Anti-cancer copper salicylaldoxime complex inhibits topoisomerase II catalytic activity

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Topoisomerase II (topo II) is a potential cellular target for a number of anti-cancer drugs. To elucidate the possible mechanism for anti-proliferation activity of copper salicylaldoxime (CuSAL), which was earlier shown to effectively inhibit the L1210 leukaemia cell proliferation, effect of the drug on topo II relaxation activity was studied. The results show that the relaxation activity of topo II was completely inhibited at 300 μM CuSAL concentration. To characterize the mode of inhibition by CuSAL, drug-induced topo II cleavage assay was conducted. The results show that CuSAL inhibits the enzyme activity through induction of enzyme-linked single-strand breaks in the DNA. Our data suggest that CuSAL may inhibit topo II dimerization, which may lead to the formation of single-strand breaks.

TOPOISOMERASE II (topo II) is a nuclear enzyme which is crucial for resolving DNA knots in the chromosomes during replication, transcription and cell division during chromosome segregation. It is a 170 kDa protein, which is a homodimer in its active form. The enzyme catalyses a number of ATP-dependent DNA topoisomerization reactions, including relaxation of both positive and negative supercoiled DNA, catenation/decatenation and knotting/unknotting via a double-strand-passage mechanism[1–5].

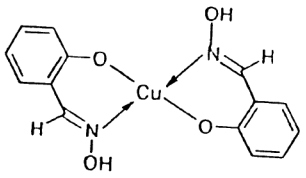
Topo II is one of the highly expressed enzymes in cancerous cells. Many clinically useful anti-tumour drugs target this enzyme[6]. Topo II-targetting drugs are broadly classified into two groups, viz. topo II poisons and topo II inhibitors[7]. The major class of the topo II-targetting drugs induce the enzyme to form single- and double-strand breaks and turn the enzyme into a DNA-damaging agent, e.g. m-AMSA, etoposide, etc. Such drugs interfere with the enzyme activity through the formation of a cleavage complex containing DNA–drug–enzyme, and are known as topo II poisons. Another class of drugs inhibits the catalytic activity of topo II without aiding the cleavage complex formation and is known as topo II inhibitors, e.g. quinolones and coumarins.

Copper complexes are known to have a broad spectrum of biological actions[8]. Many copper complexes are used as anti-inflammatory, anti-arthritic, anti-ulcer, anti-convulsant and anti-tumour agents[9,10]. It has been shown that copper accumulates in tumours due to the selective permeability of cancer cell membranes to copper compounds[11]. Because of this, a number of copper complexes have been screened for anti-cancer activity and some of them were found active both *in vivo* and *in vitro*[12]. Recently, it was shown that copper complex of 2-furaldehyde oxime poisons topo II activity in a manner similar to etoposide and teniposide, and shows better activity than these drugs by blocking the phosphorylation activation of topo II[13].

In the present study, we report an anti-tumour copper salicylaldoxime (CuSAL) complex which induces topo II to form single-strand nicks in DNA and poisons its activity, which could be one of the possible mechanisms for the anti-cancer activity of this complex.

Topo II was purified from rat testis by following the procedure of Galande and Muniyappa[14]. The concentration was determined by colorimetric assay of Bradford[15]. The negatively-supercoiled pBR322 DNA was prepared as described by Wang and Rossman[16]. [γ ^{32}P] ATP was supplied by BARC, India. PEI-Cellulose-F sheets were from Merck. M-AMSA, etoposide and calf thymus DNA from Sigma. NADH, pyruvate kinase, lactate dehydrogenase and phosphoenol pyruvate were from Boehringer–Mannheim. Other chemicals and biochemicals used were of standard grade.

The complex was synthesized according to the procedure given by Lumme *et al.*[17]. 0.745 g (10 mmol) of salicylaldoxime 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) diacetate monohydrate in 50 ml of 0.01 M HCl was added slowly. The solution turned greyish-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. The stoichiometry of the compound obtained was analysed using CHN analysis and atomic absorption spectrometry; the results verify well with the chemical structure given in Scheme 1.



Scheme 1. Chemical structure of CuSAL.

The coordination of the imine nitrogen atom to the Cu(II) ion is indicated by the displacement of the band assigned to the ν(C=N) stretching vibration. The spectrum (Figure 1) of the complex exhibits downward shift of ν(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 ν(M–O) bond, confirming the complex formation.

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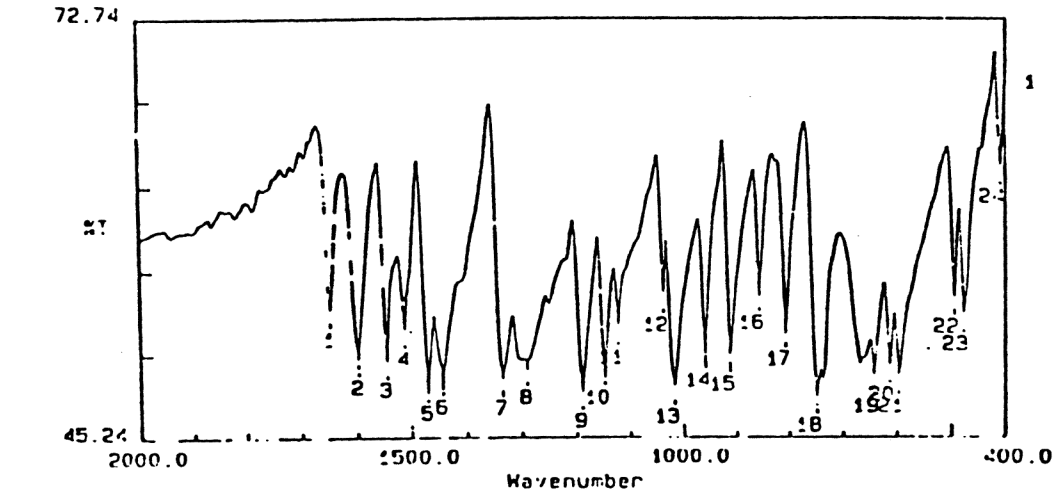
For the enzyme-catalysed relaxation assay, the reaction mixture of 20 µl contained 50 mM Tris-HCl pH 8.0, 120 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 30 µg/ml BSA, 1 mM ATP and 0.6 µg of negatively-supercoiled pBR322 plasmid DNA. The reaction was initiated by addition of topo II to the final concentration of 8 nM and incubated at 30°C for 20 min. The reaction was stopped by addition of SDS to the 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-HCl pH 8.0) was added, and the products were separated on 1% agarose gel in 0.5 × Tris-acetate buffer (40 mM Tris acetate pH 8.3 and 2 mM EDTA) at 50 V for 16 h. The gel was stained in ethidium bromide (10 µg/ml) and visualized under UV and photographed.

In the double-strand DNA cleavage reaction, the assay was done using the method of Lee *et al.*[18]. The reaction was performed in 20 µl of solution containing 10 mM Tris-HCl pH 7.0, 10 mM $MgCl_2$, 50 mM KCl, 1 mM ATP, 50 µg/ml BSA, 0.3 µg of pRYG supercoiled plasmid and 5 units of topo II. The reaction mixture was incubated at 30°C for 10 min and the reaction was terminated by adding SDS to 1% and proteinase K to 100 µg/ml. Incubation was continued at 45°C for 30 min, followed by the addition of 3 ml of 0.67% SDS, 67 mM EDTA, 26.7% sucrose, 0.067% bromophenol blue and 0.067% xylene cyanol FF.

For single-strand DNA cleavage reaction, the procedure of Lee *et al.*[18] was adopted. Briefly, the reaction was performed in 20 µl of solution containing 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 µM KCl, 200 mM NaCl, 12 mM EDTA, 1 mM AMP–PNP, 50 µg/ml BSA, 0.3 µg of pRYG supercoiled plasmid and 5 units of topo II. The reaction mixture was incubated at 30°C for 10 min and terminated by adding SDS to 1% and proteinase K to 100 µg/ml.

The ATPase activity of DNA topo II was measured by two methods. In the TLC assay, the reaction mixture of 20 µl containing the assay buffer (as given in relaxation assay) with 1 mM [γ^{32}P] ATP (0.025 Ci/mmol) and 8 nM of topo II and 0.6 µg of pBR322 supercoiled DNA was incubated at 30°C for 15 min. The reaction was stopped by adding 2 µl of 250 mM EDTA and kept on ice. The reaction mixture was spotted on cellulose plastic sheets coated with polyethyleneimine (DC-Plastikfolien PEI-Cellulose F, Merck) and chromatographed in freshly-made 1 M lithium chloride. The bands were monitored with reflecting UV at 366 nm in a Photodyne gel documentation system. In the lithium chloride solution, $^{32}P_i$ migrates first followed by ADP and ATP. The areas corresponding to the reaction products were cut out of the chromatogram and counted in a Wallac scintillation counter using toluene-based scintillation fluid[19].

In the spectrophotometric assay, rapid conversion of ADP back to ATP by pyruvate kinase and phosphoenolpyruvate, a reaction coupled to NADH oxidation, was used to measure the rate of ATP hydrolysis. The procedure described by Morrical *et al.*[20] was followed. Under steady-state conditions, the rate of ATP hydrolysis is directly proportional to the rate of the decrease in absorbance observed at 340 nm. The assay was performed in a



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:	1199.19 (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:	750.30 (48.5)	19:	642.35 (49.8)	20:	613.42 (50.5)
21:	596.06 (49.0)	22:	493.82 (55.0)	23:	476.46 (53.8)	24:	408.95 (63.5)

Figure 1. Infrared spectrum of CuSAL.

Shimadzu UV-650A spectrophotometer. The reaction mixture of 1 ml containing reaction buffer (20 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 and 8 nM topo II for 30 min and absorbance was recorded at 340 nm.

For DNA–CuSAL binding studies the following methods were adopted: (i) Melting temperature studies were done by dissolving calf thymus DNA 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 mM). This DNA was used in melting temperature studies in the presence of drugs. The concentration of metal complexes was adjusted such that CuSAL 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 CuSAL–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 minute and the increase in absorbance was recorded. The absorbance was recorded from 40 to 90°C. T_m was determined from these absorbance values and the data were plotted.

(ii) Circular dichroic spectra of pBR322 DNA (20 μg) were monitored in the presence of 20 mM of CuSAL in a Jasco J-715 spectropolarimeter. The DNA and CuSAL concentrations proportionately 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. Two mM of m-AMSA corresponding to 75 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 were represented graphically as molar ellipticity ($[\theta]*10^{-3}$ deg-cm^{-2}/dmol) versus wavelength (nanometres).

Figure 2 depicts the results of an experiment in which the relaxation activity of topo II in the presence of increased concentrations of inhibitor was examined. CuSAL shows complete inhibition of relaxation activity of topo II at 300 μM CuSAL concentration (lane 11).

Double-stranded DNA cleavage reaction was carried out to see if CuSAL could form CuSAL-induced ternary complex of DNA–drug–topo II, called the cleavage complex. If this ternary complex involves cleavage of both the strands of DNA, the proteinase K treatment would lead to the formation of a linear DNA. If CuSAL can only induce single-stranded cleavage, it would facilitate the formation of nicked, circular DNA as intermediate. The results of double-stranded DNA cleavage reaction as in Figure 3, suggest that CuSAL may not induce double-strand breaks in DNA, as it is evident from the absence of linear DNA formation.

To examine whether CuSAL induced a single-strand cleavage by topo II, we have conducted single-strand cleavage reaction according to the procedure of Lee *et al.*[18]. Figure 4 *a* suggests the formation of nicked, circular DNA by topo II in the presence of CuSAL. Further to verify the presence of CuSAL-induced DNA protein cross-links, we have conducted the experiments in the presence of 200 and 250 mM of CuSAL, with and without proteinase K treatment. The results of these experiments (Figure 4 *b*) clearly suggest that CuSAL induces DNA–protein cross-links. These cross-links could be single-stranded DNA-specific.

It has been shown that some of the topo II-targetting drugs induce the enzyme-linked single-strand breaks due to the formation of a 'non-covalent cleavage' complex[18]. This type of single-stranded cleavage complex formation

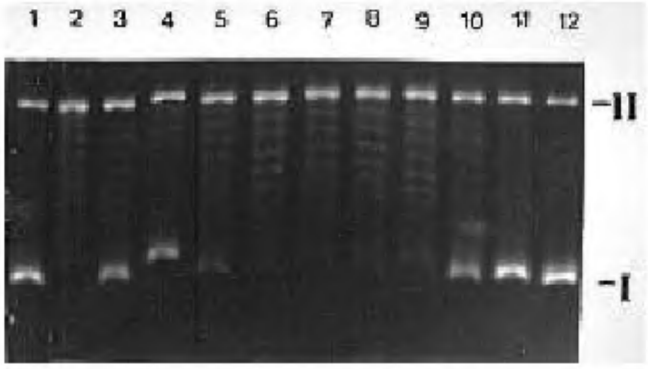


Figure 2. Effect of CuSAL on topo II-catalysed DNA relaxation activity. Supercoiled pBR322 DNA (lane 1) was incubated with topo II in the absence (lane 2) or presence of 50 and 100 μM m-AMSA (lanes 3 and 4) and 50, 100, 150, 200, 250 and 300 μM CuSAL (lanes 5–11), without enzyme but with 300 μM CuSAL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

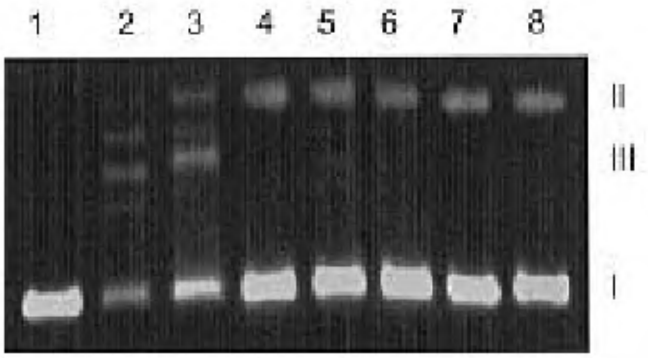


Figure 3. Double-stranded DNA cleavage mediated by topo II in presence of CuSAL. Double-stranded DNA cleavage reaction was carried out as described in the text with increasing concentration of CuSAL. Reaction product after proteinase K treatment was analysed by 1% agarose gel in presence of rhidium bromide at 0.5 μg/ml. Supercoiled DNA (lane 1) with topo II in the absence (lane 2) and presence (lane 3) of 100 μM etoposide, and 50, 100, 150, 200 and 250 μM CuSAL (lanes 4–8). Labels beside gel denote supercoiled (I); nicked, circular (II); and linear (III).

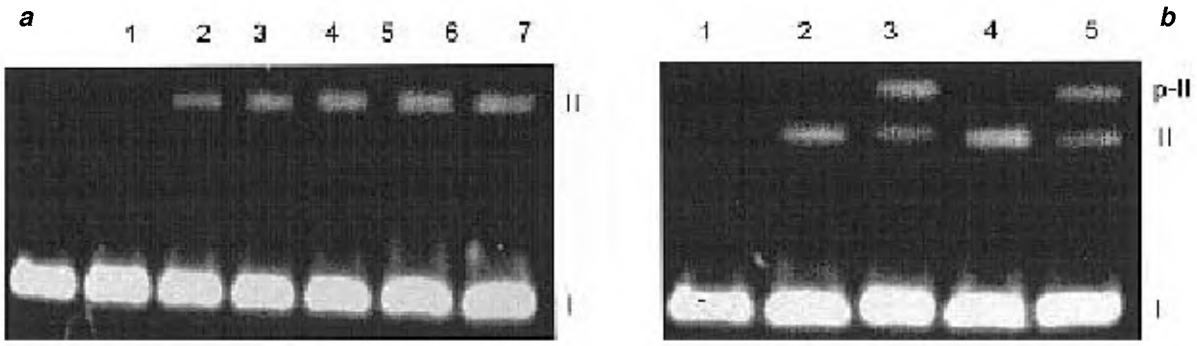


Figure 4. *a*. Single-strand DNA cleavage mediated by topo II in presence of CuSAL. Single-stranded DNA cleavage reaction was carried out as described in the text with increasing concentration of CuSAL. Reaction products after proteinase K treatment were analysed by 1% agarose gel in the presence of ethedium bromide at 0.5 µg/ml. Supercoiled DNA (lane 1) with topo II in the absence (lane 2) and presence of 50, 100, 150, 200 and 250 µM CuSAL (lanes 3–7). Labels beside gel denote supercoiled (I); nicked, circular (II); and protein–DNA crosslinks (p-II); *b*, Supercoiled DNA (lane 1) with topo II in the presence of CuSAL, 200 µM (lanes 2 and 3) and 250 µM (lanes 4 and 5). DNA samples treated with proteinase K (lanes 2 and 4) or without proteinase K (lanes 3 and 5). Labels beside gel denote supercoiled (I); nicked, circular (II); and protein–DNA crosslinks (p-II).

can be seen in the presence of m-AMSA and some quinolone derivatives under special reaction conditions[21]. Another possibility is that the cleavage reaction performed in the presence of drugs which can disrupt the coordination between the topo II monomers (in dimer formation) induces this non-covalent cleavage complex, similar to that of topo I cleavage complex[18].

Drugs like genistein and flavon-based compounds that enhance DNA breakage also strongly inhibit the ATPase activity of topo II[22]. To test whether CuSAL also interferes with the ATP hydrolysis action of the enzyme, 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 5 *a*). We also carried out a spectrophotometric assay (pyruvate-coupled) to examine the inhibition of topo II-induced ATP hydrolysis by CuSAL. The results of this assay correlate well with those of the TLC assay; 300 µM CuSAL could inhibit ~ 85% of DNA-stimulated ATP hydrolysis (Figure 5 *b*). Further, when the assay was conducted at 400 µM, there was no significant change in inhibition of DNA-stimulated ATP hydrolysis of the enzyme.

The following DNA-binding studies were made. (i) DNA melting temperature experiments were carried out by incubating calf thymus DNA with increasing concentrations of CuSAL. The absorbancy of nucleotide bases was monitored at 260 nm with increasing temperatures, which shows that CuSAL protects melting of calf thymus DNA (Figure 6 *a*). CuSAL increases the T_m of DNA from 57° to 65°C at a stoichiometric ratio of 5 nucleotides per 2 CuSAL molecules (Figure 6 *b*). The concentration of nucleotide was calculated based on the absorbancy measurements.

The curve widths of the T_m curves were measured at different CuSAL to nucleotide ratios according to Kelly *et al.*[23] and the data were plotted, which shows that

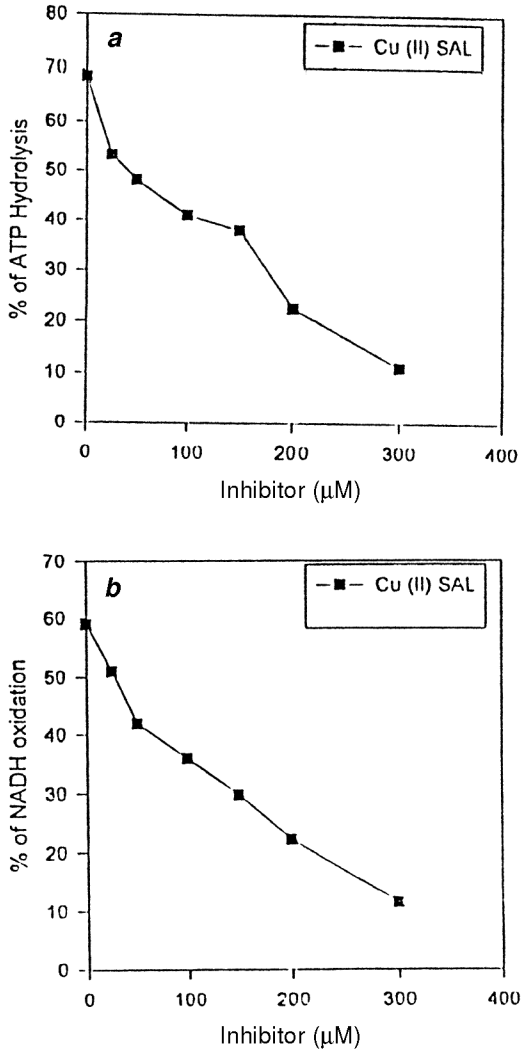


Figure 5. Inhibition of ATPase activity of topo II by CuSAL. ATP hydrolysis in presence of increasing concentration of inhibitors is presented as mean of three experiments. *a*, Percentage of ATP hydrolysed versus concentration of CuSAL in µM; and *b*, percentage of NADH oxidation versus concentration of CuSAL in µM.

CuSAL possesses low affinity in DNA interaction (Figure 6 *c*). The increase in T_m by 8°C may be due to weak interaction of the central metal ion with the anionic DNA phosphate. This could be also due to the presence of non-specific interaction of CuSAL with the DNA phosphate.

(ii) The circular dichroic spectrum of DNA in presence of CuSAL shows that CuSAL does not induce any change in the spectrum (Figure 6 *d*). This suggests that CuSAL does not induce any conformational change in DNA through its weak interaction.

Copper is one of the trace elements present in living systems[24]. It is a component of several enzymes and copper compounds are 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 anti-tumour agents were extensively investigated[25].

In this study, we present one of the possible mechanisms responsible for the anti-tumour activity of a salicylaldoxime complex of copper, CuSAL. The ligand salicylaldoxime structurally resembles pyridoxal, a component of vitamin B_6. Salicylaldoxime is an extremely powerful inhibitor of pyridoxal kinase[26]. Vitamin B_6 deficiency and B_6 antagonists are known to have growth-inhibitory and anti-tumour activity[27]. Since the side effects of CuSAL (weight loss and acrodynia) resemble those of B_6-deficiency and B_6-antagonism[28], it appears

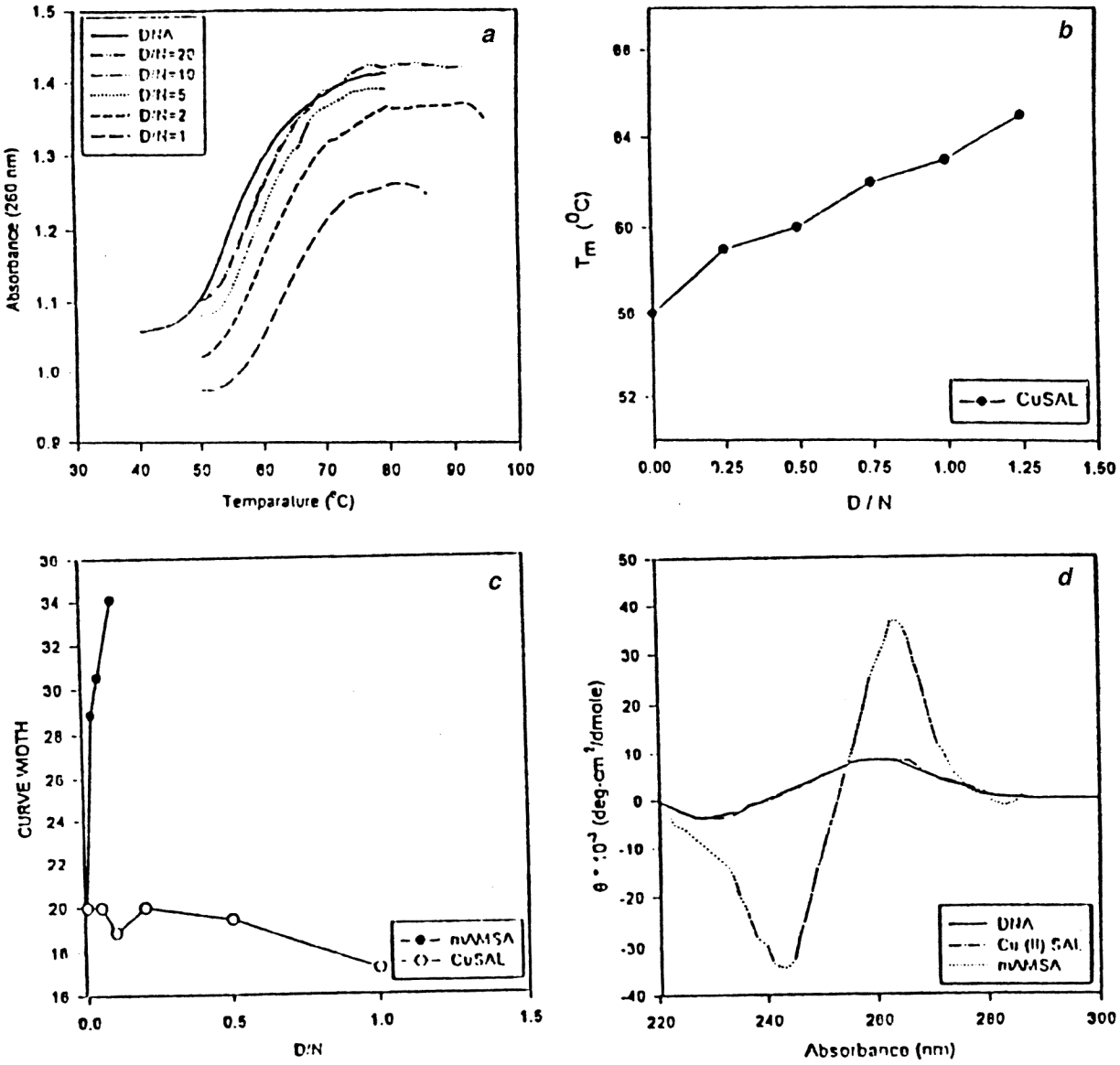


Figure 6. CuSAL–DNA binding studies. *a*, CuSAL increases the T_m of calf thymus DNA from 57°C for DNA control (—) to 59, 60, 62, 63 and 66°C for DNA nucleotide to CuSAL ratios of 20 : 1 (- · · -), 10 : 1 (-· -), 5 : 1 (····), 2 : 1 (---) and 1 : 1 (- -), respectively; *b*, *D/N* (CuSAL/nucleotide) was plotted against increase in T_m by CuSAL (●) to determine specific CuSAL 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 (○) 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 spectra. But m-AMSA (····) shows a very prominent change at a concentration less than 5 times that of the copper complex.

that pyridoxal antagonism might be involved in the mechanism of action of CuSAL. However, the pyridoxal theory alone cannot explain the different antiproliferative activities of copper, cobalt and nickel salicylaldoxime complexes[29].

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[30–32]. Topo 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 topo II poisons[33]. Nelson *et al.*[34] have reported that covalent complexes of pulse-labelled DNA with topo II can be trapped by the topo II inhibitor, tenopiside, in a 'cleavage complex'. Our results show that CuSAL induces single-stranded protein-associated DNA breaks like DPC-forming agents, where the protein linked 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 coordination between the two monomers of the dimeric topo II or those which bind to the ATP-binding site of the enzyme. The intensity of the nicked, circular DNA was increased with increasing CuSAL concentration, suggesting that CuSAL could act like the topo II-targetting drugs which inhibit topo II activity by either disrupting the topo II dimer formation or by binding to ATP-binding site of the enzyme.

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

To analyse if CuSAL causes DNA cleavage complex formation by bi-directionally interacting with DNA and topo II or only by interacting with the enzyme, we conducted the DNA-binding studies. Our results from these studies show that the CuSAL protects melting of calf thymus DNA to a small extent (8°C) through a weak interaction with the DNA. This interaction may stabilize the ionic environment around the DNA, which protects melting of DNA to a certain extent. The CD spectral studies show that CuSAL does not change DNA conformation. These results suggest that CuSAL interacts only with topo II and forms the cleavage complex.

In summary, our results strongly suggest that CuSAL inhibits catalytic activity of topo II by formation of monomeric topo II cleavage complex. This may be taking place as CuSAL associated disruption of the topo II monomers to form the dimeric enzyme. This process may be involved in the formation of CuSAL-induced enzyme-linked single-stranded DNA breaks.

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