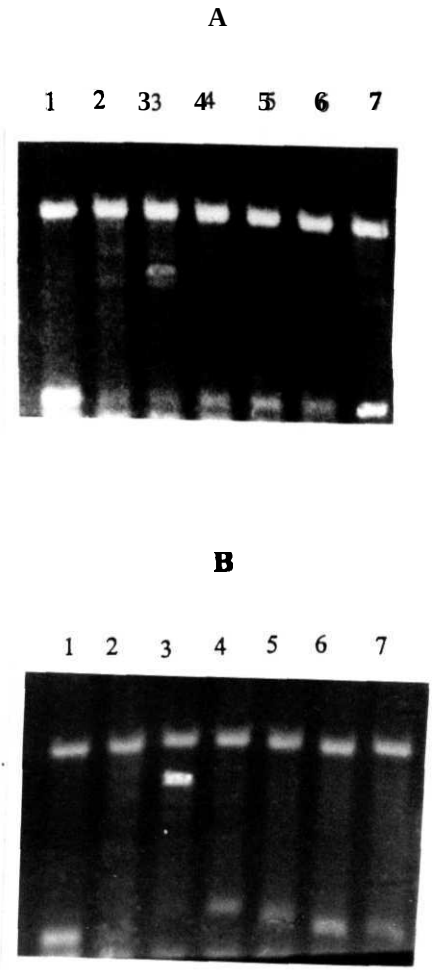


Figure 12: Protection of drug-induced cleavage complex by topoisomerase II poisons etoposide and m-AMSA in presence of cisplatin. (A) Cleavage reaction was conducted by incubating pBR322 DNA (lane 1) with topoisomerase II (lane 2) in presence of 100 μM of etoposide (lane 3) and 50, 100, 200 & 300μM of cisplatin plus 100 μM of etoposide (lanes 4 -7). (B) Cleavage reaction was conducted by incubating pBR322 DNA (lane 1) with topoisomerase II (lane 2) in presence of 75 μM of m-AMSA (lane 3) and 50, 100, 200 & 300μM of cisplatin plus 75 μM of m-AMSA (lanes 4 -7)

the cleavage complex formed by etoposide and m-AMSA were inhibited in the presence of cisplatin suggesting that the cisplatin may inhibit the topoisomerase II to form double-strand breaks in DNA, thus protecting mAMSA and etoposide induced cleavage complex by the enzyme.

Discussion

Cisplatin is a drug extensively used in cancer chemotherapy. It was widely accepted that DNA is the main target for its anticancer activity. Numerous reports suggest that the drug interferes with DNA replication through the action on some of the enzymes involved in replication (Deboeah *et ah*, 1995). To find out the possible cellular targets for its anticancer activity we studied the effect of the drug on topoisomerase II, an enzyme which plays crucial roles in resolving DNA knots in the chromosomes during replication, transcription and cell division during chromosome segregation.

Studies of cisplatin action on catalytic activity of topoisomerase II show that it inhibits the enzyme-catalyzed relaxation activity of supercoiled pBR322 at 300 μM concentration while transplatin, an inactive isomer of the drug does not. But it was observed that the drug does not induce the cleavage complex. The results from the DNA-stimulated ATPase activity studies show that the cisplatin inhibits the ATPase activity of topoisomerase II.

The antineoplastic activity of cisplatin in comparison with the inactive transplatin is due to the bifunctional coordination of cisplatin to DNA e. g., intrastrand cross-linksing of two adjacent guanine or cytosine bases and interstrand cross-linking. This type of coordination is not possible in the case of transplatin. DNA cleavage by topoisomerase II requires adjacent purine and pyramidine bases. Thus the cross-linking of cisplatin to DNA may inhibit the topoisomerase II cleavage of DNA. The same is confirmed by the protection of

drug-induced DNA double-strand breaks by topoisomerase II poisons in the presence of cisplatin.

In summary, cisplatin inhibits topoisomerase II catalyzed relaxation of supercoiled DNA, where as transplatin could not show such an activity indicating that topoisomerase II may be one of the targets for the anti-proliferative activity of cisplatin.

Section B

Single-Strand DNA cleavage by Topoisomerase II in presence of Anticancer Copper salicylaldoxime complex.

Introduction

The second anticancer metal complex studied was copper salicylaldoxime (CuSAL), which was earlier shown to inhibit L1210 leukemia cell proliferation (Lumme *et al.,* 1984). Copper complexes are known to have a broad spectrum of biological actions (Couch *et al.,* 1986). Many copper complexes are used as anti-inflammatory, anti-arthritic, anti-ulcer, anti-convulsant and anti-tumor agents (Sorenson *et al.,* 1984; Sorenson, 1984). It has been shown that copper accumulates in tumors due to selective permeability of cancer cell membranes to copper compounds (Apelgot *et al.,* 1986). Because of this a number of copper complexes have been screened for anticancer activity and some of them were found active both *in-vitro* and *in-vivo* (Renade *et al.,* 1984). Recently it was shown that the copper complex of 2-furaldehyde oxime poisons topoisomerase II activity in a manner similar to that by etoposide and teniposide, and also shows a better activity than these drugs by blocking the phosphorylation activation of topoisomerase II (Hall *et al.*, 1997).

In the present study we report that CuSAL induces topoisomerase II to form single strand nicks in DNA and poisons its activity, which could be one of the possible mechanisms for the anticancer activity of this complex.

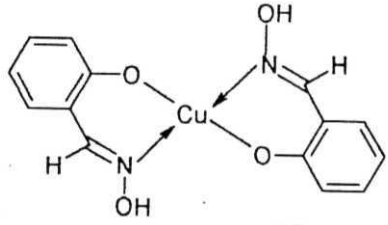
Materials and methods:

Biochemicals:

$\gamma^{32}P$ATP was supplied by BARC, India. PEI Cellulose-F sheets were from Merck. Calf thymus DNA was from Sigma. Other chemicals and biochemicals used were of standard grade.

Synthesis of copper salicylaldoxime (CuSAL).

The complex was synthesized according to the procedure given by Lumme *et al.* (1984). Salicylaldoxime (0.745 g, 10 mmol) was dissolved in 175 ml of absolute ethanol containing 25 ml of 0.01 *M* HCl. The solution was heated to 60 °C. To this, a solution which was prepared by dissolving 0.9989 g (5 mmol) of copper(II)di-acetate monohydrate in 50 ml of 0.01 M HCl was added slowly. The solution turned grayish green and a dark precipitate separated. The solution was allowed to cool slowly. After standing for one week, the solution was filtered, the precipitate washed three times with water and allowed to dry on a sinter (structure III).



Structure III

ir spectra

The coordination of the inline nitrogen atom to the Cu (II) ion is indicated by the displacement of the band assigned to the v(C=N) stretching vibration. The spectra of the complex exhibits a downward shift of v(C=N) from ca. 1618 cm^{-1} for the ligand to approximately 1597 cm^{-1} and a stretching band appears at 1512 cm^{-1} corresponding to the v(M-O) bond confirming the complex formation (Spectrum HI).

Results

Effect of CuSAL on Relaxation activity of topoisomerase II:

Figure. 13 depicts the results of an experiment in which the relaxation activity of topoisomerase II in the presence of increasing concentrations of drug was examined. CuSAL shows complete inhibition of relaxation activity of topoII at 300 μM drug concentration (lane 11).

Effect of CuSAL on DNA cleavage mediated by topoisomerase II:

This assay was carried out to see if CuSAL could form drug-induced ternary complex of DNA-drug-topoisomerase II, called the Cleavage complex. The formation of a topoisomerase II-drug-DNA cleavage complex can be seen by the formation of linear DNA. The result shows that CuSAL affects the ATP-stimulated cleavage/religation of DNA-mediated by topoisomerase II and forms protein linked single-strand breaks (Figure 14). It has been shown that some of the topoisomerase II targeting drugs induce enzyme linked single-strand breaks due to the formation of a 'non-covalent cleavage' complex (Maxwell *et al.,* 1989). This type of single-strand cleavage complex formation can be seen in presence of m-AMSA and some quinolone derivatives under special reaction conditions (Muller *et al.,* 1988). Another possibility is that the cleavage reaction performed in the presence of drugs which can disrupt the co-ordination between the topoisomerase II monomers (in dimer formation) induces this non-covalent cleavage complex, similar to that of topo I cleavage complex (Maxwell *et al.,* 1989). To see if the nicked circular DNA formed in presence of drug contains the enzyme-linked to nicked termini, one set of

Figure 13

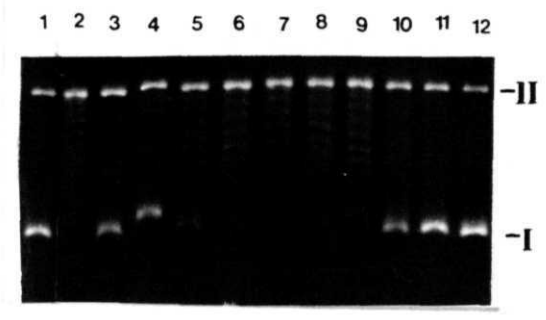


Figure 13: Effect of CuSAL on topoisomerase II catalyzed DNA relaxation activity. Supercoiled pBR322 DNA (lane 1) was incubated with topoisomerase II in the absence (lane 2) or presence of 50 and 100 μM m-AMSA (lane 3-4) and 25, 50, 100, 150, 200, 250 & 300 μM CuSAL (lanes 5-11), without enzyme but with 300 μM CuSAL (lane 12). The positions of supercoiled (form 1) and nicked circular (form 2) DNA are indicated by I and II

Figure 14

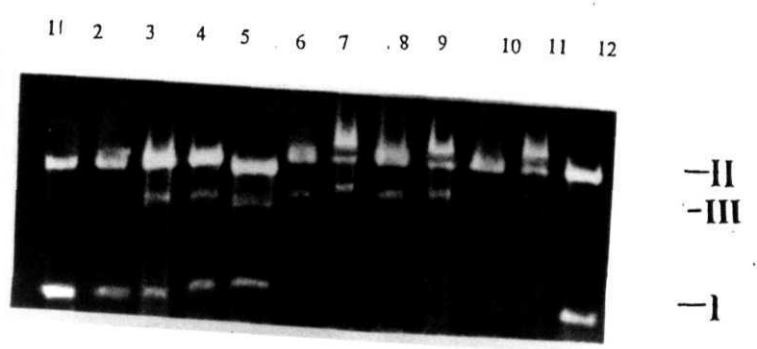


Figure 14: (A) Cleavage reaction was conducted by incubating pBR322 DNA (lane 1) with topoisomerase II (lane 2) in presence of 50 & 75 μM m-AMSA (lane 3-4), 100 μM Etoposide (lane 5) and 100, 200 & 300μM of CuSAL with proteinase K treatment (lanes 6, 8 & 10) and without proteinase K treatment (lanes 7, 9& 11); without enzyme but with 300 μM of CuSAL (lane 12). The positions of supercoiled, nicked circular and linear (form 3) DNA are indicated by L II and III.

samples were treated with proteinase K. The mobility of nicked circular DNA is shown in lanes 6, 8 and 10. Another set of samples which were not treated with proteinase K show a small shift in the mobility of nicked circular DNA, due to the presence of enzyme-linked to the nicked termini (lanes 7, 9 & 11). These results suggests that the nicked circular DNA formed in presence of drug is due to single-strand DNA cleavage by topoisomerase II

Effect of CuSAL on Enzyme-mediated ATP Hydrolysis:

Drugs like genistein and flavone-based compounds that enhance DNA breakage also strongly inhibit the ATPase activity of topoisomerase II (Okura *et al.*, 1988). To test whether CuSAL also interferes with the DNA stimulated ATP hydrolysis of the enzyme, an ATPase assay was performed.

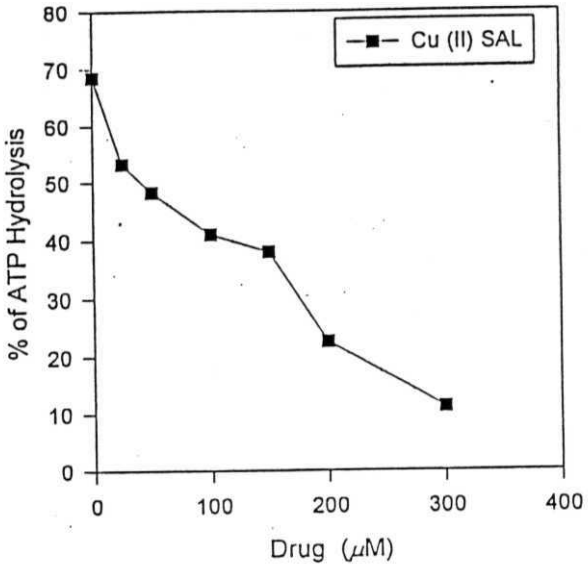
A TLC assay was conducted in the presence of increasing concentrations of CuSAL. The result shows that CuSAL inhibits ~ 80% of the DNA dependent ATP hydrolysis at 300 μM concentration (Figure 15A). A pyruvate coupled spectrophotometric assay was carried out to examine the extent of inhibition of topoisomerase II induced ATP hydrolysis by the drug. The results of this assay correlate well with those of the TLC assay in that 300 μM drug could inhibit ~85% of ATP hydrolysis (Figure 15B).

DNA binding studies:

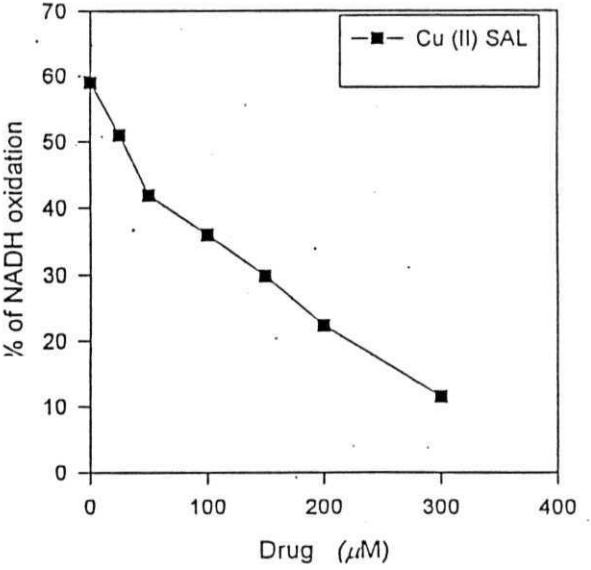
(a) DNA melting temperature experiments were carried out by incubating calf thymus DNA with increasing concentrations of CuSAL. The absorbance of nucleotide bases was monitored at 260 run with increasing temperatures which shows that CuSAL protects

Figure 15: Inhibition of ATPase activity of topoisomerase II by CuSAL. ATP hydrolysis in presence of increasing concentration of the drugs are presented as mean of three experiments; plot A shows the percentage of ATP hydrolyzed versus concentration of drug in μM and plot B shows the percentage of NADH oxidation versus concentration of drug in μM.

A


Cu (II) SAL
% of ATP Hydrolysis
0
10
20
30
40
50
60
70
80
0
100
200
300
400
Drug (μM)

B

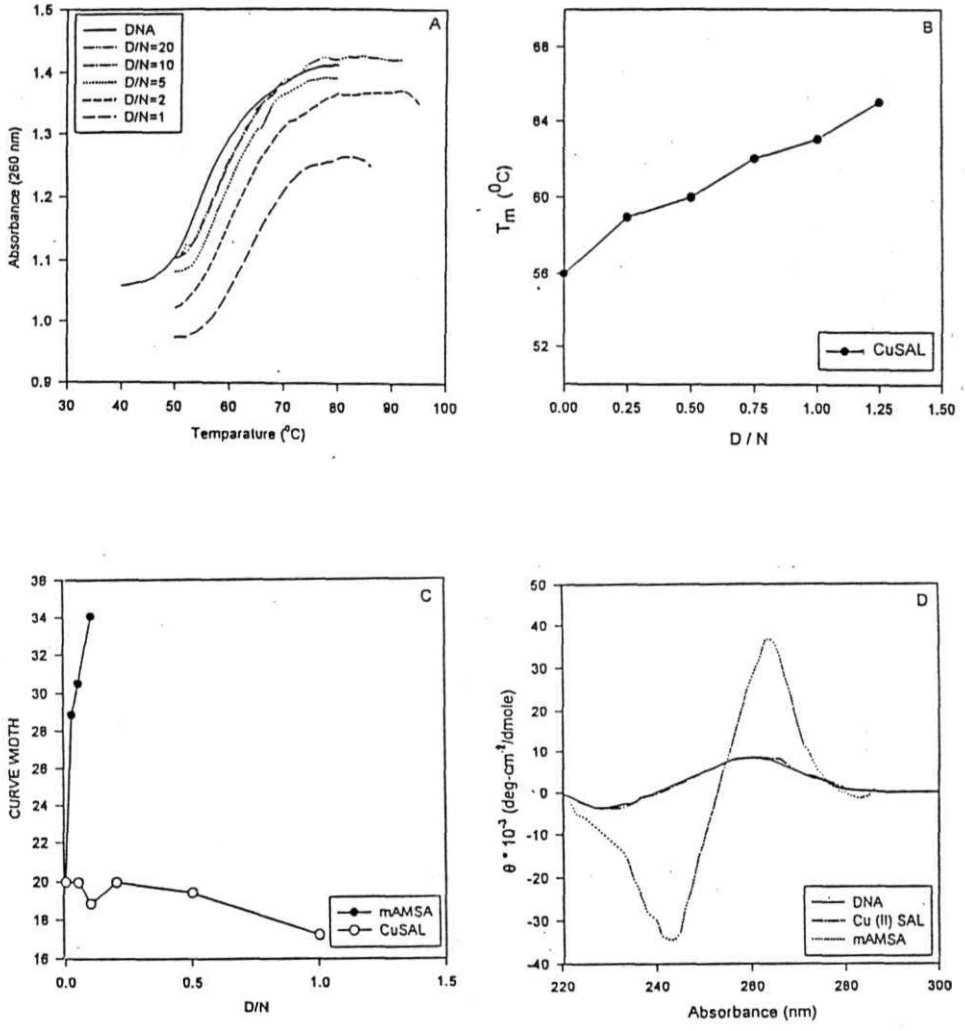

Cu (II) SAL
% of NADH oxidation
0
10
20
30
40
50
60
70
0
100
200
300
400
Drug (μM)

melting of calf thymus DNA (Figure 16A). CuSAL increases the T_m of DNA from 57 °C to 65 °C at a stoichiometric ratio of 5 nucleotides per 2 drug molecules (Figure 16B). Following the procedure of Kelly *et al*. (1985), the curve widths of the T_m curves were measured at different drug to nucleotide ratios and the data were plotted. The results show that CuSAL does not bind to DNA, but only protects melting (Fig 16C).

(b) The circular dichroic spectra of DNA in presence of CuSAL shows that the drug does not induce any change in CD spectrum (Figure 16D). This suggests that CuSAL does not produce any conformational change in DNA.

Figure 16: Drug-DNA binding studies. (A) CuSAL increases the T_m of calf thymus DNA from 57 °C for DNA control(—) to 59, 60, 62, 63 and 66 °C for DNA nucleotide to drug ratios of 20:1(-··-), 10:1(-·-), 5:1(....) 2:1(—) and 1:1(—) respectively (B) D/N (drug/nucleotide) was plotted against increase in T_m by CuSAL (•) to determine specific drug binding to DNA nucleotides from the slopes of the curves. (C) D/N plotted against curve width shows a characteristic increase in curve width by m-AMSA (•), a characteristic DNA intercalator and CuSAL (0) shows a curve width similar to that of DNA non binders. (D) The Circular Dichroism spectra of pBR322 DNA (—) in presence of CuSAL (—) does not change the CD spectra. But m-AMSA(....) shows a very prominent change at a concentration less than 5 times that of the copper complex.

Figure 16


A
DNA
D/N=20
D/N=10
D/N=5
D/N=2
D/N=1
Absorbance (260 nm)
Temparature (°C)
B
Tm (°C)
CuSAL
D / N
C
CURVE WIDTH
mAMSA
CuSAL
D/N
D
θ * 10⁻³ (deg-cm²/dmole)
DNA
Cu (II) SAL
mAMSA
Absorbance (nm)

Copper is one of the trace elements present in living systems (Underwood *et al.,* 1977). It is a component of several enzymes and copper compounds known to have a broad spectrum of biological activities. Copper implication in neoplastic diseases and the possible use of this element and its compounds as antitumor agents have been extensively investigated (Willingham *et al.,* 1986).

In the present study, one of the possible mechanisms responsible for the antitumor activity of a salicylaldoxime complex of copper, CuSAL is presented. The ligand salicylaldoxime structurally resembles pyridoxal, a component of vitamin B_6. Salicylaldoxime is an extremely powerful inhibitor of pyridoxal kinase (Churchich *et al.,* 1981). Vitamin B_6 deficiency and B_6 antagonists are known to have growth-inhibitory and antitumor activity (Korytnyk *et al.,* 1970). Since the side effects of CuSAL (weight loss and acrodynia) resemble those of B_6 deficiency and B_6 antagonism (Emerson, 1967), it appears that pyridoxal antagonism might be involved in the mechanism of action of CuSAL. However, the pyridoxal theory alone cannot explain the greatly different anti-proliferative activities of copper, cobalt and nickel salicylaldoxime complexes (Elo *et al.,* 1987).

It has been proposed that Cu^{2+} plays an important role in the stabilization and maintenance of nuclear matrix organization and DNA folding, acting through metalloproteins (Dijkwel *et al.,* 1986; Berrios *et al.,* 1987; Earnshaw *et al.,* 1985). Topoisomerase II is a major component of the nuclear matrix and a likely candidate for DPC (DNA-Protein cross-links) formation when cells are exposed to topoisomerase II poisons (Chui et al., 1992)). Nelson *et al* (1986) have reported that covalent complexes of

pulse-labeled DNA with topoisomerase II can be trapped by the topoisomerase II inhibitor, tenopiside in a 'cleavage complex'. Our results show that CuSAL induces single-strand protein associated DNA breaks like DPC forming agents, where the protein bound to nicked termini of DNA is trapped in a non-covalent cleavage complex. This type of complex formation is induced only by the drugs which can disrupt the co-ordination between the two monomers of the dimeric topoisomerase II or those which bind to the ATP binding site of the enzyme. The intensity of the nicked circular DNA was increased with increasing drug concentration, suggesting that CuSAL could act like the topoisomerase II targeting drugs which inhibit topoisomerase II activity by either disrupting the topoisomerase II dimer formation or by binding to ATP binding site of the enzyme.

To test whether this complex binds to topoisomerase II and interferes with its catalytic activity we have conducted the DNA-stimulated ATPase activity assay. Both TLC and pyruvate kinase coupled reactions show that CuSAL inhibits the DNA-depended ATPase activity of topoisomerase II.

To analyze if CuSAL causes DNA cleavage complex formation by bi-directionally interacting with DNA and topoisomerase II or only by interacting with the enzyme, we conducted the DNA binding studies. Our results from these studies show that the drug protects melting of calf thymus DNA to a small extent (9 °C) suggesting that it does not bind to DNA but may only stabilize the ionic environment around DNA which protects DNA melting to a certain extent. The CD spectral studies show that CuSAL does not change DNA conformation. These results suggest that CuSAL interacts only with topoisomerase II and forms the cleavage complex.

In summary, our results strongly suggest that inhibition of topoisomerase II by CuSAL occurs by the binding of CuSAL to the enzyme in DNA-topoisomerase II complex and disruption of the monomers from the dimeric enzyme and this induces single strand protein linked breaks in DNA (DPC formation).

CHAPTER IV

Topoisomerase II Is a Cellular Target for Anti-proliferative Cobalt Salicylaldoxime Complex.

The analysis of action of the metal complexes, cisplatin and CuSAL, on catalytic activity of topoisomerase II shows that both complexes could inhibit topoisomerase II activity. CuSAL does not bind to DNA but it induces enzyme-linked single-strand breaks and forms non-covalent cleavage complex. Cisplatin binds to DNA but does not seem to show a stronger interaction with the enzyme, The drugs which can bind both DNA and enzyme may show potent topoisomerase II inhibition by poisoning the enzyme activity resulting in covalent cleavage complex formation. With this idea, cobalt was selected for these studies due to the following reasons:

- Cobalt (HI) complexes bind specifically to N7 and N1 nitrogen atoms of adenine bases through covalent bond formation (Theophilus *et al.,* 1976).
- Cobalt has the ability to alter the configuration of ligands attached to it.
- Cobalt has the capacity to form 2, 4, 5 and 6 coordination bonds based on its reaction environment.
- The complexes of Co(III) are kinetically inert octahedral coordination complexes. This inertness is due to the d^6 lowspin electronic configuration of trivalent cobalt (Cotton *et al.,* 1972). Kinetically inert transition metal complexes undergo the water exchange reaction relatively slowly with a half life of about 24 h. (Kettle *et al,* 1969). The biological consequences of kinetic inertness is that Co(III) complexes will remain intact when added to a culture medium or injected into animals and should arrive at their cellular target with their ligand configuration intact.

In this study it is shown that topoisomerase II is a cellular target for the anti-proliferation activity of cobalt salicylaldoxime (CoSAL) and it poisons topoisomerase II activity by forming a covalent cleavage complex.

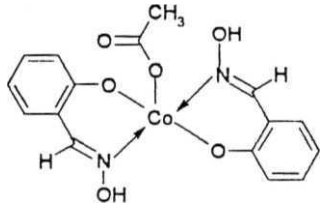
Materials and methods :

Biochemicals, Enzyme and DNA:

RPMI 1640 medium, ^{3}H thymidine were supplied by BARC, India. Other chemicals and bio-chemicals used were of standard grade.

Synthesis of the cobalt Compounds: (a) Cobalt(III) Salicylaldoxime (CoSAL):

Dry cobalt acetate was added to salicylaldoxime in dry methanol slowly under dry nitrogen gas with constant stirring at 60 °C for 6 h and the solution was left undisturbed overnight. The complex formed was vacuum dried and re-crystallized in dry methanol (structure IV).



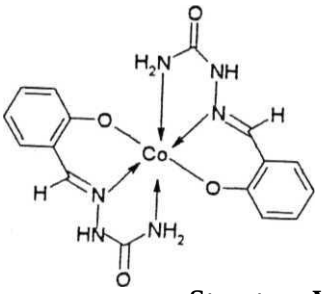
Structure IV

ir spectra

The coordination of the amine nitrogen atom to the metal (III) ion is indicated by the shift of the v (C=N) stretching vibrations. The spectra of the complex exhibits downward shift of v (C=N) from *ca.* 1618 cm^{-1} for the ligand to approximately 1599 cm^{-1} and a stretching band appears at 1471 cm^{-1} corresponding to v (M-O), which confirms the formation of the complex (Spectrum IV).

(b) Cobalt(II)Salicylalsemicarbazone (CoSSC):

Semicarbazide hydrochloride (1 mM) and a few drops of tri-methyl amine were added to 1 mM salicylaldehyde in dry methanol and stirred under a slow stream of dry nitrogen gas at 60 °C for 3 h. This was vacuum dried and re-crystallized in dry methanol to form salicylalsemicarbazone. This ligand was added to dry cobalt(II)acetate in dry methanol slowly under dry nitrogen atmosphere with constant stirring at 60 °C for 6 h. This yielded a yellow colored precipitate which was left undisturbed overnight. The precipitate was filtered, vacuum dried and re-crystallized in dry methanol (structure V).



Structure V

ir spectra

The coordination of the imine nitrogen atom to the metal (II) ion is indicated by the shift of the v (C=N) stretching vibrations. The spectra of the complex exhibits downward shift of v (C=N) for the ligand from *ca.* 1616 cm^{-1} to approximately 1599 cm^{-1}. A stretching band appears at 1469 cm^{-1} corresponding to v (M-O), and another stretching band appears at 3360 cm^{-1} corresponding to NH_2 in bound form, which confirms the complex formation (Spectrum V).

In vitro anti-proliferation assay:

^{3}H-Thymidine incorporation assays were carried out to examine the effect of the cobalt complexes on the proliferative response of HL-60 (Human T-cell leukemia) cancer cells. The cells were grown in RPMI-1640 medium supplemented with 20% fetal calf serum. 0.2 million cells in 200 μl were distributed in a 96 well microtitre tissue culture plate. Increasing concentrations of the two cobalt drugs were added to the cells. The cells were incubated for 48 h in a CO_2 incubator at 37 °C maintaining 5% CO2. The cultures were then pulsed with 0.5 μCi of ^{3}H-thymidine. Incubation was continued for 6 h to allow thymidine incorporation by cells. The cells were harvested on glass microfibre strips using a Skatron automated cell harvester. Radioactivity was measured in a Wallac liquid scintillation counter.

Oxidation state of Cobalt in the DNA bound drugs:

The Cyclic Staircase Voltametry (C V) spectra of complexes was recorded both in the presence and absence of DNA using the system of DMSO/TABP/GIass carbon working electrode/ Ag^{+}-AgCl. The concentration of linear DNA, pBR322 DNA and complex were maintained as given in cleavage assay.

Results

***Anti-Proliferation** Activity:*

The ^{3}H thymidine incorporation experiments were conducted with HL60 T-cell leukemia cells with increasing concentrations of the cobalt drugs. The results show that 60 μM of CoSAL could inhibit 50% of cell proliferation, while CoSSC shows only 20% of inhibition at the same concentration (Figure. 17).

DNA - Drug Binding Studies

(a) DNA melting temperature experiments were carried out by incubating calf thymus DNA with increasing concentrations of Cobalt complexes. The absorbance of nucleotide bases at 260 nm was monitored by increasing temperatures from 40 °C to 90 °C. The results show that both Cobalt complexes protect melting of calf thymus DNA (Figure 18A & B). CoSAL increases the T_m of DNA from 56 °C to 74 °C, and shows a DNA binding stoichiometric ratio of five nucleotides per two drug molecules, where as CoSSC increases the T_m up to 72 °C at a stoichiomeatric ratio of five nucleotides per one drug molecules (Figure 18C).

The curve width of the T_m curves were measured at different drug to nucleotide ratios according to the procedure of Kelly *et al* (1985) and the results are presented in Figure 18D.

(b) The circular dichroic spectra of these complexes show that CoSAL induces a small hypochromic shift in the positive [8]M of DNA, similar to that of groove binding

Figure 17

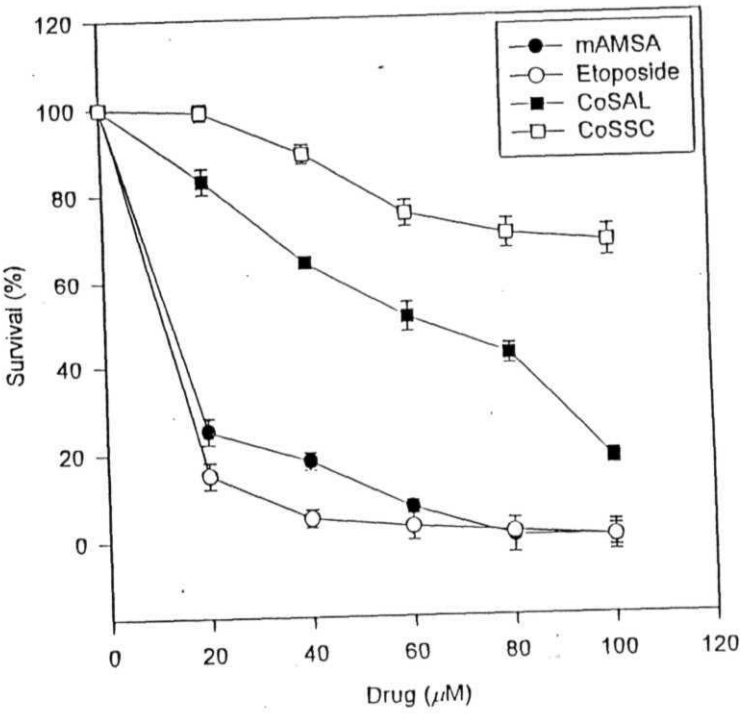


Figure. 17: HL 60 Human leukemic cells were incubated with increasing concentrations of CoSAL and CoSSC. ^{3}H thymidine incorporation during the last 6 h of incubation was measured as described in methods. Values are presented as mean of three independent experiments Data is graphically expressed as percentage increase in inhibition versus concentration of CoSAL (•) and CoSSC (D) in μM. mAMSA (•) and Etoposide (O) (positive controls) are also shown.

Figure 18: Drug-DNA binding studies. (A) CoSAL increases the T_m of calf thymus DNA from 57 °C for DNA control(—) to 68, 70, 74, 72 and 74 °C for DNA nucleotide to drug ratios of 20:1(–··–), 10:1(—), 5:1(····) 2:1(—) and 1:1(~) respectively. (B) CoSSC shows an increase in T_m of 67, 70.7, 73.8, 71 and 72 °C for the same drug to DNA ratios. (C) D/N (drug/nucleotide) plotted against increase in T_m by CoSAL (●)and CoSSC (0) to determine specific drug binding to DNA nucleotides from the slopes of the curves. (D) D/N plotted against curve width shows a characteristic increase in curve width by m-AMSA (•), a characteristic DNA intercalator and an intermediate increase by CoSAL (•) and CoSSC (D) similar to a DNA groove binder.

Figure 18

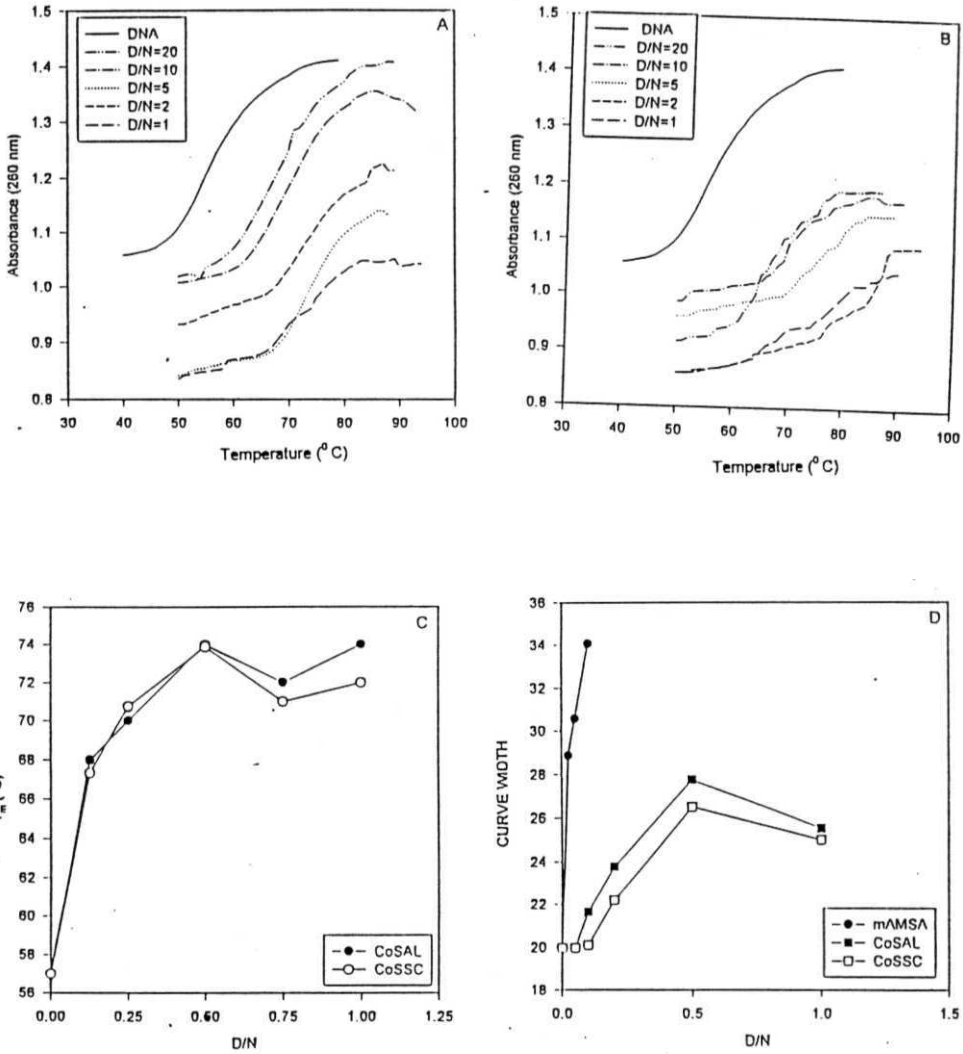

A
DNA
D/N=20
D/N=10
D/N=5
D/N=2
D/N=1
Absorbance (260 nm)
Temperature (°C)
B
DNA
D/N=20
D/N=10
D/N=5
D/N=2
D/N=1
Absorbance (260 nm)
Temperature (°C)
C
CoSAL
CoSSC
T_m (°C)
D/N
D
mAMSA
CoSAL
CoSSC
CURVE WIDTH
D/N

Figure 19

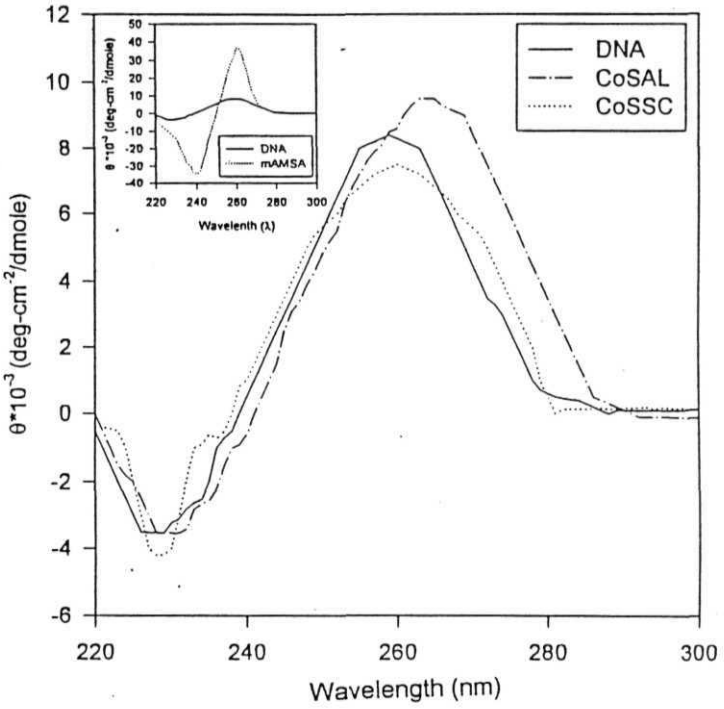


Figure 19: The Circular Dichroism spectra of pBR322 DNA (—) in presence of CoSAL (—) and CoSSC (….). CoSAL shows a small hypochromic shift in CD spectra of DNA while CoSSC shows a small negative shift . But **m-AMSA** (see in **insert**) shows a very prominent change at a concentration less than 5 times that of the cobalt complexes.

molecules, while CoSSC shows a small negative shift in CD spectra (Figure 19). These observations show that both complexes bind DNA with similar affinity with a small difference in their mode of interaction.

Action of Cobalt drugs on the DNA Relaxation Activity of Topoisomerase II

CoSAL inhibits the topoisomerase II catalysed relaxation activity in a dose dependent manner and shows complete inhibition at 600 μM concentration (Figure 20A) while CoSSC does not affect the DNA relaxation activity of topoisomerase II (Figure 20B).

Formation of cleavage complex

The cleavage assay was conducted to see if cobalt drugs could form drug-induced ternary complex of DNA-drug-topoisomerase n, called the cleavage complex. The formation of topoisomerase II-drug-DNA cleavage complex can be seen by the formation of linear DNA. The results show that CoSAL induces cleavage complex formation (Figure 21 A) while CoSSC does not. Density analysis of DNA bands in the agarose gels shows an increase in intensity of linear DNA formation with increasing drug concentration (Figure 21B)

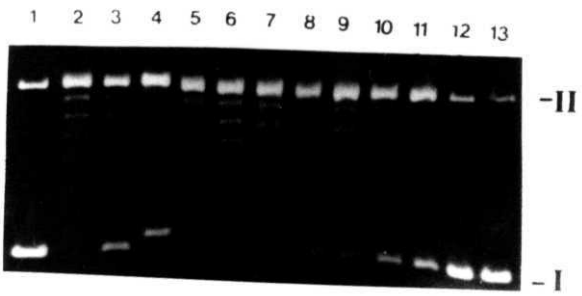
Action of the cobalt compounds on ATPase activity of topoisomerase II

To see whether these complexes can inhibit DNA-depended ATPase activity of topoisomerase II which is necessary for the enzyme's relaxation activity, ATPase assay was performed in presence of increasing concentration of the drugs using $\gamma^{32}P$ ATP. The

Figure 20: Effect of CoSAL (A) on topoisomerase II catalyzed DNA relaxation activity. Supercoiled pBR322 DNA (lane 1) was incubated with topoisomerase II in the absence (lane 2) or presence of 50 and 100 μM m-AMSA (lane 3-4) and 25, 50, 100, 200, 300, 400, 500 & 600 μM CoSAL (Panel A. lanes 5-12), without enzyme but with 600 μM CoSAL (lane 13). Panel B shows DNA alone (lane 1) with topoII (lane 2) in presence of 50 & 100 μM m-AMSA (lanes 3-4) and 50, 100, 200, 300, 400, 500 & 600 μM of CoSSC (lanes 5-11) and without enzyme but with 600 μM CoSSC (lane 12). The positions of supercoiled (form 1) and nicked circular (form 2) DNA are indicated by I and II

Figure 20

A



B

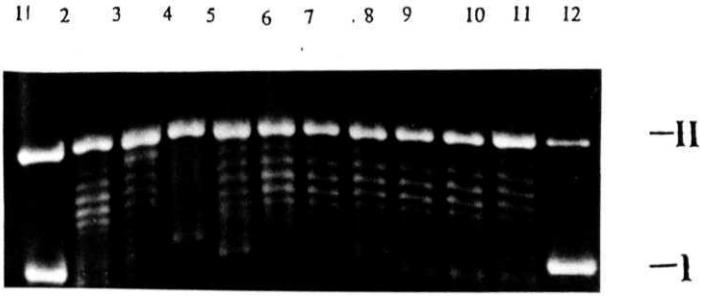
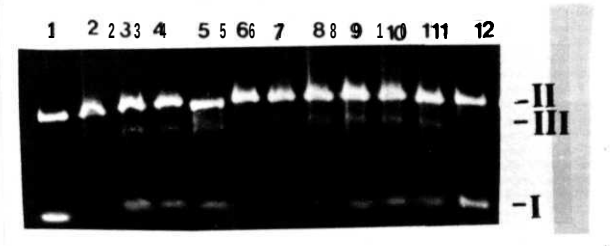


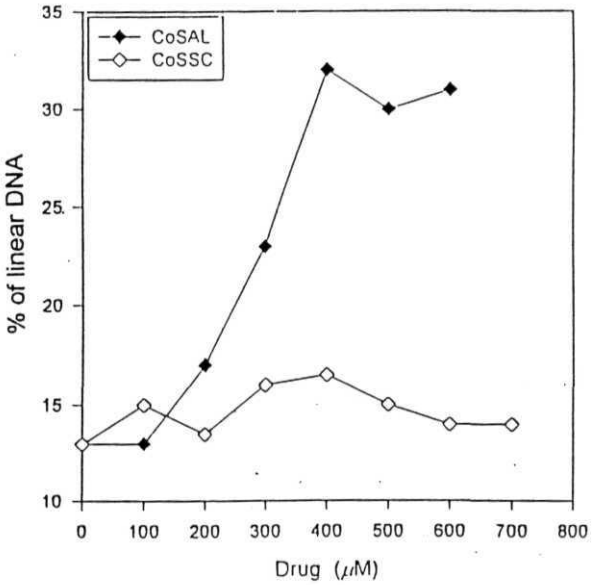
Figure 21: (A) Cleavage reaction was conducted by incubating pBR322 DNA **(lane** 1) with topoisomerase II (lane 2) in presence of 50 & 75 μM m-AMSA (lane 3-4), 100 μM Etoposide (lane 5) and 100, 200, 300, 400, 500 & 600 μM of CoSAL (lanes 6-11), **and** without enzyme but with 600 μM of CoSAL (lane 13). The positions of supercoiled, nicked circular and linear (form 3) DNA are indicated by I, II and HI. (B) The plot shows the percentage of linear DNA formed with increasing concentration of CoSAL.

Figure 21

A


1 2 2 3 3 4 5 5 6 6 7 8 8 9 1 10 11 12
-II
-III
-I

B


CoSAL
CoSSC
% of linear DNA
35
30
25
20
15
10
0
100
200
300
400
500
600
700
800
Drug (μM)

products were separated on PEI-cellulose sheets in 1 M LiCl. ^{32}Pi and unhydrolysed $\gamma^{32}P$ ATP in the samples were quantified. The results show that CoSAL inhibits the DNA-dependent ATPase activity of topoisomerase II while CoSSC shows a marginal inhibition of ATPase activity (Figure 22).

***Presence** of **drug in cleavage** complex*

To confirm that the cleavage complex formed in presence of CoSAL actually contains CoSAL, an immunoprecipitation assay with anti-topoisomerase II antibody was performed. The results show that 75% of drug is present in the cleavage reaction. The control experiments with drug + DNA and drug + topoisomerase II were also carried out to monitor any non-specific interaction of drug. Both the experiments show negligible amount of non-specific interaction by the drug (Table 1).

The experiment was repeated in the presence of CoSSC. The results show that a 10 fold lower concentration of drug is present in cleavage complex (7.65%). This may be nonspecific interaction of drug with both enzyme and DNA (Table 1).

***Analysis of oxidation state of cobalt in DNA bound** CoSAL*

The CV spectra of CoSAL (Figure 23A) shows that cobalt is present in +3 oxidation state. In higher oxidation state, cobalt may undergo reduction while oxidizing DNA, thus resulting in nonspecific cleavage and unwinding of supercoiled DNA. To confirm this, we recorded CV spectra of the complex in presence of linear calf thymus DNA (Figure 23B) and pBR322 supercoiled DNA (Figure 23C). The results show that cobalt remains in +3

Figure 22

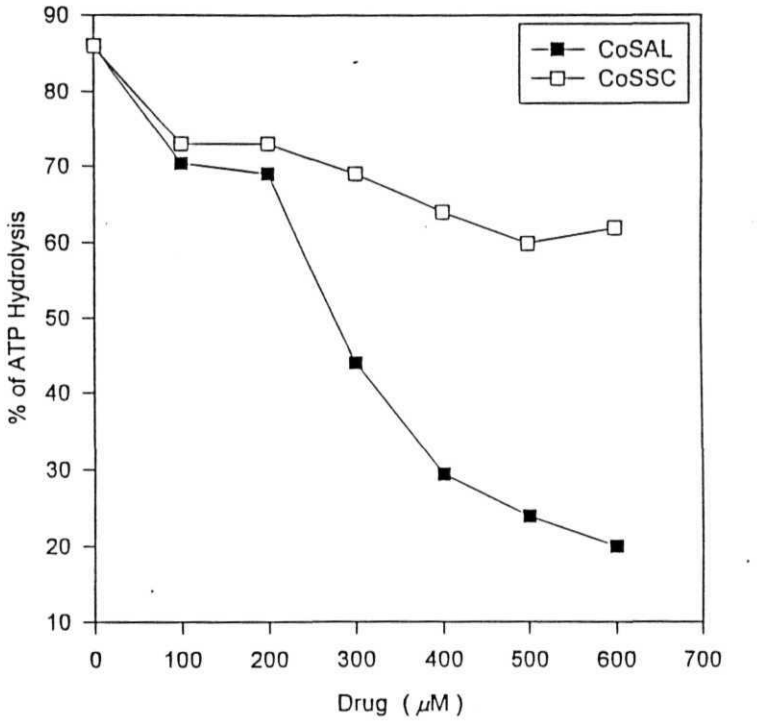


Figure 22: Inhibition of ATPase activity of topoisomerase II by CoSAL (•) and CoSSC (•). ATP hydrolysis in presence of increasing concentration of the drugs are presented as mean of three experiments; data is plotted as the percentage of ATP hydrolyzed versus concentration of drug in μM.

Table. 1 Presence of cobalt drugs in cleavage complex

	CoSAL	CoSSC
topo II + DNA	0	0
drug	<2	<2
drug + DNA	<2	<2
topo II + drug	5 ±0.6	4 ±0.75
drug + DNA + topo II	75 ±0.78	7.65 ±0.52

Note. The data is an average of three independent experiments conducted in triplicates (± error).

Figure 23

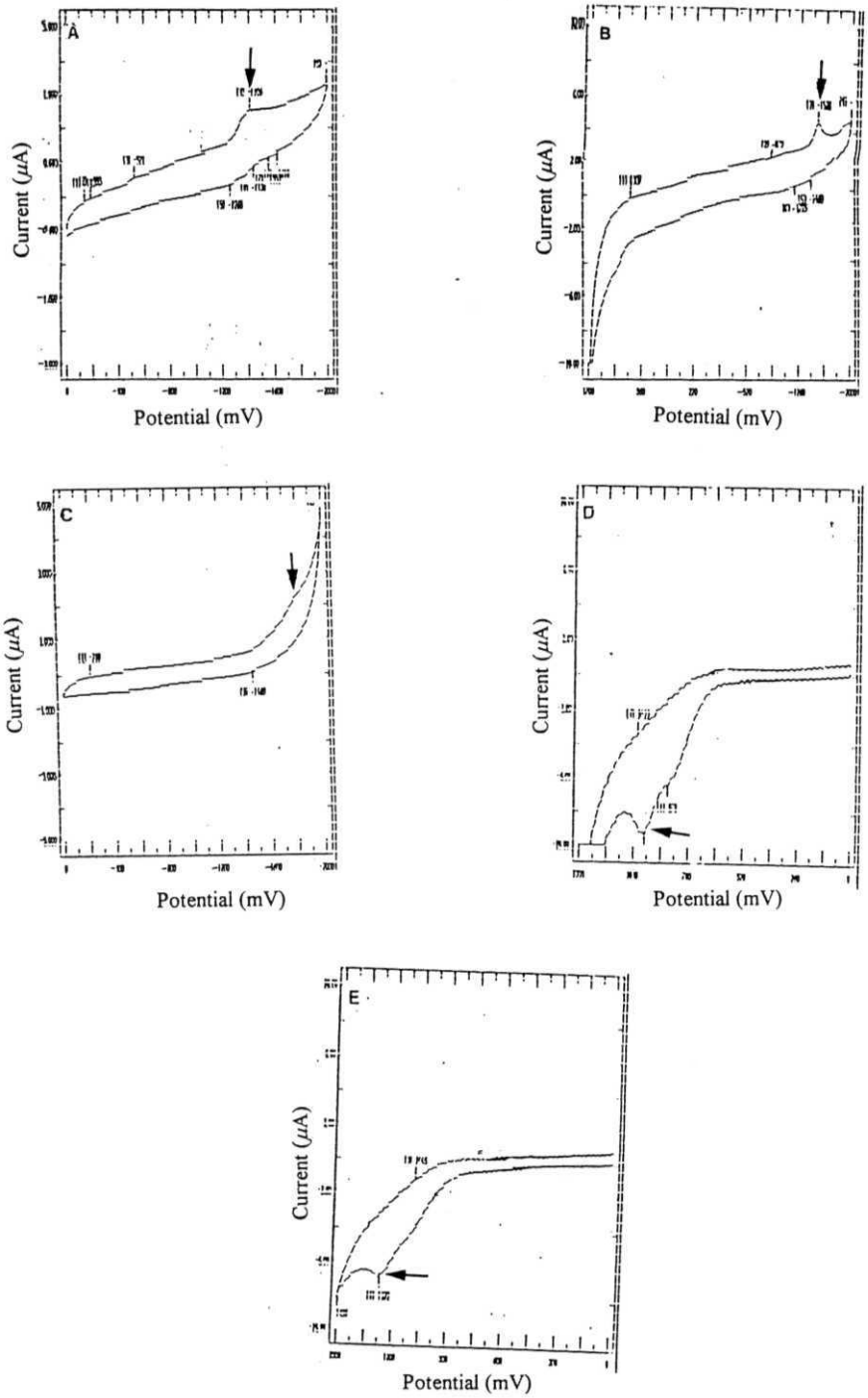


Figure 23: Cyclic voltametric spectra of CoSAL (A) in presence of linear DNA (B), supercoiled pBR 322 DNA (C) and CV spectra of CoSSC (D) in presence of linear DNA (F) (arrows show the peaks of the complexes).

state in DNA bound form and so, does not oxidise DNA. The CV spectra of CoSSC suggests that it is in +2 state in presence and also absence of DNA (Figure 23D & E).

DISCUSSION

Cobalt and Copper metal complexes with salicylaldoxime ligands have earlier been shown to possess anti-proliferative properties (Lumme *et al.,* 1984). The ligand salicylaldoxime resembles pyridoxal, a well-known vitamin B_6 analog and an extremely powerful inhibitor of pyridoxal kinase (Churichich *et al.,* 1981). Since vitamin B_6 deficiency and B_6 antagonists are known to have growth-inhibitory and anti-tumor activities (Korytnyk *et al.*, 1970), the side effects of Copper salicylaldoxime (CuSAL) (weight loss and acrodynia) resemble those of B_6 deficiency and B_6 antagonism (Emerson, 1967). It appears that pyridoxal antagonism might be involved in the antitumor action of CuSAL. However, the pyridoxal theory alone cannot explain the greatly different anti-proliferative activities of copper, cobalt and nickel salicylaldoxime complexes (Elo *et al.*, 1987).

In the present study, it is shown that salicylaldoxime complex of cobalt inhibits the proliferation of HL60 leukemia cells. The complex shows 50% inhibition of cell proliferation at 60 μM concentration. The molecular structure of this complex may enable it to bind to DNA and exhibit antitumor effects in more than one way. It has been reported that some cobalt metal complexes bind tightly and site specifically to DNA at N(7), N(1) of adenosines (Sorrel *et al.,* 1977; Beverly *et al.*, 1990). To examine the DNA binding ability of CoSAL we conducted DNA thermal denaturation studies. The results show that

CoSAL binds to calf thymus DNA with a stoichiometric ratio of two molecules per five nucleotide base pairs. Upon binding, it causes a small hypochromic shift like that of DNA groove binding molecules as shown by CD spectral analysis. Also the complex does not change the mobility of supercoiled pBR322 DNA (Figureure 26, panel A, lane 13) but it seems to protect the conversion of form I to form II DNA probably due to the strong binding of CoSAL to form I DNA. These results suggest that CoSal does not intercalate to DNA like m-AMSA or form DNA adducts like Cisplatin. It seems to bind to DNA by covalent or non-covalent interactions without greatly affecting the topology of DNA.

Studies on DNA binding anticancer compounds have shown that the anticancer ability of these compounds arises from their ability to poison the catalytic activity of topoisomerase II especially by cleavage complex formation (D'Arpa *et ai,* 1989). This prompted us to examine if CoSAL inhibits topoisomerase II catalytic activity. Our results show that the CoSAL inhibits topoisomerase II catalysed DNA relaxation activity in a dose dependent manner. The drug seems to inhibit this activity through the formation of the enzyme-drug-DNA cleavage complex as shown by the cleavage assay. Inhibition of topoisomerase II activity by formation of cleavage complex is the most potent form of topoisomerase II poisoning (Zechiedrich *et ai,* 1989). This is because the cleavage complexes harbor enzyme associated DNA double stranded breaks. When the replication or transcriptional machinery comes across these cleavage complexes, the complex breaks down, releasing the enzyme from the broken strands of DNA without religating the strands. Such accumulation of DNA strand breaks in large numbers may prompt the cells to undergo apoptosis or necrosis leading to effective killing of cancer cells (D'Arpa *et ai,* 1989).

It has been reported that the hydroxyl (OH) groups at specific positions are required for topoisomerase II inhibition and anti-proliferation activity for topoisomerase II targeting drugs (Skladanowski *et al.*, 1996). To see the effect of substitution of hydroxyl group present on the imine nitrogen, we synthesized an analogue of CoSAL, where the hydroxyl group of CoSAL was replaced with semicarbazone. This complex shows marginal anti-proliferation activity, does not inhibit topoisomerase II catalyzed relaxation activity and does not form cleavage complex. This strongly suggests that hydroxyl group of CoSAL is involved in topoisomerase II inhibition.

The complex shows DNA binding affinity similar to CoSAL and protects the conversion of pBR322 form I DNA to form II (Figureure 26, panel B, lane 12). The CD spectra of DNA in presence of CoSSC shows a negligible change in DNA conformation. These results suggest that the two complexes exhibit more or less the same DNA binding affinity. The common entities in the two complexes, cobalt and salicylal groups, may be involved in DNA binding. The shift in redox peaks of cobalt metal in both the complexes in the presence of calf thymus DNA as evidenced by CV spectra suggests the involvement of metal ion in DNA interaction. The difference in topoisomerase II inhibition by these complexes could be due to the structural differences of the ligands induced by the removal of hydroxyl groups in CoSSC. In CoSAL, the free hydroxyl groups may interact with DNA bound topoisomerase ft, and form a stable ternary cleavage complex consisting of enzyme, drug and DNA. This hypothesis is strengthened by the inability of CoSSC to inhibit the enzyme's activity, which may be due to the substitution of hydroxyl groups with semicarbazone groups. Semicarbazone forms a closed pentacyclic ring structure by

bonding to the metal atom with the free NH_2 group, and cannot contribute free active groups like OH, NH etc. for interacting with enzyme.

The ATPase assay provides additional confirmation for the difference in the topoisomerase II inhibition by the cobalt complexes. The inhibition of DNA stimulated ATPase activity by CoSAL and almost no inhibition by CoSSC corroborates well with the relaxation and cleavage assays.

Our hypothesis was further evidenced by the immnoprecipitation assay. The results show that 75% of complex is present in cleavage complex formed in presence of CoSAL where as only 7.65% of complex is present when reaction is conducted in presence of CoSSC. However, both complexes do not bind to topoisomerase II alone, suggesting that CoSAL requires the presence of DNA for poisoning topoisomerase II.

From our results, the following mechanism for the anti-proliferation activity of CoSAL is proposed. The central metal ion (cobalt) may interact either covalently or non-covalently with nucleotide bases and the salicylal groups of the ligands may get oriented in the DNA grooves. The free OH groups may form bonds with DNA bound topoisomerase II and inhibit the domain movements required for strand passage activity of topoisomerase II by forming a ternary cleavage complex containing DNA, drug and topoisomerase II.

In summary, the inhibition of topoisomerase II activity by CoSAL may be due to the bi-directional mode of interaction of drug with enzyme and DNA which could aid in forming a stable cleavage complex.

CHAPTER V

Topoisomerase II poisoning by the structural analogues of cobalt salicylaldoxime: Elucidation of molecular mechanism of action using DNA interaction and computer simulation studies.

Introduction

During the last three decades, the complexes of many transition metals have been tested both in cell culture and animal models for antitumor activity (Kopf-maier, 1987; Lumme et al., 1987, Kopf-Maier *et al.*, 1989). Only the complexes of platinum however, are currently in routine clinical use. These platinum complexes are highly toxic, especially causing nephrotoxicity (Lumme *et al,* 1987). It would be useful to search for complexes with metal centers, which could minimize the toxicity-associated with the therapeutic use of such complexes. Also, the development of metal complexes which specifically antagonize a molecular target involved in the progression of cancer would go a long way in reducing *in vivo* cytotoxicity.

The identification of such intracellular targets through which active drugs exert their selective anticancer action would facilitate the design of more effective antineoplastic drugs. In the past decade, the molecular basis of the antitumor activity of many DNA binding agents has been recognized to rest on the interference of topoisomerase II mediated DNA breakage-reunion catalytic cycle (Beck, 1989; Liu. L. F, 1989; Zwelling, L. A, 1989). Topoisomerase II (topoisomerase II) is required for cell division and also plays important roles in transcription (Osheroff, 1990), DNA replication (Brill *et al,* 1987), condensation and segregation of chromosomes (Newport *et al.*, 1987). It has been shown that the topoisomerase II levels and activity is highly regulated in the cell cycle of normal cells. In normal dividing cells the enzyme levels are in the order of G1<S<M<G2 and activity is in the order G1<S<G2<M at different phases of cell cycle respectively. But in cancerous cells the levels and activity are maintained constantly high irrespective of the

phase of cell cycle (Schneider *et al.,* 1990). The importance of topoisomerase II in the cell cycle of fast growing neoplastic cells stirred the development of numerous molecules which antagonize this enzyme. The biochemical actions of these drugs on topoisomerase II are of two types (a) inhibition of enzyme catalysed DNA double-strand passage and (b) stabilization of the DNA-protein intermediate in the strand passage action known as the "cleavage complex", that is these drugs trap the transient cleaved DNA-topoisomerase II complexes in which the cleaved DNA strands are covalently-linked to the topoisomerase II subunits. This latter action of the drugs can be rapidly quantified in cellular and biochemical systems (Osheroff, 1989; Robinson *et al.,* 1990). Numerous evidences suggest that the production of such stabilized configurations (cleavage complexes) initiate a cascade of events which lead to cell death (reviewed by Zwelling, L. A. 1989 and D'Arpa *et al.,* 1989). Hence, the development of cleavage complex forming drugs is very important in specific targeting and effective anticancer action.

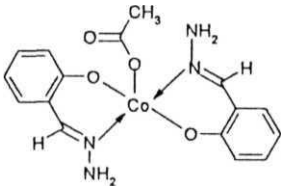
In the present study, five derivatives of cobalt (III) salicylaldoxime (Structures VI -X) were designed which are Cobalt (III) *N*-aminosalicylaldimine, cobalt (II) salicylalthiosemicarbazone, cobalt (III) *N*-phenylsalicylaldimine, cobalt (III) *N*-phenylaminosalicylaldimine and cobalt (III) (2-4, dinitro) *N*-phenylaminosalicylaldimine in order to poison the activity of topoisomerase II with a similar mechanism of action but with a higher potency of cleavage complex formation.

Materials and ***methods :***

Biochemicals, *Enzyme and DNA:* Salicylaldehyde, Hydrazine hydrochloride, Phenylamine, Phenylhydrazine hydrochloride, Thiosemicarbazone and 2,4-dinitrophenylhydrazine were from Aldrich. *y* ^{32}P ATP and ^{3}H thymidine were supplied by BARC, India. Polyethyleneimine (PEI) Cellulose-F sheets were from Merck. Other chemicals and biochemicals used were of standard grade.

Synthesis of the cobalt Compounds:

(a) Cobalt(III) N-Amino Salicylaldimine (CoASI) [Cobalt (III) (*N*-aminosalicylaldimine)$_2$ (acetato)]: 1 mM Hydrazine hydrochloride and a few drops of trimethyl amine were added to 1 mM salicylaldehyde in dry methanol and stirred under a slow stream of dry nitrogen gas at 60 °C for 3 h. This was vacuum dried and re-crystallized in dry methanol to form *N*-Aminosalicylaldimine. This ligand was added to dry cobalt(II)acetate in dry methanol slowly under dry nitrogen atmosphere with constant stirring at 60 ^{0}C and refluxed for 6 h. This yielded a pale yellow colored precipitate which was left undisturbed overnight. The precipitate was filtered, vacuum dried and re-crystallized in dry methanol (Structure VI).

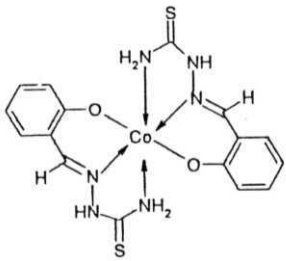


Structure **VI**

ir spectra

The coordination of the amine nitrogen atom to the metal (III) ion is indicated by the shift of the v (C=N) stretching vibrations. The spectra of the complex exhibits downward shift of v (C=N) from *ca.* 1624 cm^{-1} for the ligand to approximately 1618 cm^{-1}, a stretching band appears at 1471 cm^{-1} corresponding to v (M-O) and a stretching band appears at 3294 cm^{-1} corresponding to free NH_2 group, which confirms the complex formation (Spectrum VI).

(b) Cobalt(II)Salicylalthiosemicarbazone (CoSTSC) [Cobalt (II) (salicylalthiosemicarbazone) $_2$]: 1 mM Thiosemicarbazide hydrochloride and a few drops oftri-methyl amine were added to 1 mM salicylaldehyde in dry methanol and stirred under a slow stream of dry nitrogen gas at 60 ^{0}C for 3 h. This was vacuum dried and re-crystallized in dry methanol to form salicylalthiosemicarbazone. This ligand was added to dry cobalt(II)acetate in dry methanol slowly under dry nitrogen atmosphere with constant stirring at 60 C for 3 h. This yielded a dark brown colored precipitate which was left undisturbed overnight. The precipitate was filtered, vacuum dried and re-crystallized in dry methanol (Structure VII).

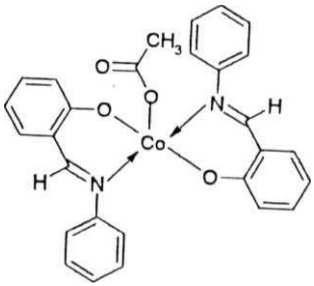


Structure VII

ir spectra

The coordination of the imine nitrogen atom to the metal (II) ion is indicated by the displacement of the v (C=N) stretching vibrations. The spectra of the complex exhibits downward shift of v (C=N) from *ca.* 1616 cm^{-1} for the ligand to approximately 1612 cm^{-1}. A stretching band appears at 1427 cm^{-1} corresponding to v (M-O), and a stretching band appears at 3011 cm^{-1} corresponding to NH_2 in bound form, which confirms the complex formation (Spectrum VII).

(c) Cobalt(II)N-Phenylsalicylaldimine (CoPSI) [Cobalt (III) (*N*-phenylsalicylaldimine)$_2$ (acetato)]: 1 mM Phenylamine was added to 1 mM salicylaldehyde in dry methanol and stirred under a slow stream of dry nitrogen gas at 60 ^{0}C for 5 h. This was vacuum dried and re-crystallized in dry methanol to form *N*-Phenyl Salicylaldimine. This ligand was added to dry cobalt(II)acetate in dry methanol slowly under dry nitrogen atmosphere with constant stirring at 60 ^{0}C and refluxed for 12 h. This yielded a brick red colored precipitate which was left undisturbed overnight. The precipitate was filtered, vacuum dried and re-crystallized in dry methanol(Structure VIII).

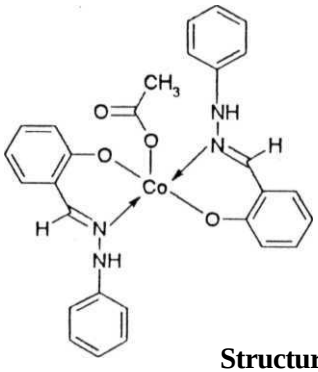


Structure VIII

ir spectra

The coordination of the amine nitrogen atom to the metal **(III)** ion is indicated by the shift of the v (C=N) stretching vibrations. The spectra of the complex exhibits downward shift of v (C=N) from *ca.* **1618 cm^{-1}** for the ligand to approximately **1612 cm^{-1}**, stretching band appears at **1572 cm^{-1}** corresponding to v **(M-O)** and stretching bands appear at 1427, **1344** and 1292 **cm^{-1}** corresponds to acetate group, which confirms the complex formation (Spectrum V11I).

(d) Cobalt(II)N-Phenylaminosalicylaldimine (CoAPSI) [Cobalt **(III)** **(*N*-phenylaminosalicylaldimine)$_2$** (acetato)]: 1 **mM** Phenyl hydrazine and a few drops of trimethyl amine were added to 1 mM salicylaldehyde in dry methanol and stirred under a slow stream of dry nitrogen gas at 60 °C for 7 h. This was vacuum dried and re-crystallized in dry methanol to form *N*-Phenyl Amino Salicylaldimine. This ligand was added to dry **cobalt(II)acetate** in dry methanol slowly under dry nitrogen atmosphere with constant stirring at 60 **^{0}C** for **15** h. This yielded a dark brown colored precipitate which was left undisturbed overnight. The precipitate was filtered, vacuum dried and re-crystallized in dry methanol (Structure IX).

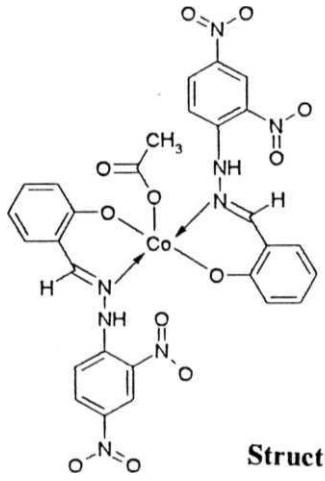


Structure IX

ir spectra

The coordination of the amine nitrogen atom to the metal (III) ion is indicated by the shift of the v (C=N) stretching vibrations. The spectra of the complex exhibits downward shift of v (C=N) from *ca.* 1618 cm^{-1} for the ligand to approximately 1602 cm^{-1}, stretching band appears at 1566 cm^{-1} corresponding to v (M-O) and stretching bands appear at 1486, 1358 and 1302 cm^{-1} corresponds to acetate group, which confirms the complex formation (Spectrum IX).

(e) Cobalt(III)(2,4-di Nitro)N-Phenylaminosalicylaldimine(CoDNPSI) [Cobalt (III) ((2-4, dinitro)*N*-phenylaminosalicylaldimine)$_2$ (acetato)]: 1 mM 2,4-di NitroPhenyl hydrazine was added to 1 mM salicylaldehyde in dry DMSO and stirred under a slow stream of dry nitrogen gas at 60 °C for 3 h. To this to dry cobalt(II)acetate was added slowly under dry nitrogen atmosphere with constant stirring at 60 ^{0}C for 24 h Diethyl ether was added to form a green colored precipitate which was left undisturbed overnight. The precipitate was filtered, vacuum dried and re-crystallized in dry methanol (Structure X).



Structure X

ir spectra

The coordination of the amine nitrogen atom to the metal (III) ion is indicated by the shift of the v (C=N) stretching vibrations. The spectra of the complex exhibits a stretching band at 1651 cm^{-1} corresponding to NO_2 groups, a stretching band appears at 1496 cm^{-1} corresponding to v (M-O) and stretching bands appear at 1439, 1386 and 1331 cm^{-} corresponds to acetate group, , which confirms the complex formation (Spectrum X)

In vitro anti-proliferation assay:

^{3}H-Thymidine incorporation assays were carried out to examine the effect of the cobalt complexes on the proliferative response of HL-60 (Human T-cell leukemia) cancer cells. The cells were grown in RPMI-1640 medium supplemented with 20% fetal calf serum. 0.2 million cells in 200 μl were distributed in a 96 well microtitre tissue culture plate. Increasing concentrations of the two cobalt drugs were added to the cells. The cells were incubated for 48 h in a CO_2 incubator at 37 °C maintaining 5% CO_2. The cultures were then pulsed with 0.5 μCi of H-thymidine. Incubation was continued for 6 h to allow thymidine incorporation by cells. The cells were harvested on glass microfibre strips using a Skatron automated cell harvester. Radioactivity was measured in a Wallac liquid scintillation counter.

DNA- Drug Binding Studies:

Absorption titration experiments were performed in a buffer containing 5 mM tris pH 7.0, 50 mM NaCl, with 25 μM of metal complex to which 10 μM increments of calf thymus DNA solution were added. The concentration of the metal complex used was 25

μM and that of DNA ranged between 0-100 micromolar equivalents (base pairs). After the addition of DNA to the metal complex, the resulting solution was allowed to equilibrate for 5-10 min. at 25 °C. The absorption readings (corresponding to the changes in absorption maxima of the drug used) were noted. The data were then fit into the following equation to obtain the intrinsic binding constant K_b (Wolfe et al., 1987).

$$[DNA]/(\varepsilon_a - \varepsilon_f) - [DNA]/(\varepsilon_b - \varepsilon_f) + 1/Kb\ (\varepsilon_b - \varepsilon_f)$$

Where ε_a, ε_f, and ε_b are the apparent, free and bound metal complex extinction coefficients respectively. A plot of $[DNA]/(\varepsilon_a - \varepsilon_f)$ versus [DNA] gave a slope of $1/(\varepsilon_b - \varepsilon_f)$ and a Y intercept equal to $K_b/(\varepsilon_b$ - Ef); K_b is the ratio of slope to Y intercept.

Oxidation state of Cobalt in the DNA bound drugs:

The Cyclic Staircase Voltametry (CV) spectra of complexes was recorded in absence and presence of DNA using the system of DMSO/TB AP/glass carbon working electrode/ Ag^+-AgCl. The concentration of drug to DNA was maintained two drug molecules per five nucleotide bases.

Molecular modeling analysis:

Drug conformations of the cobalt salicylaldoxime and its analogues were examined by computer-aided molecular modeling techniques. The software package SPARTAN (version 4.1, Wave Functions Inc., California, USA) was utilized.

For each complex the following tasks were performed.

1)To build and optimize 3-dimensional model structures of the molecules, SPARTAN graphic interface free-valence geometry force-field and semi-empirical I IF program was used for energy minimization. The partial atomic charges needed for the coulombic

electrostatic potentials were obtained by RHF PM_3 (tm) calculations. PM3 (tm) is a semi-empirical method when the parameters of different systems are calibrated with a large set of experimental results. This kind of method is known as a parameterized method.

2) Frequency vibration calculations were carried out to analyze the nature of the optimized structures to show all the structures to be at a stable minima.

3) From the 3-D structures the distance of the active groups from the central metal ion was calculated.

All molecular modeling calculations were performed on an EBMRS/6000 model 530 UNIX workstation.

Results

Anti-Proliferation Activity:

The ^{3}H thymidine incorporation experiments were conducted with HL60 T-cell leukemia cells with increasing concentrations of the cobalt drugs. The results show that 35, 30 and 20 μM of CoPSl, CoPASl, and CoDNPSl could inhibit 50% of the cancer cell proliferation, while CoASl and CoTSSC shows negligible anti-proliferation activity (Figurc. 24).

Action of Cobalt drugs on the DNA Relaxation Activity of Topoisomerase 11:

CoPSl, CoAPSl and CoDNPSl inhibits the topoisomerase 11 catalysed relaxation activity in a dose dependent manner and shows complete inhibition at 200, 175 and 150 μM concentration respectively (Figure. 25 panel D lane 7, panel E lane 6 and panel F lane 6) while CoASI and CoTSSC does not affect the DNA relaxation activity of topoisomerase ll (Figure. 25B & C).

Formation of cleavage complex:

The cleavage assay was conducted to see if cobalt drugs could form drug-induced ternary complex of DNA-drug-topoisomerase ll, called the cleavage complex. The formation of topoisomerase ll-drug-DNA cleavage complex can be visualised by the appearance of linear DNA upon treatment of the cleavage complex with SDS and proteinasc K. The results show that phenyl derivatives induced the cleavage complex formation (Figure. 26

Figure 24

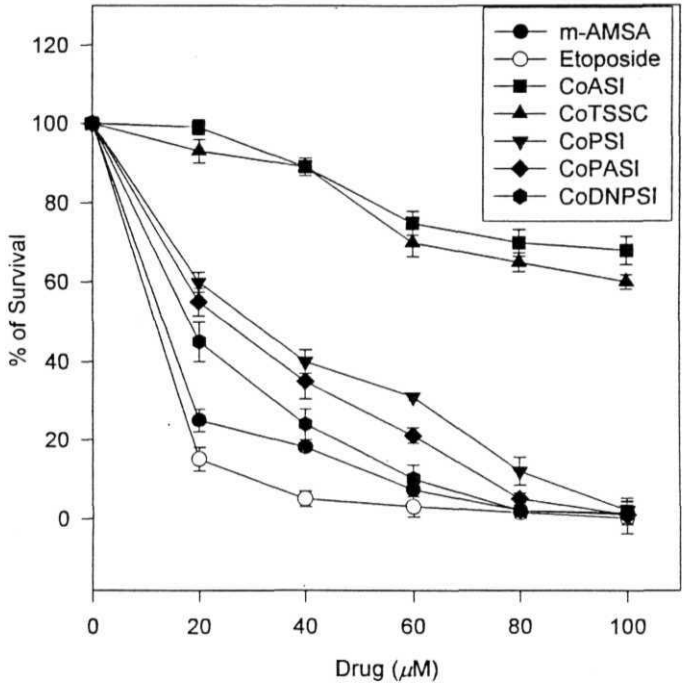


Figure 24: HL 60 Human leukemic cells were incubated with increasing concentrations of CoASI, CoTSSC, CoPSI, CoAPSI & CoDNPSI. ^{3}H thymidine incorporation during the last 6 h of incubation was measured as described in methods. Values are presented as mean of three independent experiments. Data is graphically expressed as percentage increase in inhibition versus concentration of CoASI (•), CoTSSC (A), CoPSI (T), CoAPSI (•) & CoDNPSI (•) in μM. mAMSA (•) and Etoposide (0) (positive controls) are also shown.

Figure 25: Effect of CoASI (B), CoTSSC (C), CoPSI (D), CoAPSI (E) & CoDNPSI (F) on topoisomerase II catalyzed DNA relaxation activity. Supercoiled pBR 322 DNA (panel A, lane 1) was incubated with topoisomerase II in the absence (panel A, lane 2) or presence of 50 and 100 μM m-AMSA (panel A, lane 3 - 4) and 50, 75, 100, 125, 150, 175 & 200, μM CoASI, CoTSSC & CoPSI (panel B, C & D. lanes 1-7), without enzyme but with 200 μM CoASI, CoTSSC & CoPSI (lane 8). Panel E & F show DNA with topoisomerase II in presence of 25, 50, 75, 100, 125, 150, 175 & 200 μM of COAPSI & CoDNPSI (lanes 1-8) and without enzyme but with 200 μM CoAPSI & CoDNPSI (lane 9). The positions of supercoiled (form 1) and nicked circular (form 2) DNA are indicated by I and II.

Figure 25

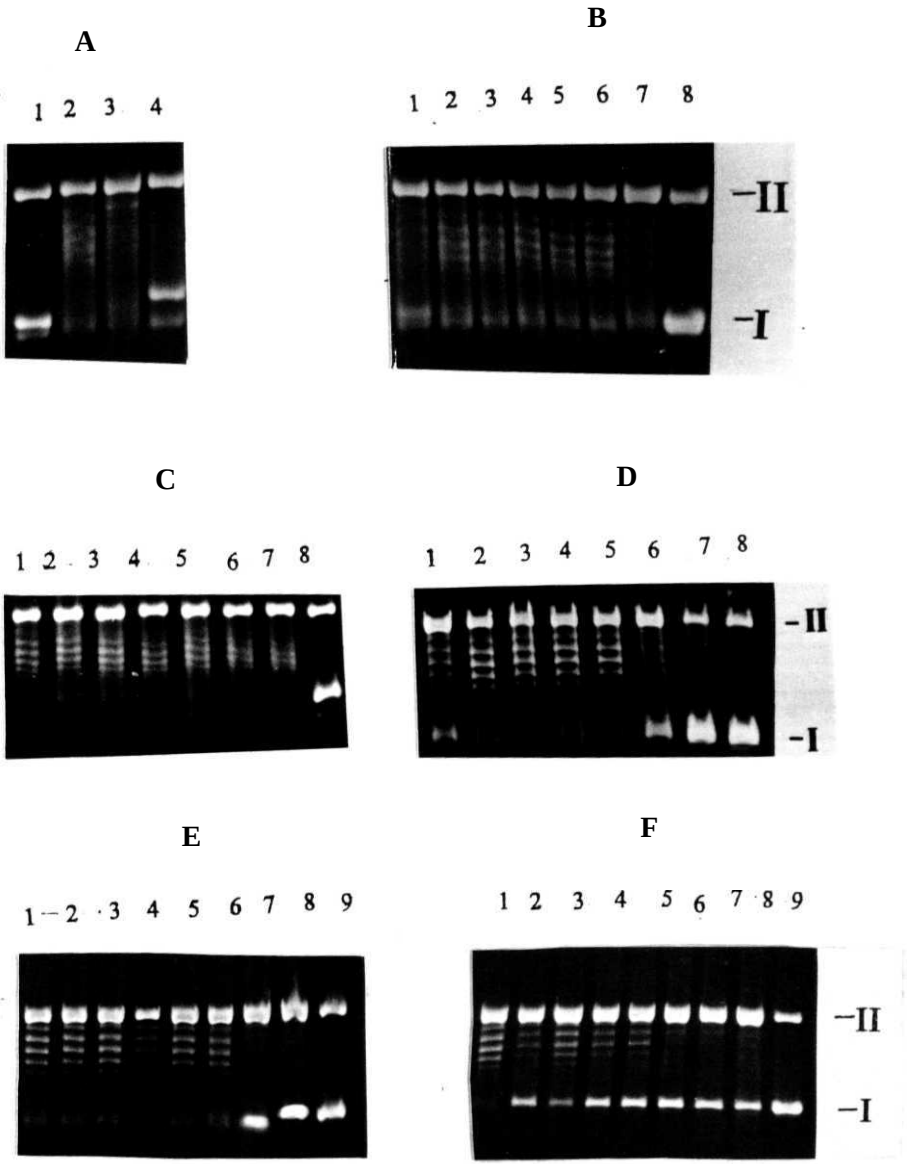
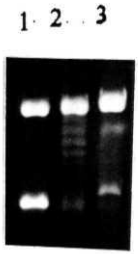

A
1 2 3 4
B
1 2 3 4 5 6 7 8
-II
-I
C
1 2 3 4 5 6 7 8
D
1 2 3 4 5 6 7 8
-II
-I
E
1 2 3 4 5 6 7 8 9
F
1 2 3 4 5 6 7 8 9
-II
-I

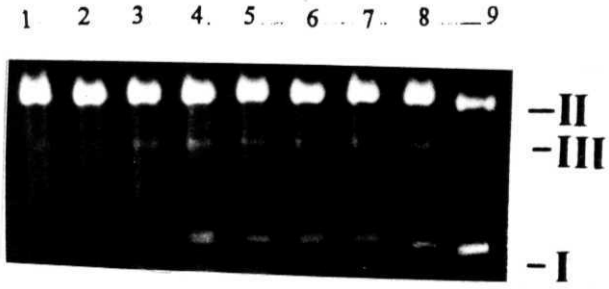
Figure 26: (A) Cleavage reaction was conducted by incubating pBR322 DNA (lane 1) with topoisomerase II (lane 2) in presence of 60 μM m-AMSA (lane 3) and 25, 50, 75, 100, 125, 150, 175 & 200 μM of CoPSI, CoAPSI & CoDNPSI (panel B, C & D lanes 1-8), and without enzyme but with 200 μM of CoPSI, CoAPSI & CoDNPSI (lane 9). The positions of supercoiled, nicked circular and linear (form 3) DNA are indicated by I, II and HI. (E) The plot shows the percentage of linear DNA formed with increasing concentration of CoPSI, CoAPSI & CoDNPSI

Figure 26

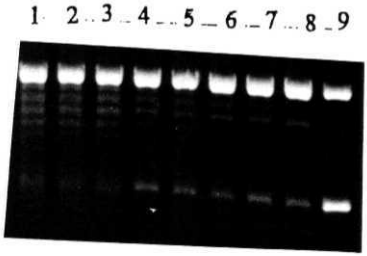
A



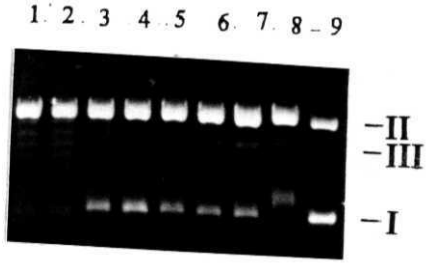
B



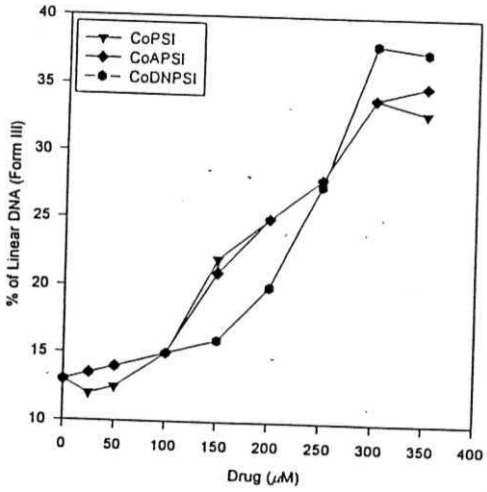
C



D



E



B, C and D) while CoASI and CoTSSC do not. Density analysis of DNA bands in the agarose gels shows the increase in intensity of linear DNA formation with increasing drug concentration in a dose dependent manner (Figure. 26E)

Action of the cobalt compounds on ATPase activity of topoisomerase II;

To see if these complexes can inhibit DNA-stimulated ATPase activity of topoisomerase II which is necessary for the enzyme's relaxation activity, ATPase assay was performed in presence of increasing concentration of the drugs using $\gamma^{32}P$ ATP. The products were separated on PEI-cellulose sheets in 1 M LiCl. ^{32}Pi and unhydrolysed $\gamma^{32}P$ ATP in the samples were quantified. The results show that phenyl derivatives inhibit the dependent activity of topoisomerase II while CoASI and CoTSSC show a marginal inhibition of ATPase activity (Figure 27).

Presence of drug in cleavage complex:

To confirm that the cleavage complex formed in presence of phenyl derivatives actually contains the drug, an immunoprecipitation assay was performed. The results show that 32 %, 36 % and 44 % of CoPSI, CoPASI and CoDNPSI are present in the cleavage complex. The control experiments with drug + DNA and drug + topoisomerase II were carried out to monitor any non-specific interaction of drug. Both the experiments show negligible amount of non-specific interaction by any of these drugs (Table 2).

The experiment was repeated in presence of CoASI and CoTSSC. The results show that a 4 fold lower concentration of drug is present in the cleavage complex (8% & 6%) (Table. 2).

Figure 27

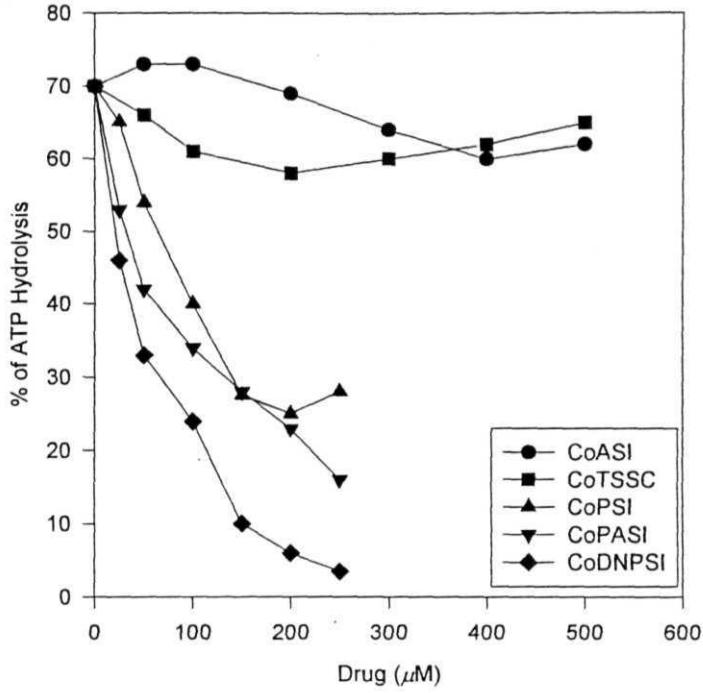


Figure 27: Inhibition of ATPase activity of topoisomerase II by CoASI (•), CoTSSC (•), CoPSI (A), CoAPSI (T) & CoDNPSI (•). ATP hydrolysis in presence of increasing concentration of the drugs are presented as mean of three experiments, data is plotted as the percentage of ATP hydrolyzed versus concentration of drug in μM.

Table. 2 Presence of cobalt drugs in cleavage complex.

	CoASI	CoTSSC	CoPSI	CoAPSI	CoDNPSI
topo II + DNA	0	0	0	0	0
drug	<2	<3	<2	<1	<2
drug + DNA	<1	<2	<1	<2	<3
topo II+ DNA	5 + 0.6	8 + 0.6	10 + 0.6	14 + 0.6	16 + 0.6
drug + DNA + topo II	7+0.78	11+0.78	32 + 0.78	38+0.78	45+0.78

Note. The data is an average of three independent experiments conducted in triplicates (+ error).

DNA - ***Drug Binding Studies:***

(a) DNA melting temperature experiments were carried out by incubating calf thymus DNA with increasing concentrations of Cobalt complexes. The absorbance of nucleotide bases at 260 nm was monitored by increasing temperatures from 50 ^{0}C to 90 ^{0}C. The results show that both Cobalt complexes protect melting of calf thymus DNA (Figure 28 a, b, c, d & e). The phenyl derivatives (CoPSI, CoPASI and CoDNPSI) increase the T_m of DNA from 56 ^{0}C to 78, 79 and 80 ^{0}C respectively, where as amine and thiosemicarbazone derivatives (CoASI and CoTSSC) increase the T_m up to 74 and 72 ^{0}C.

To understand the mode of binding to DNA, curve width of the T_m curves were measured at different drug to nucleotide ratios according to the procedure of Kelly *et al* (1985) and were plotted (Figure. 29a) and Figure 29b shows the T_m verses D/N. The results show that these complexes bind DNA in a manner similar to major groove binding molecules eg. polypyridyl liganded metal complexes.

The degree of binding was established using a scatchard plot analysis of spectrophotometric data obtained by the method described by Peacocke *et al* (1956). A typical scatchard plot analysis of binding of cobalt complexes to DNA is shown in Figure 30 as r / m versus r. The letter r represents the number of moles of cobalt complexes bound per mole of DNA base pairs, and m is the concentration of unbound complex. A value of ~0.6 was determined for n, which is the number of moles of cobalt complexes bound per mole of DNA base pairs (Table 3).

Figure 28: Drug-DNA binding studies. (a) CoASI increases the T_m of calf thymus DNA from 57 °C for DNA control (—) to70, 72, 73, 72 and 74 °C , (b) CoTSSC increases the T_m of calf thymus DNA from 57 °C for DNA control (—) to68, 70.76, 73.87, 75 and 75.5 °C , (c) CoPSI increases the T_m of calf thymus DNA from 57 °C for DNA control(—) to70.15, 73.01, 76.13, 77.19 and 78 °C , (d) CoAPSI increases the T_m of calf thymus DNA from 57 °C for DNA control(—) to67, 69, 73, 76 and 79 °C , and (e) CoDNPSI increases the T_m of calf thymus DNA from 57 °C for DNA control(—) to 65, 66, 71, 75 and 80 °C . In all cases, DNA nucleotide to drug ratios of 20:1 (——), 10:1(—), 5:1(....) 2:1(---) and 1:1(--) respectively were used.

Figure 28

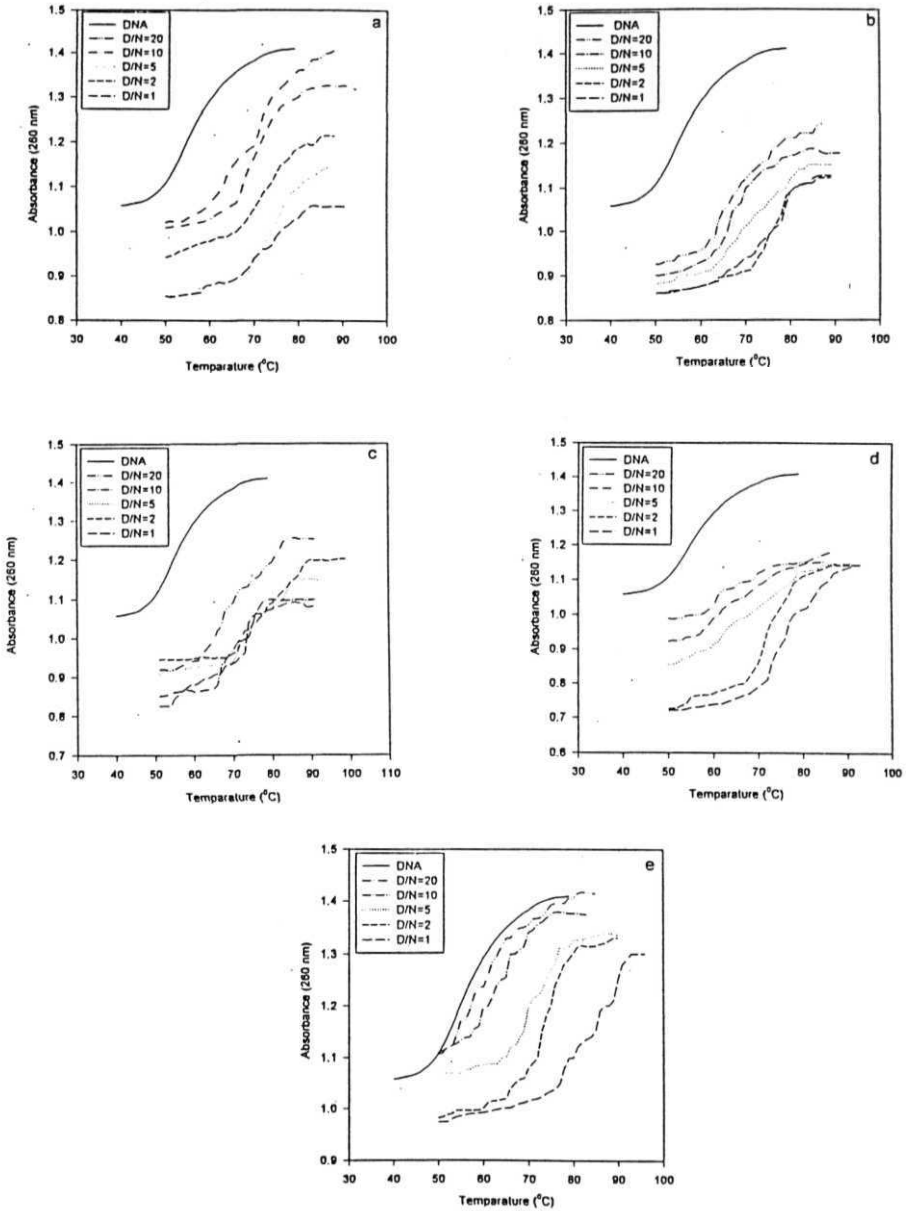

a
b
c
d
e
DNA
D/N=20
D/N=10
D/N=5
D/N=2
D/N=1
Absorbance (260 nm)
Temparature (°C)

Figure 29: (a) D/N plotted against curve width shows a characteristic increase in curve width by m-AMSA (•) which is a characteristic DNA intercalator and an intermediate increase by CoPSI (T) CoAPSI (•) and CoDNPSI( ). CoASI (•) and CoTSSC (A) show a change in curve width which is similar to an external binding agent of DNA. (b) D/N (drug/nucleotide) plotted against show an increase in T_m by CoASI (•) CoTSSC (•), CoPSI (A), CoAPSI (•) and CoDNPSI ( ).

Figure 29

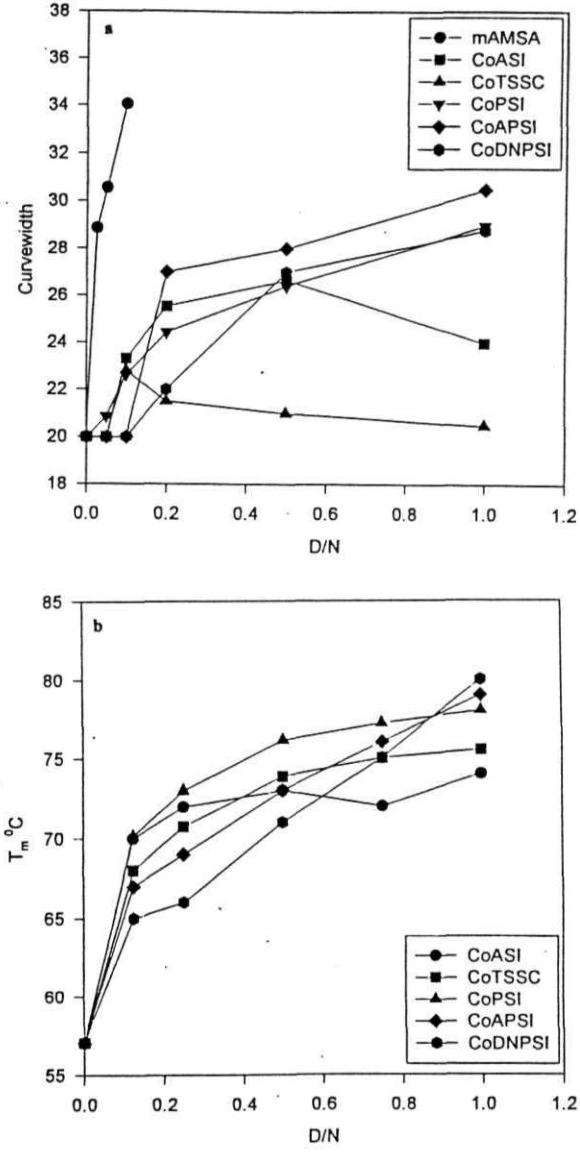

a
mAMSA
CoASI
CoTSSC
CoPSI
CoAPSI
CoDNPSI
Curvewidth
D/N
b
CoASI
CoTSSC
CoPSI
CoAPSI
CoDNPSI
T_m °C
D/N

Figure 30

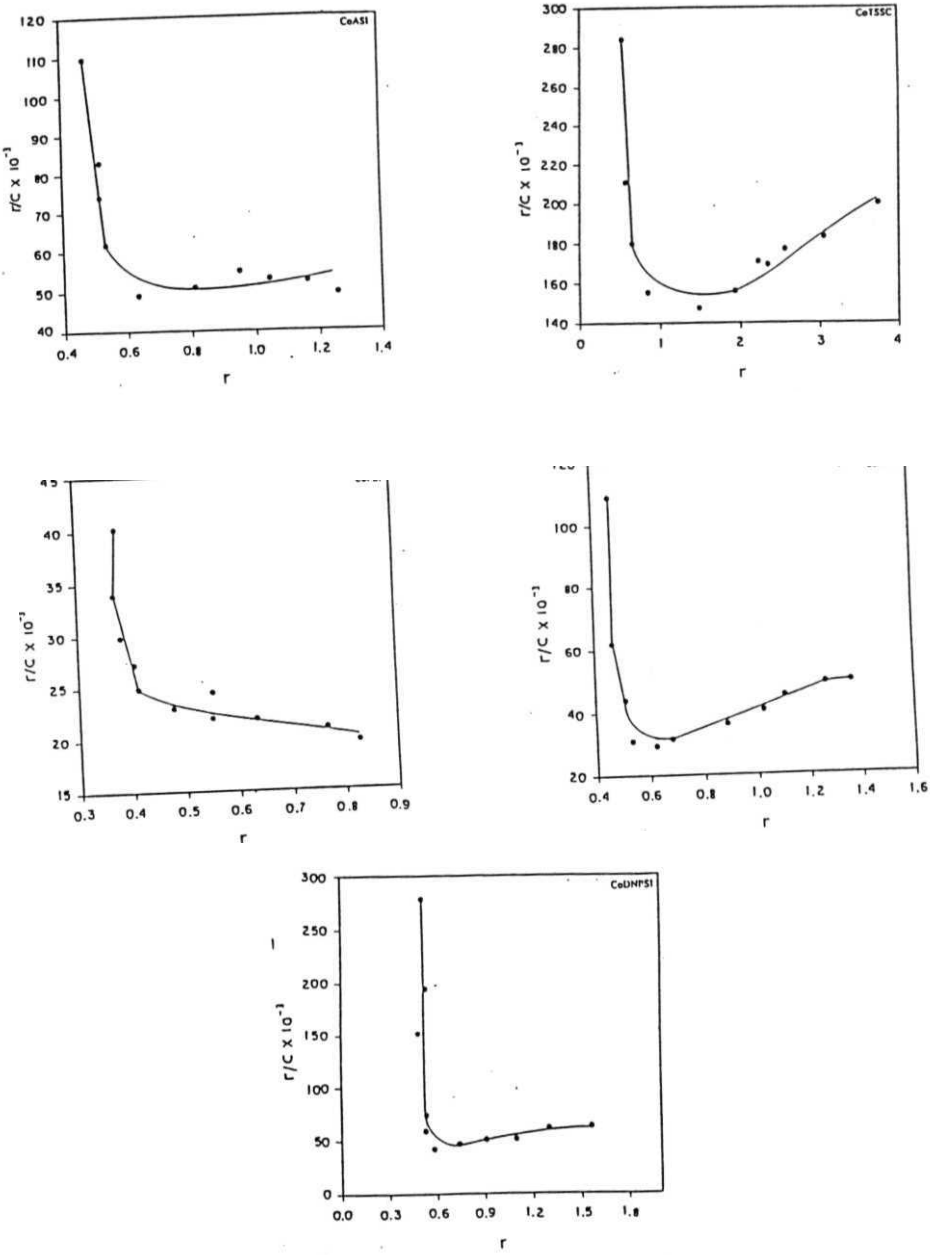


Figure 30: Scatchard plots showing the binding of the Cobalt complexes to calf thymus DNA.

Table. 3 Results of absorption titrations (Kb), thermal denaturation (Tm) and scatchard studies carried out in presence of DNA.

	K_b, M^{-1}	n (mole)	T_m $^0C^a$
CoSAL	0.23 X 10^2	0.50	74
CoASI	0.75 X 10^2	0.56	74
CoTSSC	0.30 X10^2	0.80	75
CoPSI	1.15 X 10^2	0.47	78
CoAPSI	0.76 X 10^2	0.50	79
CoDNPSI	0.68 X 10^2	0.57	80
m-AMSA	1.40 X 10^2		82
DNA			57

Note a: [Drug]/[nucleotide] • 20

(b) The binding constant (Kb) was determined from the spectroscopic titration experiments. In the presence of increasing amounts of calf thymus DNA. the phenyl derivatives CoPSI, CoAPSI and CoDNPSI show a bathochromic shift (7+1 nm, 3+1 nm & 5±1nm) and a hypsochromism of 74+2%, 85+4% and 77+3% respectively in the UV-visible spectra (Fig. 31). CoASI and CoTSSC do not show any change in UV-visible spectra (Figure. 31). The binding constant values for the cobalt drugs, CoASI, CoTSSC, CoPSI, CoAPSI and CoDNPSI were determined and/the|are given in Table 3. CoPSI shows a very high binding constant followed by CoDNPSI, CoAPSI, CoASI and CoTSSC in the decreasing order. The DNA intercalating drug m-AMSA shows the highest binding constant.

(c) The circular dichroic spectra of the phenyl derivatives CoPSI, CoPASI and CoDNPSI show that these complexes induce a hyperchromic shift in the positive [0]M of DNA and a hypochromic shift in negative [0]M suggesting that the binding of these drugs may induce unwinding, possibly by intercalation. CoASI does not change CD spectra but CoTSSC shows a small negative shift in CD spectra (Figure. 32). These observations show that all these complexes bind DNA with similar affinity with small differences in their mode of interaction.

Analysis of oxidation state of cobalt in DNA bound Complexes:

The CV spectra of cobalt complexes CoASI, CoPSI, CoAPSI and CoDNPSI (Figure. 33a, b, c & d) shows that cobalt is present in +3 oxidation state. In higher oxidation state, cobalt may undergo reduction while oxidizing DNA, thus resulting in nonspecific cleavage and unwinding of supercoiled DNA. To confirm this, the CV spectra of these complexes in presence of linear calf thymus DNA was recorded (Figure 33e, f, g & h). The re:

Figure31

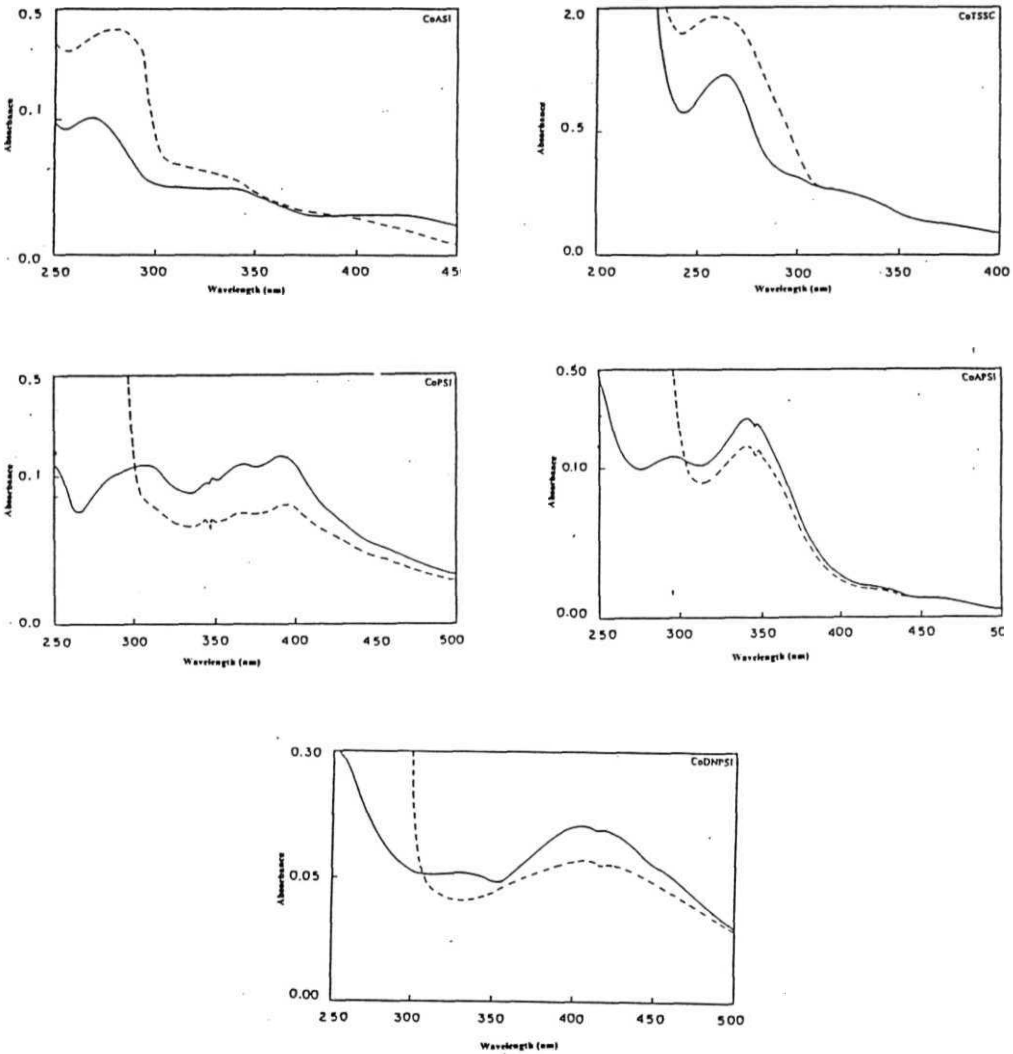


Figure 31: Panel A, B, C, D and E are the UV-visible spectra of CoASI, CoTSSC, CoPSI, CoAPSI & CoDNPSI (25 μM) in absence (—) and presence of increasing concentrations ofcalf thymus DNA (——). The DNA doping concentration shown in the figures is 100 μM base pairs.

Figure 32

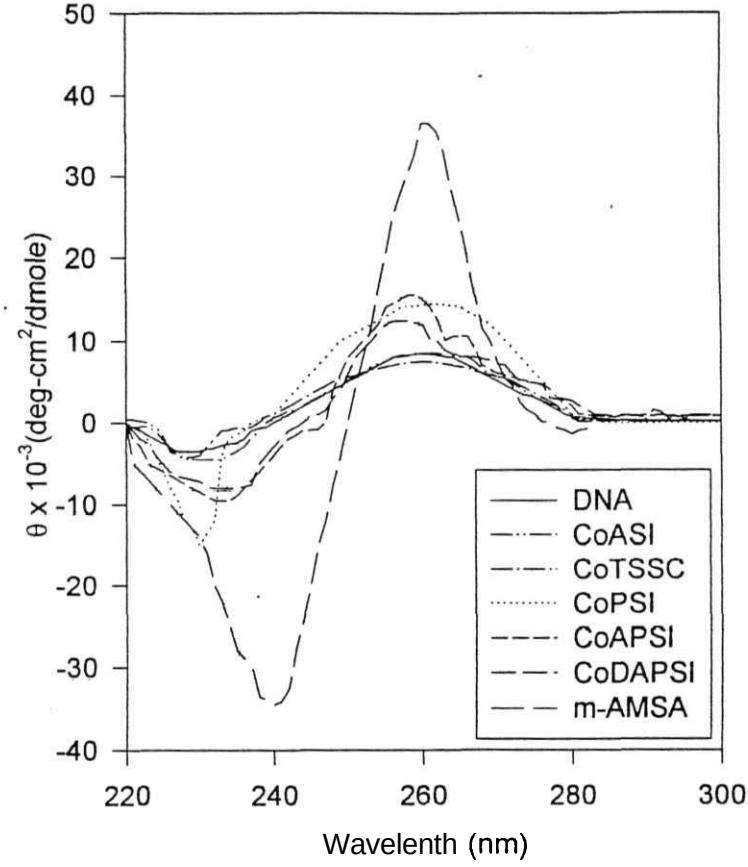


Figure 32: The Circular Dichroism spectra of pBR322 DNA (—) in presence of CoASI (——), CoTSSC (—), CoPSI (….), CoASI (---) and CoDNPSI (——). CoPSI, CoAPSI and CoDNPSI show a hyperchromic shift in the positive [9] and hypochromic shift in the negative [θ] in the CD spectra of DNA, while CoASI and CoTSSC show a small negative shift in the positive signal. But m-AMSA(——) shows a very prominent change at a concentration less than 5 times that of the cobalt complexes.

Figure 33

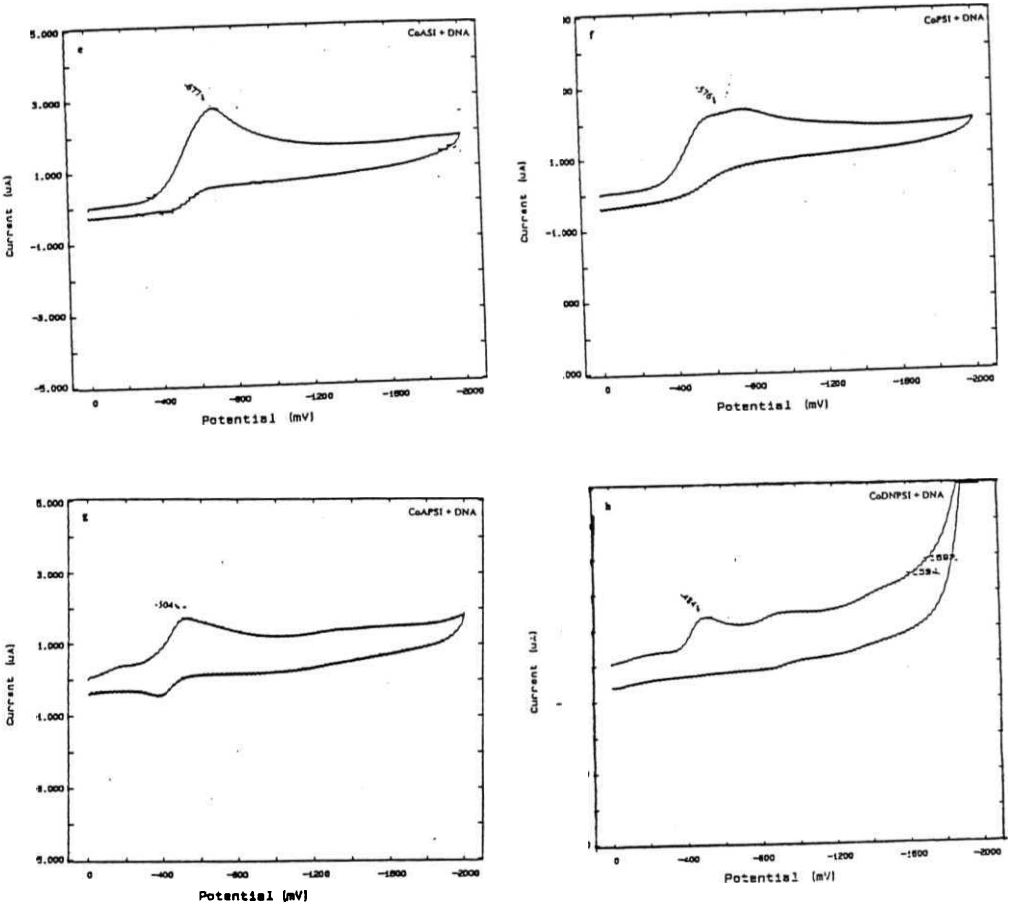
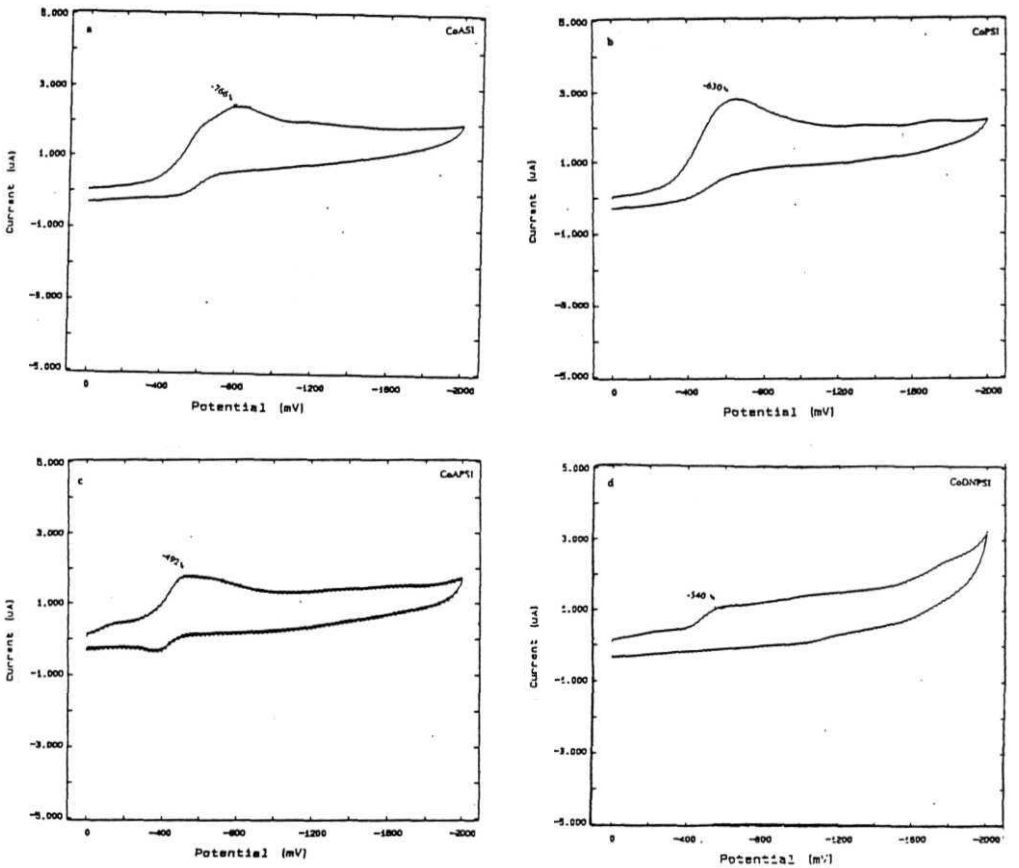


Figure 33: Cyclic voltametric spectra of CoASI (a), CoPSI (b), CoAPSI (c) and CoDNPSI (d) in the presence of linear DNA (e, f, g and h) respectively. (arrows show the peaks of the complexes).

Figure 33



show that the +3 oxidation state of cobalt in the complexes remains unaltered even when bound to DNA and so, it does not oxidise DNA. The CV spectra of CoTSSC suggests that it is in +2 state in presence and absence of DNA (Figure. 34a & b).

Molecular modeling analysis.

To identify the structural elements in the drug that play a role in the DNA interaction and topoisomerase II poisoning, molecular modeling studies using the transition metal modeling software SPRAT AN (version 4.1) were conducted. Minimized Energy and charge around the central metal ion are given in Table 4. The frequency vibration calculations (Table 5) of the complexes show that these conformations impart stability to the structures. The 3 D structures (Figure. 35) show that the salicilyl groups of the ligands form two identical domains around the central metal atom in a single plane and the substitutions on the imine nitrogen (amine, phenyl, aminophenyl and dinitro aminophenyl) are present perpendicular to the salicylal groups in different planes. While in CoTSSC, the thiosemicarbazone forms a closed pentacyclic ring structure by bonding to the metal atom with the free NH_2 group.

Figure 34

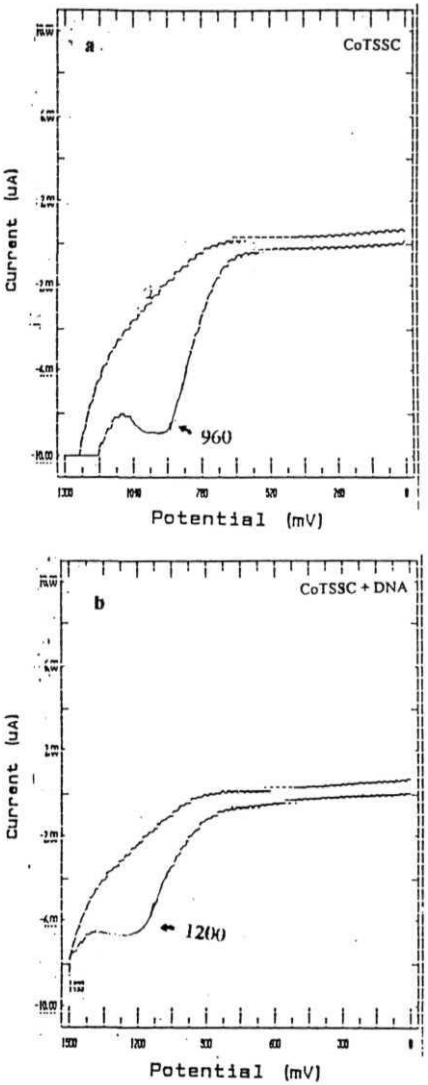


Figure 34: CV spectra of CoTSSC (a) in presence of linear DNA (b) (arrows show **the** peaks of the complexes).

Table. 4

	CoSAL	CoASI	CoTSSC	CoPSI	CoAPSI	CoDNPSI
Charge	-0.11	-0.16	+0.034	+0.065	+0.049	+0.032
Minimum energy (kcal/mol)	-1701.13	-1705.1	-1408.26	-1669.79	-1525.05	-1634.08
Distance between cobalt and possible topoisomerase II interacting group (A^0)	2.9	2.0	1.9	2.9	4.5	~9.0

Table.5 Vibrational frequencies (cm')

CoSAL	CoASI	CoTSSC	CoPSI	CoAPSI	CoDNPSI
18.89.32.35,43.33	-54.36, 31.65, 51.83	32.3, 42.00, 45.03	16.42, 35.35, 41.92	10.60, 19.03, 22.13	8.55, 13.32, 15.76
65.79,77.09,100.80	61.74,77.15,94.58	58.89,68.47,76.07	43.82, 51.30, 52.65	28.41,32.72,37.26	19.58,21.8,24.9
104.62.143.48.150.71	99.1, 115.26. 135.66	81.87.141.34.176.49	60.45, 68.10, 76.50	37.86, 50.17, 55.14	25.82, 26.5, 35.75
168.08.170.40,183.49	146.32,174,180.25	183.36,198.51,224.82	79.97,95.2.103.30	66.56, 78.29, 97.31	45.94, 50.3, 52.27
196 03, 206.66, 246.04	187.5, 201.04, 211.8	225.39, 255.88, 265.18	129.3, 154 3. 173.4	106.6, 123.80. 126.5	58.6.69.75,71.7
254.56.281.32.294.44	226.4.258.4,261.78	283.06.283.54,299.64	177.3, 202.9, 212.1	137.9,152.88.158	81.7,86.1,101.66
309.82,330.92,339.58	289.3,292.7,333.57	304.74, 318.51, 329.84	221.3, 235.5, 240.2	177.93, 179.6, 199.7	113.8, 119.1, 122..7
356.53,367.25,395.29	340.7,375.1,387.23	363.12,364.37,391.32	250.4, 268.2, 271.6	212.94, 226.53 234.1	128.7, 134.9, 141.91
421.55,443.03,449.83	402.59,418.7,422.7	402.28.427.43,443.93	285.8,289.1,297.8	258., 262.5, 266.52	150.2, 154.3, 178.45
468.95,479.84,489.9	450.58,454.5,458.5	447.16,465.83,480.46	323.6,347.6,367.48	296.04.329,253.78	180.8, 185.5, 192.2

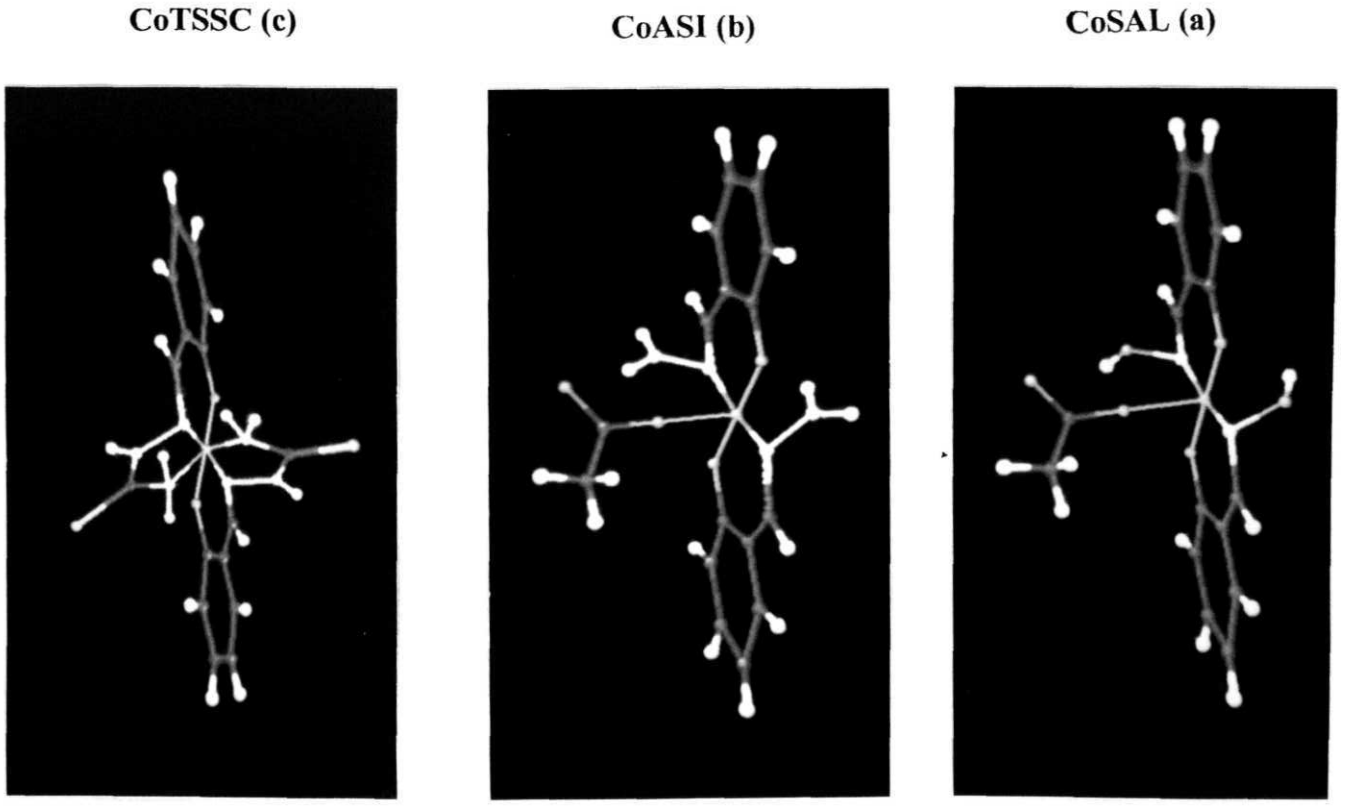


Figure 35: 3-D molecular structures of the cobalt complexes.

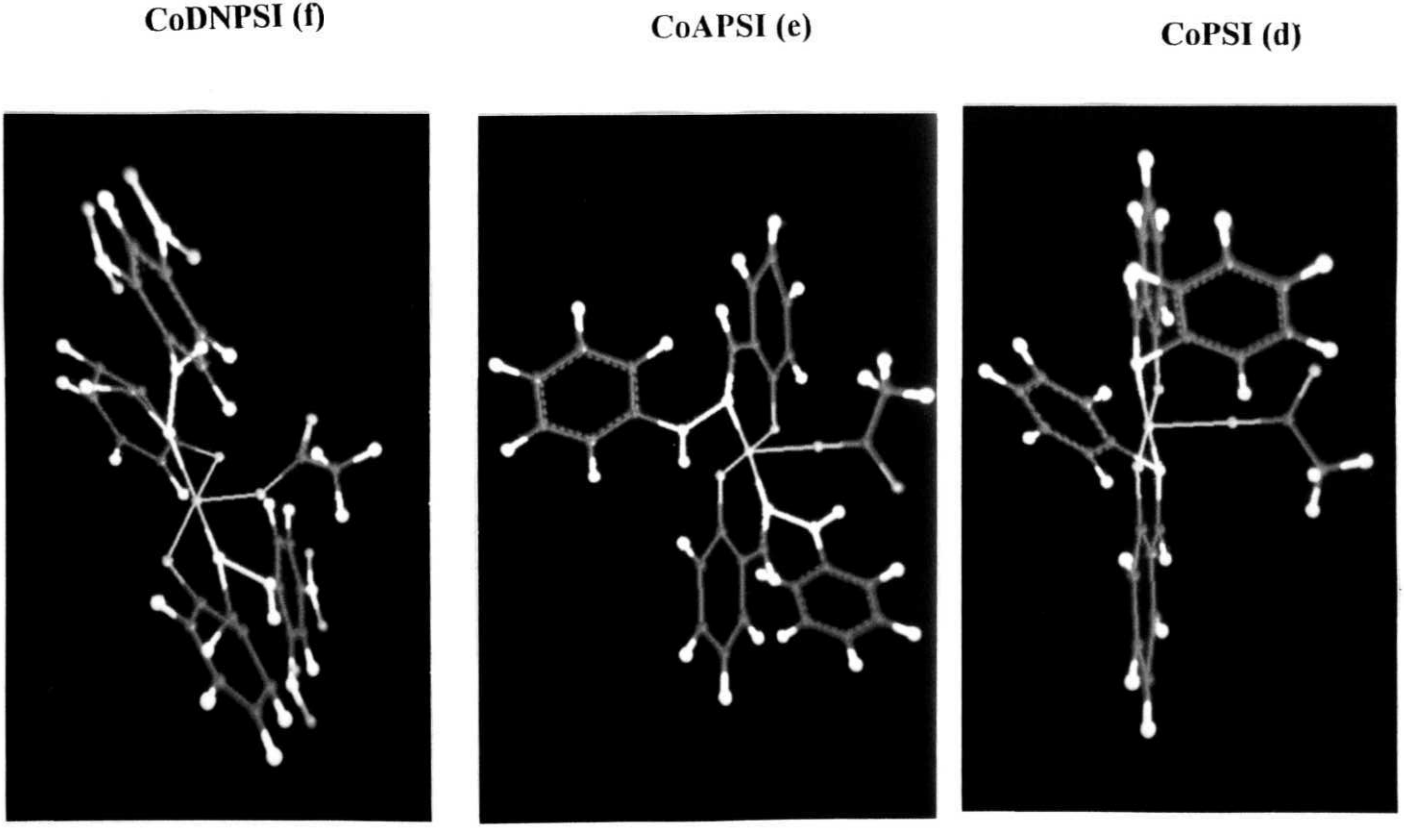


Figure 35: 3-D molecular structures of the cobalt complexes.

Discussion

A wide variety of complexes of numerous metals have undergone pre-clinical testing as anticancer agents (kopf-maier *et al.*, 1989). The anticancer metal complexes are broadly classified in to two groups. The first class consists of the metal complexes, like the classical inorganic anticancer agent cisplatin which potentially forms intra-strand and inter-strand DNA adducts and the other class consists of metal complexes which cleave DNA and are known as antitumor chelating agents. Some of the DNA binding antitumor complexes bind site-specifically to DNA through the metal center and its ligands are involved in interaction with cellular targets. The development of such metal complexes with a higher potency of action has received considerable attention in the development of anticancer agents which have a specific cellular target.

During the last few years, studies have indicated that the antitumor activity of DNA binding drugs and antibiotics depends in most cases on their capacity to interfere with the catalytic activity of topoisomerases rather than on their ability to bind DNA *sensu stricto* (Wang, 1985; Chen *et al.,* 1994 & Ralph *et al.*, 1994). This information has been exploited by medical chemists to create new categories of antitumor drugs. On the one hand, hundreds of sequence selective ligands derived from anti-viral antibiotics netropsin and distamysin have been synthesized (Kopka *et al.,* 1992 & Lown *et al.,* 1994). On the other hand, a growing variety of topoisomerase II inhibitors have been developed (Chen *et al.,*

1994 & Su *et al.,* 1992). With a view to develop novel antitumor agents which bind to DNA through the metal atom and interact with topoisomerase II through the ligands attached to the metal, cobalt was selected as the central metal atom due to the following reasons.

(1) Cobalt (III) complexes bind specifically to N7 and N1 nitrogen atoms of adenine bases through covalent bond formation (Theophilus *et al.*, 1976).

(2) Cobalt has the ability to alter the configuration of ligands attached to it.

(3) Cobalt has the capacity to form 2, 4, 5 and 6 coordination bonds based on the reaction environment it is present in (Parsons et al., 1997).

(4) The complexes of Co(III) are kinetically inert octahedral coordination complexes. This inertness is due to the d^6 lowspin electronic configuration of trivalent cobalt (Cotton *et al.,* 1972). Kinetically inert transition metal complexes undergo the water exchange reaction relatively slowly with a half-life of about 24 h. (Kettle *et al,* 1969). The biological consequences of kinetic inertness is that Co(III) complexes will remain intact when added to a culture medium or injected to animals and should arrive at their cellular target with their ligand configuration intact.

A strategy was devised based on both topoisomerase II inhibition and DNA binding to design highly potent anticancer derivatives of cobalt salicylaldoxime. To improve topoisomerase II antagonism by the salicylaldoxime complex of cobalt, the approach was to substitute the hydroxyl group on the imine nitrogen of the salicylal groups (which was shown to be important for topoisomerase II poisoning by CoSAL) with amino, thiosemicarbazone, phenyl, amino phenyl and dinitro substituted amino phenyl groups. This was done to retain the salicylal backbone which was found to be necessary for DNA

interaction; the imine nitrogen substitutions would effectively change topoisomerase II interaction by the new cobalt complexes which should result in increase or decrease of topoisomerase 11 poisoning and the corresponding anticancer activity.

The results from *in vitro* antiproliferation studies of these cobalt complexes show that the phenyl derivatives of CoSAL inhibit 50% of cell poliferation at 35, 30 and 20 μM concentrations of CoPSI, CoAPSI and CoDNPSI respectively. But the other two derivatives CoASI and CoTSSC requires >75 μM. This suggests that the imine nitrogen substitutions play an important role in the activity of these complexes. This point was strengthened when the ability of these complexes to inhibit the DNA relaxation activity of topoisomerase II was tested. The phenyl derivatives CoPSI, CoAPSI and CoDNPSI inhibited the DNA relaxation action of topoisomerase II at 200, 175 and 150 μM respectively while CoASI and CoTSSC could not inhibit the same at any concentration tested. The ATPase assay also showed a similar inhibition profile of the ATPase activity of topoisomerase II in presence of these drugs. The three phenyl derivatives seem to inhibit this activity through the formation of the enzyme-drug-DNA cleavage complex as shown by the cleavage assay. This was further confirmed by the immunopricipitation assay which showed 35, 40 and 48% of CoPSI, CoAPSI and CoDNPSI in the drug induced topoisomerase II-DNA cleavage complex.

To find out the possible molecular mechanism of action of these analogues on topoisomerase II, the DNA binding and computer-aided molecular modeling analysis were carried out. The DNA binding studies of these complexes show different interaction modes for the two sets of complexes. The curve width analysis of the DNA T_m curves in presence of these complexes show that CoASI and CoTSSC possibly bind to DNA

externally, ie. they interact outside the DNA helix without affecting the conformation of DNA. This was verified by the CD spectral analysis which showed a negligible change in the CD spectra of DNA in presence of these two complexes. The absorbance spectra of the two complexes in the visible region in presence of DNA show no change in the absorbance maxima. The phenyl derivatives however bind more strongly to DNA and show a curve width much higher than CoASI and CoTSSC. They also induce a significant hyperchromic shift in the CD spectra of DNA. The absorbance spectra of these complexes doped with increasing concentrations of DNA shows a decrease in the absorbance maxima. The scatchard plot analysis of the phenyl derivatives shows a two mode interaction, one seems to be an intercalative mode at low r value and the other seems to be an electrostatic interaction at the higher r value. This along with the CD spectral data and the curve width analysis suggest a weak intercalative DNA interaction by these complexes. This difference of DNA interaction by the phenyl derivatives compared to the amine and the thiosemicarbazone derivatives definitely seems to be induced by the phenyl groups.

The differential inhibition of topoisomerase II by the complexes could be explained in terms of the three-dimensional structures of these complexes by.

- charge around the central metal atom.
- size of their ligand
- distance between the metal center and the possible enzyme interacting groups of the metal complex.

Molecular modeling analysis of the cobalt complexes showed that the two salicylal groups in CoTSSC and CoASI are oriented in a single plane opposite to each other around the central cobalt atom. In CoTSSC, two co-ordinations are occupied by thiosemicarbazone

groups which form pentacyclic rings oriented at 120 degrees to the salicylal backbone. In CoASI, CoPSI, CoAPSI and CoDNPSI there is a single acetate co-ordination, a possible leaving group. In CoASI, CoPSI and CoAPSI, the nitrogen on each salicylal group is covalently bonded to amine, phenyl and amino phenyl groups which are oriented in opposite directions of the salicylal backbone in different planes. In case of CoDNPSI, the nitrogens on the salicylal groups are covalently linked to 2,4-dinitro amino phenyl groups. The introduction of the phenyl rings in CoPSI and CoAPSI imparts a conformational change in the salicylal groups, orienting them in two slightly different planes. In CoDNPSI, due the presence of the nitro groups, there is an enormous change in the salicylal conformations which orient themselves in two distinct planes.

In CoASI, the electronegative NH_2 groups render a partial negative charge on the cobalt atom. This could prevents a strong interaction by the molecule to the negatively charged DNA. In case of CoTSSC, the cobalt atom is essentially uncharged. The rings formed by the thiosemicarbazone groups may sterically hinder a strong interaction of the cobalt atom to DNA. The planar salicylal groups in both the complexes may not involve in any interaction with DNA. But in the phenyl derivatives, the presence of the phenyl rings decreases the negative charge on the cobalt atom compared to CoASI and CoSAL rendering a partial positive charge on the metal atom. This could result in an ionic or covalent association of the complex with DNA through the cobalt atom. The proximity resulting due to this interaction may enable a stacking interaction of one of the phenyl rings of the complex with DNA bases which could lead to an intercalative mode of DNA binding by these complexes.

CoASI does not poison topoisomerase II activity probably because the amino groups may not interact with the enzyme as strongly as the hydroxyl groups of CoSAL (Jayaraju et al., 1999). Also, the distance of the hydroxyl oxygen from the cobalt atom in CoSAL is 2.9 A^0 while in CoASI, the distance of the amino nitrogen from the metal atom is only 2.0 A^0. Due to this, the DNA bound drug may not be able to interact with the enzyme with the amino group. In CoTSSC, the amino groups of the thiosemicarbazones co-ordinate to the cobalt atom and are hence unavailable for enzyme interaction. In both CoASI and CoTSSC, the salicylal groups do not seem to exert any influence either on DNA binding or enzyme interaction due to their orientation in a single plane. The phenyl derivatives poison topoisomerase II in the order of CoPSI<CoAPSI<CoDNPSI. This could be definitely due to the presence of the phenyl ring. On the one hand, the distance of the phenyl groups from the metal atom is clearly more compared to CoASI and CoSAL which could account for a greater interaction with the enzyme. On the other hand, the phenyl groups (especially the DNP-phenyl) change the ligand environment significantly, which could favor a stronger interaction with DNA and enzyme, thus increasing the potency of cleavage complex formation compared to CoSAL.

The above data suggests that the phenyl derivatives of cobalt salicylaldoxime show a bidirectional interaction with DNA and topoisomerase II by sandwiching between the two. The cobalt atom and one of the phenyl rings may bind to DNA electrostatically and through intercalation respectively while the other phenyl ring and the salicylal groups may interact with topoisomerase II. This could effectively result in a stable cleavage complex comprised of the enzyme, drug and DNA.

CONCLUSIONS

1. The work on the known anticancer metal complexes, cisplatin and copper salicylaldoxime (CuSAL) suggests that topoisomerase II antagonism may be one of the possible mechanism for their anticancer activity.
2. The metal center plays a very important role in topoisomerase II poisoning as seen by the mechanism of topoisomerase II inhibition by CuSAL and cobalt salicylaldoxime (CoSAL). Though both drugs have the same ligand (salicylaldoxime), CuSAL forms a non-covalent cleavage complex while CoSAL forms covalent cleavage complex by poisoning enzyme activity.
3. In CoSAL, the -OH group of oxime is involved in topoisomerase II inhibition. This was revealed by the amine, semicarbazone and thio semicarbazone derivatives of the complex, where the -OH group is replaced. These drugs could not inhibit the enzyme activity
4. When the -OH was substituted with strong interacting groups (phenyl, phenyl amine and dinitro phenyl amine), the enzyme inhibition was increased. This suggests that large interacting domains increase the topoisomerase II inhibition by the cobalt complexes.

5. The studies reveal that two domains are required for topoisomerase poisoning by the cobalt complexes - A DNA binding domain and enzyme interacting domain.
6. The studies on the cobalt metal complexes as potential topoisomerase II poisons open a new avenue for designing potent anticancer drugs possessing a metal center.

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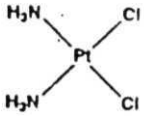
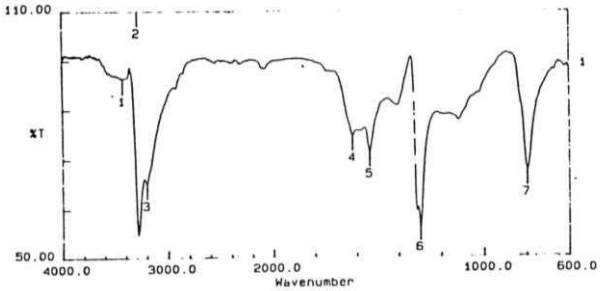
Spectrum I

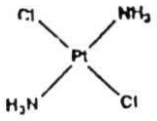
IR spectra of cisplatin

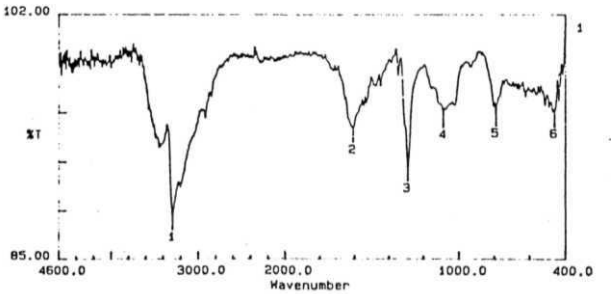




Spectrum II

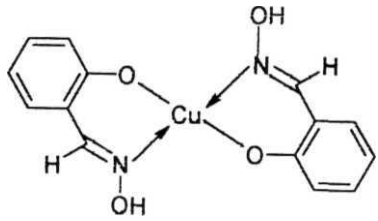
IR spectra of transplatin

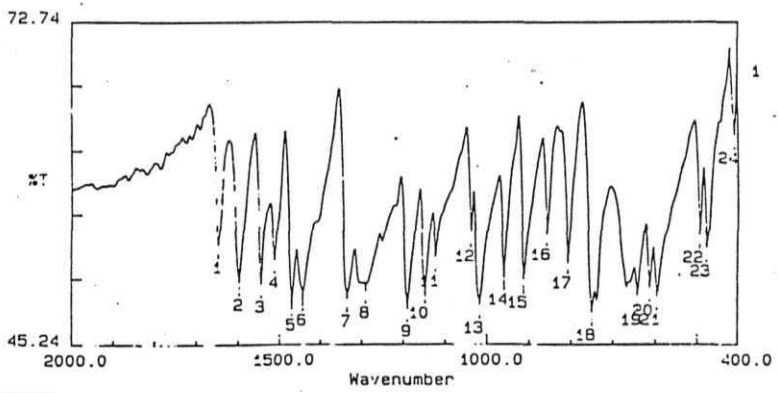




Spectrum III

IR spectra of Copper (II) salicylaldoxime





Condition

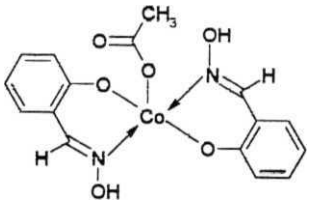
upper 72.74 lower 45.23 depth 2.00

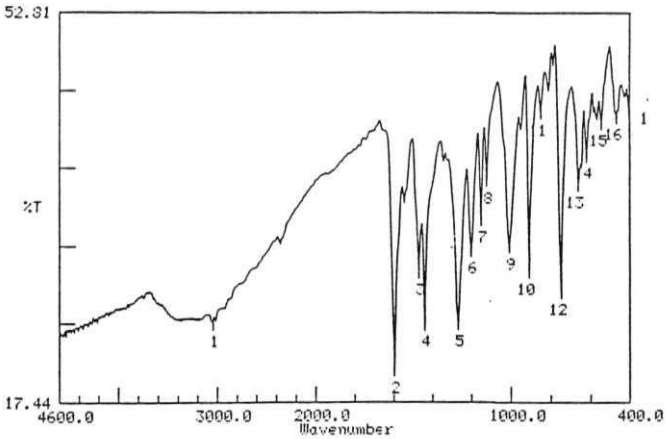
Peak table

1:	1647.36 (54.2)	2:	1597.20 (51.0)	3:	1545.12 (50.9)	4:	1512.33 (52.9)
5:	1469.89 (49.3)	6:	1442.88 (49.7)	7:	1334.86 (49.6)	8:	1290.49 (50.3)
9:	1190.19 (48.8)	10:	1147.75 (50.3)	11:	1122.67 (53.0)	12:	1037.80 (55.1)
13:	1016.58 (49.1)	14:	958.71 (51.4)	15:	912.41 (51.2)	16:	856.47 (55.2)
17:	806.32 (52.8)	18:	750.38 (48.5)	19:	642.35 (49.8)	20:	613.42 (50.5)
21:	596.06 (49.8)	22:	493.82 (55.0)	23:	476.46 (53.8)	24:	408.95 (63.5)

Spectrum IV

IR spectra of Cobalt (HI) salicylaldoxime





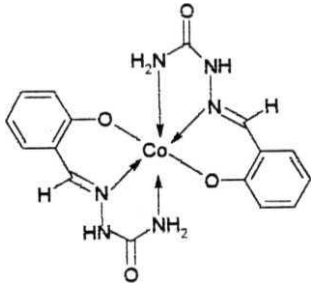
Condition
upper 52.80 lower 17.44 depth 2.00

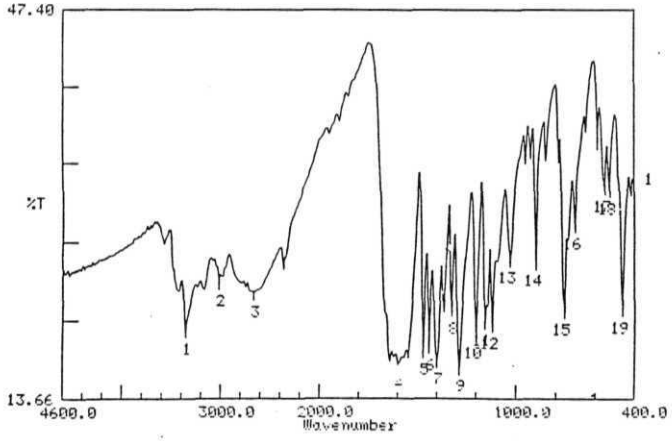
Peak table

1:	3049.73(	24.6)	2:	1599.13(	20.4	3:	1471.82(	29.4)
4:	1442.88(	24.6)	5:	1269.28(	24.6)	6:	1203.69(	31.3)
7:	1151.61(	34.1)	8:	1124.60(	37.6)	9:	1010.79(	31.7)
10:	910.48(	29.4)	11:	850.68(	43.9)	12:	750.38(	27.5)
13:	663.57(	37.7)	14:	621.13(	40.0)	15:	545.90(	43.0)
16:	468.74(	43.3						

Spectrum V

IR spectra of Cobalt (II) salicylalsemicarbazone





Condition

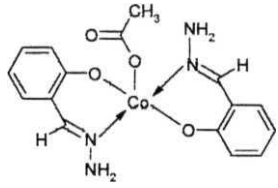
upper 47.39 lower 13.66 depth 2.00

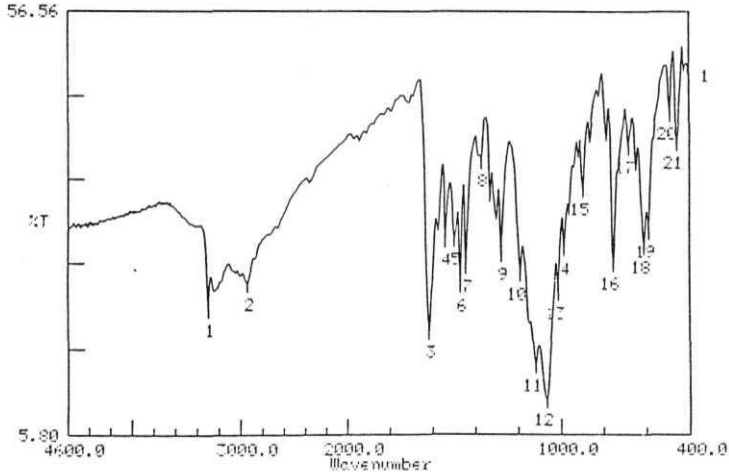
Peak table

1:	3360.30(19.7)	2:	3016.94(23.8)	3:	2663.93(22.8)
4:	1599.13(16.7)	5:	1469.89(17.9)	6:	1439.03(18.3)
7:	1400.45(17.1)	8:	1323.29(21.4)	9:	1286.64(16.5)
10:	1199.83(19.3)	11:	1155.46(20.4)	12:	1118.81(20.1)
13:	1028.15(25.7)	14:	888.31(25.4)	15:	758.09(21.7)
16:	702.15(21.7)	17:	549.76(31.8)	18:	524.69(31.6),
19:	461.03(21.1)				

Spectrum VI

IR spectra of Cobalt (III) *N*-aminosalicylaldimine





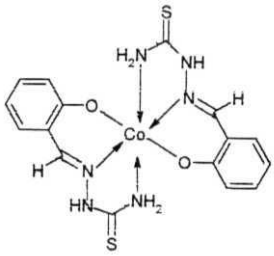
Condition
upper 56.56 lower 5.79 depth 2.00

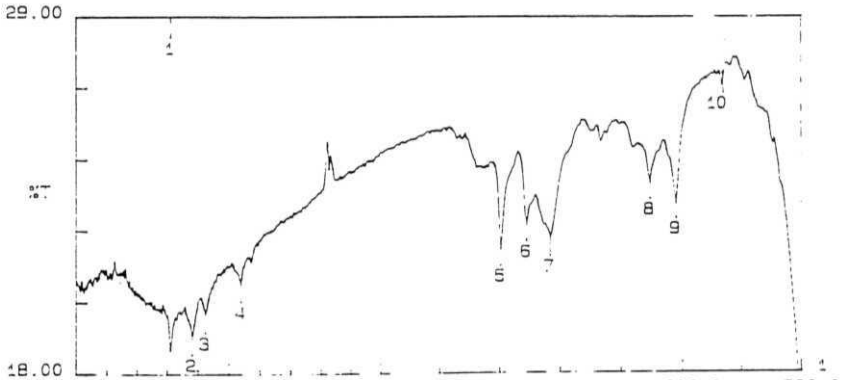
Peak table

1:	3294.71(20.7)	2:	2935.92(23.7)	3:	1618.42(18.1)
4:	1541.26(29.0)	5:	1500.75(29.1)	6:	1471.82(23.6)
7:	1446.74(25.8)	8:	1373.44(38.6)	9:	1280.85(27.3)
10:	1192.12(24.9)	11:	1120.74(14.1)	12:	1068.66(10.0)
13:	1012.72(22.7)	14:	985.71(27.9)	15:	895.05(35.1)
16:	754.23(26.2)	17:	682.86(40.1)	18:	609.56(28.2)
19:	588.34(30.1)	20:	488.03(44.7)	21:	457.17(40.7)

Spectrum VII

IR spectra of Cobalt (II) salicylalthiosemicarbazone





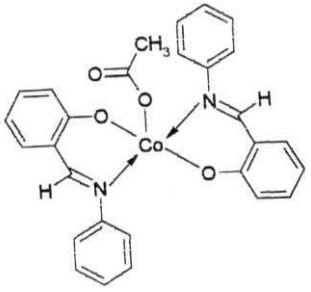
Condition
upper 29.00 lower 18.00 depth 2.00

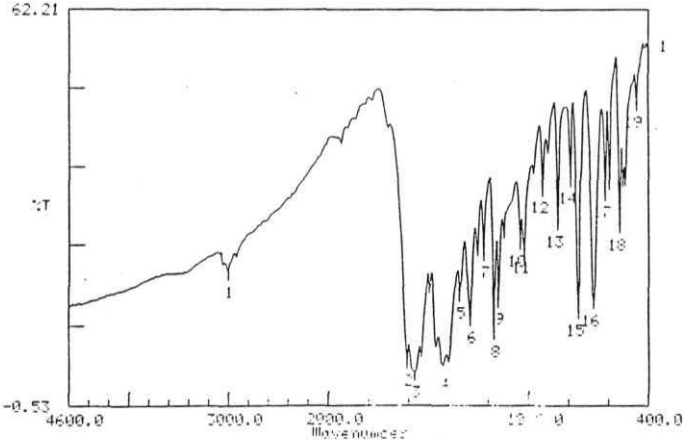
Peak table

1:	3377.66 (18.7)	2:	3238.77 (19.3)	3:	3151.97 (20.0)	4:	2922.42 (20.9)
5:	1597.20 (22.0)	6:	1510.40 (22.7)	7:	1433.24 (22.2)	8:	1105.31 (24.0)
9:	1020.44 (23.4)	10:	860.33 (27.3)				

Spectrum VIII

IR spectra of Cobalt (III)*N*-phenylsalicylaldimine





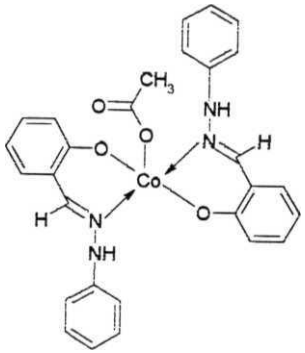
Condition
upper 62.21 lower -0.53 depth 2.00

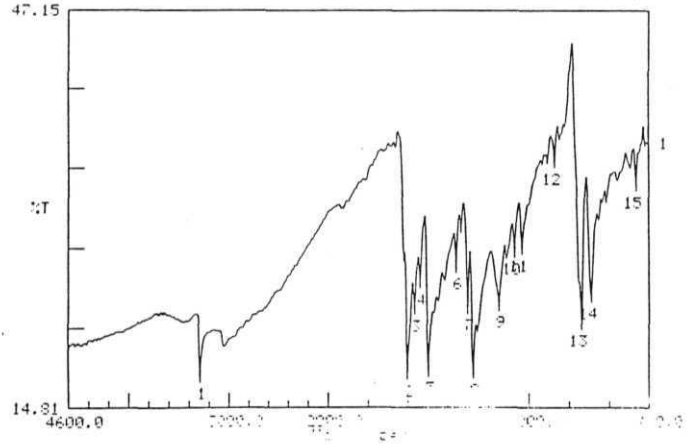
Peak table

1:	3011.15	20.3	2:	1612.64	6.5	3:	1572.13	4.7
4:	1427.45	6.0	5:	1344.51	17.1	6:	1292.42	13.2
7:	1224.91	23.4	8:	1174.75	11.3	9:	1151.61	16.1
10:	1043.58	25.1	11:	1024.29	24.2	12:	929.77	33.6
13:	856.47	28.1	14:	798.39	35.1	15:	752.31	14.4
16:	675.15	15.9	17:	817.13	72.9	18:	545.90	27.7
19:	457.17	47.7						

Spectrum IX

IR spectra of Coba.t (III)*N*-phenylaminosalicyl





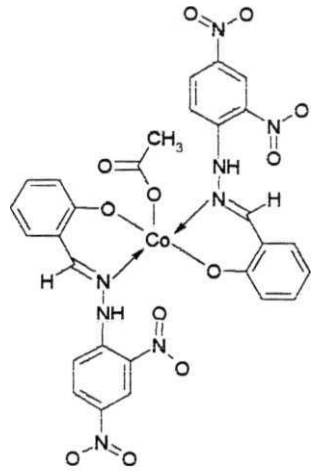
Condition

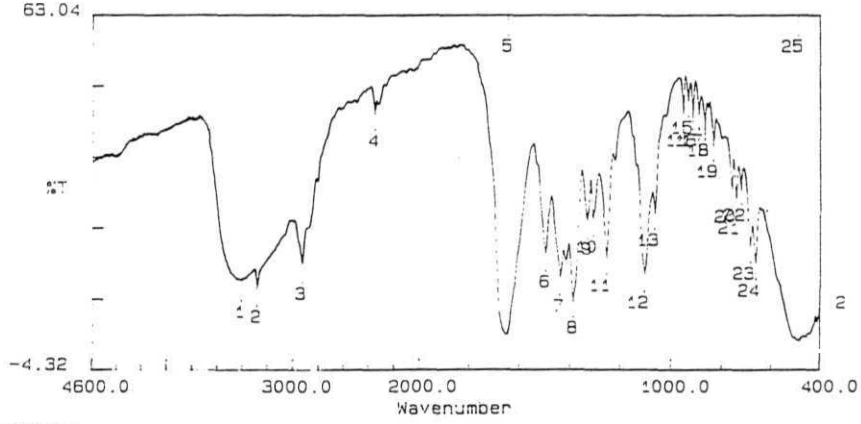
Peal table

1:	3290.66			1602.99	(17.7)	3:	1566.34 (23.0)
4:	1537.49	25.1		1[illegible].9	(18.0)	6:	1358.0 (26.4)
7:	1302.87	27.1		[illegible]	17.9)	9:	1147.15 (23.3
10:	1070.59	27.6		1033.34	(27.0)	12:	871.90 (34.9)
13:	778.10	21.		690.	(24.0)	15:	464.89 (33.0)

Spectrum X

IR spectra of Cobalt (III) (2-4, dinitro)*N*-phenylaminosalicylaldimine





Condition
upper 63.03 lower -4.32 depth 2.00

Peak table

1:	3414.31 (12.8)	2:	3287.00 (11.9)	3:	2934.00 (15.2)	4:	2361.08 (45.3)
5:	1651.22 (2.4)	6:	1496.90 (18.4)	7:	1439.03 (13.8)	8:	1386.94 (9.7)
9:	1331.00 (24.6)	10:	1307.85 (25.0)	11:	1253.84 (17.8)	12:	1103.38 (14.5)
13:	1062.87 (26.3)	14:	951.00 (45.3)	15:	931.70 (47.7)	16:	914.34 (45.3)
17:	889.25 (45.3)	18:	866.12 (43.4)	19:	831.39 (39.4)	20:	758.09 (30.8)
21:	742.66 (28.6)	22:	721.44 (31.1)	23:	684.79 (20.0)	24:	663.57 (16.8)
25:	486.11 (1.3)						

List of Publications

1. Topoisomerase II is a Cellular Target for Anti-proliferative Cobalt Salicylaldoxime Complex.

   D. Jayaraju, Y. N. Vashisht Gopal and Anand K. Kondapi.

   Archives of Biochemistry and Biophysics, August 15 (1999) Vol. 368, No.2,

Papers under communication:

1. Single Strand DNA Cleavage by Topoisomerase II in Presence of Anticancer Copper Salicylaldoxime complex.

D. Jayaraju, Y. N. Vashisht Gopal and Anand K. Kondapi.

2. Topoisomerase II poisoning by the Structural Analogues of Cobalt Salicylaldoxime: Elucidation of Molecular Mechanism of Action through DNA interaction and Computer Simulation studies.

D. Jayaraju, Y. N. Vashisht Gopal and Anand K. Kondapi.

Publications Associated with:

1. Inhibition of Topoisomerase II Catalytic Activity by Two Ruthenium Compounds: A Ligand Dependent Mode of Action.

Y. N. Vashisht Gopal, D. Jayaraju, and Anand K. Kondapi.

Biochemistry, (1999) 38, 4382-4388