Topoisomerase II poisoning by indazole and imidazole complexes of ruthenium

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Trans-imidazolium (bis imidazole) tetrachloro ruthenate (RuIm) and trans-indazolium (bis indazole) tetrachloro ruthenate (RuInd) are ruthenium coordination complexes, which were first synthesized and exploited for their anticancer activity. These molecules constitute two of the few most effective anticancer ruthenium compounds. The clinical use of these compounds however was hindered due to toxic side effects on the human body. Our present study on topoisomerase II poisoning by these compounds shows that they effectively poison the activity of topoisomerase II by forming a ternary cleavage complex of DNA, drug and topoisomerase II. The thymidine incorporation assays show that the inhibition of cancer cell proliferation correlates with topoisomerase II poisoning. The present study on topoisomerase II poisoning by these two compounds opens a new avenue for renewing further research on these compounds. This is because they could be effective lead candidates for the development of more potent and less toxic ruthenium containing topoisomerase II poisons. Specificity of action on this molecular target may reduce the toxic effects of these ruthenium-containing molecules and thus improve their therapeutic index.

1. Introduction

DNA is a very dynamic molecule and during the lifetime of a cell, it constantly undergoes various topological changes without affecting its genetic makeup. Numerous topological problems like negative/positive supercoiling and catenation arise in DNA during replication and transcription, which causes intertwining of DNA. This intertwining is resolved by a class of enzymes called topoisomerases, which thus play important roles in maintaining genome integrity (Wang 1985, 1991, 1996; Watt and Hickson 1994; Pruss and Drlica 1986). These enzymes are also involved in decatenation of DNA in the G2 phase of cell division for separation of newly replicated chromatids (Downes *et al* 1994). In the M phase, they help in chromosome condensation and segregation (Adachi *et al* 1991). Of the two types of topoisomerases (type I and type II) the type II enzymes are most impor-

tant for cell cycle progression and survival of dividing cells. The catalytic cycle of topoisomerase II (topo II) typically involves breaking both strands of a duplex DNA segment, passing another duplex segment through the gate created by the broken DNA strands and finally resealing the broken strands (Berger *et al* 1996). This strand passage reaction is central to the various functions of topo II, as well as for targeting the enzyme by anticancer chemotherapeutics called topo II poisons (Froelich-Ammon and Osheroff 1995).

An earlier study by Jayaraju *et al* (1999) showed that a salicylaldoxime complex of cobalt (CoSAL) poisons the activity of topo II by cleavage complex formation. Molecular analysis implicated the oxime groups of the salicylaldoxime ligands as the topo II interacting moieties in the molecule. In a similar study, we found that replacement of the cobalt atom with a ruthenium atom (RuSAL), abolished the topo II poisoning ability (Gopal

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et al 1999). But there was no change in the structure of the molecule by replacement of the central metal atom; also, the metal atom in CoSAL or RuSAL was not shown to interact with topo II. This was surprising because the salicylaldoxime ligands in both compounds are similar, but only those attached to the cobalt atom show topo II poisoning. In order to examine whether other coordination complexes of ruthenium with similar ligand conformation are inert towards topo II, two anticancer coordination complexes of ruthenium have been tested for topo II antagonism. The first is RuIm, in which a ruthenium atom is coordinated to two imidazole ligands and is also bonded to 4 chloride atoms. The second is RuInd, which has indazole ligands in place of the imidazoles. The synthesis and anticancer activity of these metal complexes was first described by Keppler *et al* (1989). RuInd was reported to be a more potent anticancer agent compared to RuIm. Both RuIm and RuInd possess significant anticancer activity against the Walker 256 carcinosarcoma, MAC 15A colon tumour, B16 melanoma and solid sarcoma 180 (Pruss and Drlica 1986). These compounds were more superior in their action against an autochthonous chemically-induced colorectal adenocarcinoma in rats compared to even 5-fluorouracil, which is an established cytostatic drug against human gastrointestinal carcinomas. Fruhauf and Zeller (1991) observed that RuInd brings about antitumour activity by interacting with DNA and inhibiting DNA synthesis. The present work analyses the ability of these two complexes to poison topo II.

2. Materials and methods

2.1 *Materials*

Topo II was purified from rat liver following the procedure of Galande and Muniyappa (1996). The enzyme concentration was determined using Bradford (1976) colorimetric assay. The negatively supercoiled pBR322 plasmid DNA was purified as described by Wang and Rossman (1994). N-[4-(9-acrydinylamino)-3-methoxy-phenyl]methanesulphonamide (m-AMSA), calf thymus DNA, RPMI-1640 media were from Sigma (USA), fetal calf serum, trypsin and antibiotics were from Gibco-BRL, PEI (polyethylene imine) Cellulose-F TLC sheets were from Merck, Proteinase K and ATP were from Boehringer-Mannheim, $[g^{32}P]ATP$ and [³H]-labelled thymidine were supplied by BRIT, India. Other chemicals and biochemicals used were of analytical grade.

The two ruthenium coordination compounds, RuInd and RuIm were generous gifts from Prof. B K Keppler, University of Heidelberg. RuIm was solubilized in de-ionized water and RuInd was solubilized in 50% aqueous ethanol prior to use in the assays at pH 8. The studies of Ni Dhubhghaill *et al* (1994) suggest that [RuCl₄(Him)₂]⁻

undergo hydration between pH 2·4 to 10 with a half life of 3·4 h at pH 5·6. Since our reactions are incubated the maximum time period of 30 min at pH 8, the effect of hydration on the reaction may not be significant. Also, cyclic voltammetric studies of complex in the presence of relaxation buffer have not indicated any significant change in redox state of complex. CoSAL and RuSAL were synthesized using the prescribed protocols (Gopal *et al* 1999; Jayaraju *et al* 1999). These were solubilized in dimethyl sulphoxide prior to use. The chemical structures of the compounds are shown in figure 1.

2.2 *Thymidine incorporation assay*

[³H]thymidine incorporation assays were performed to analyse the effect of the ruthenium compounds on the proliferative response of human cancer cells. The procedure of Gopal *et al* (1999) was used for these assays. Colo-205 (colon adenocarcinoma) and ZR-75-1 (breast carcinoma) cells were used in these studies. The cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum. In 96 well microtitre tissue culture plates 0·2 ×
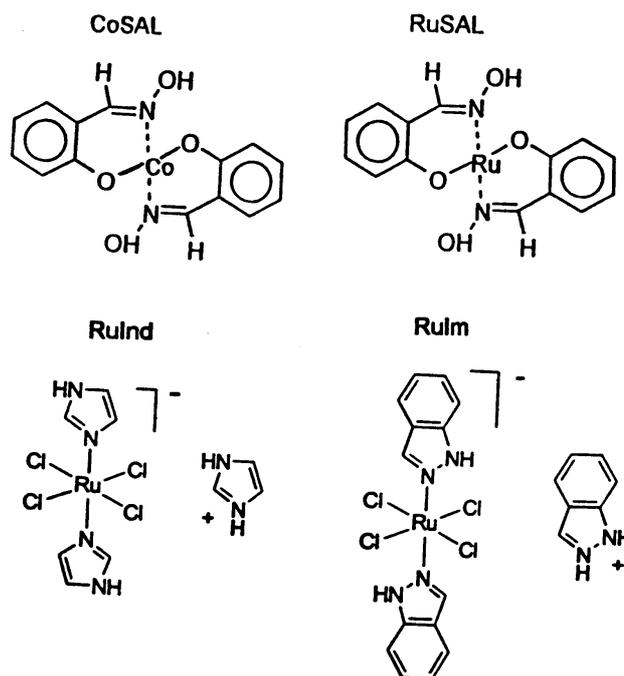

Figure 1. Chemical structures of CoSal, RuSAL, RuInd and RuIm. CoSAL and RuSAL have identical chemical structures, with different central metal atoms. The metal atoms are coordinated to two bidentate salicylaldoxime ligands. In RuInd and RuIm, the ruthenium atom is coordinated to two monodentate indazole (RuInd) and imidazole (RuIm) ligands. In chemically isolated form, the two molecules exist as anions bonded to a single cationic indazole or imidazole. These cationic ligands may be released from the main molecules in biological systems.

10^6 cells/200 μl were distributed in triplicates. The cultures were incubated for 16 h at 37°C in a CO_2 incubator (Forma Scientific) maintaining 5% CO_2 atmosphere. The ruthenium compounds were added to the cells at increasing concentrations from 10 to 60 μM. The DNA intercalating topo II poison, m-AMSA and RuSAL were used as drug controls. The drug treatment was stopped after 6 h by centrifugation and change of media. The cells were further incubated for 48 h. The cultures were then pulsed with 0·5 μCi of [³H]thymidine and incubation was continued for 4 h to allow thymidine incorporation by cells. After incubation, the medium was removed and the adhering cells were treated with 10 μl of trypsin-EDTA (0·25% trypsin, 1 mM EDTA) for 5 min at 37°C to release the cells from the adhering surface. Trypsinization was stopped by adding 10 μl of serum to the cells. The original cultures were added back to the wells and the cells were harvested on glass microfibre strips using a Skatron automated cell harvester. Radioactivity was measured in a Wallac liquid scintillation counter.

2.3 *Relaxation assay*

This assay was performed following the procedure of Osheroff *et al* (1983). The reaction mixture (20 μl) contained relaxation buffer [50 mM Tris-HCl (pH 8·0), 120 mM KCl, 0·5 mM EDTA, 0·5 mM DTT, 10 mM $MgCl_2$, 30 μg/ml BSA, 1 mM ATP], 0·6 μg of negatively supercoiled pBR322 plasmid DNA and increasing concentrations of the ruthenium drugs. The reaction was initiated by adding 2 units (~ 8 nmol) of topo II and incubated at 30°C for 15 min. The reaction was stopped by adding 2 μl of 10% SDS. To this, 3 μl of loading dye (0·5% bromo-phenol blue, 0·5% xylene cyanol, 60% sucrose, 10 mM Tris-HCl, pH 8·0) was added, and the products were separated on a 1% agarose gel in 0·5 × TAE buffer (20 mM Tris-acetate, 0·5 mM EDTA) at 50 V for 8 h. The gel was stained with ethidium bromide, visualized in a Photodyne UV transilluminator and photographed.

2.4 *ATPase assay*

This assay is a modified procedure of Osheroff *et al* (1983). The 20 μl reaction mixture contained relaxation buffer (1 mM ATP component contained 0·025 μCi [g^{32}P]ATP), 0·6 μg of pBR322 DNA and increasing concentrations of drugs. The reaction was initiated with 2 units of topo II and incubated at 30°C for 15 min. The reaction was stopped with 2 μl of 0·5 M EDTA. The reaction mixture was spotted on PEI cellulose-F TLC sheets and the sheets were subjected to thin layer chromatography in 1 M lithium chloride solution. In these conditions, [g^{32}P]$_i$ migrates first followed by ADP and [g^{32}P]ATP. After resolution, the bands were monitored under reflec-

ted UV light at 366 nm in a Photodyne transilluminator. The illuminated bands of ATP, ADP and P_i (inorganic phosphate) were cut out of the sheet and counted for [^{32}P] in a liquid scintillation counter.

2.5 *Cleavage assay*

The formation of cleavage complex was assayed following the procedure of Zechiedrich *et al* (1989). The 20 μl reaction mixture contained relaxation buffer (minus ATP), 0·6 μg of pBR322 supercoiled DNA and increasing concentrations of the drugs. The reaction was initiated by adding 10 units (40 nmol) of topo II, and incubated at 30°C for 15 min. The reaction was stopped with 2 μl of 0·5 M EDTA and 2 μl of 10% SDS. The DNA bound protein was degraded by incubating the reaction mixture with 2 μl of 1 mg/ml Proteinase K at 45°C for 1 h. The products were separated on 1% agarose gel for 8 h at 50 V in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA), stained and photographed.

3. Results

3.1 *Topoisomerase II antagonism by RuIm and RuInd*

3.1a *Inhibition of DNA relaxation activity*: Both metal complexes inhibit the supercoiled DNA relaxation activity of topo II. RuInd completely inhibits the DNA relaxation activity of topo II at a concentration of 250 μM, while RuIm inhibits the activity at a concentration of 300 μM. Figure 2 shows the dose dependent inhibition of topo II activity by these two drugs. The DNA intercalator, m-AMSA, poisons topo II at a concentration of 100 μM (lane 3 of figure 2). It also affects the migration of supercoiled DNA (form I DNA) because it is a strong DNA intercalator.

3.1b *Inhibition of the DNA stimulated ATPase activity of topoisomerase II*: The ATPase assay shows that RuIm and RuInd significantly inhibit the DNA stimulated ATP hydrolysis activity of topo II. A comparison of ATPase inhibition by the two complexes with RuSAL is shown in figure 3. These results correlate well with the inhibition of DNA relaxation activity by these complexes. RuInd inhibits 80% of the ATPase activity of topo II at the highest concentration tested, while RuIm inhibits 58% of the ATPase activity. RuSal inhibits only 12% of the ATPase activity.

3.1c *Formation of drug-induced, topoisomerase II mediated cleavage complex*: Consequent to the relaxation and ATPase inhibition by RuIm and RuInd, the next step was to check for the ability of the two complexes to freeze topo II and cleaved-DNA in a cleavage complex. Topo II
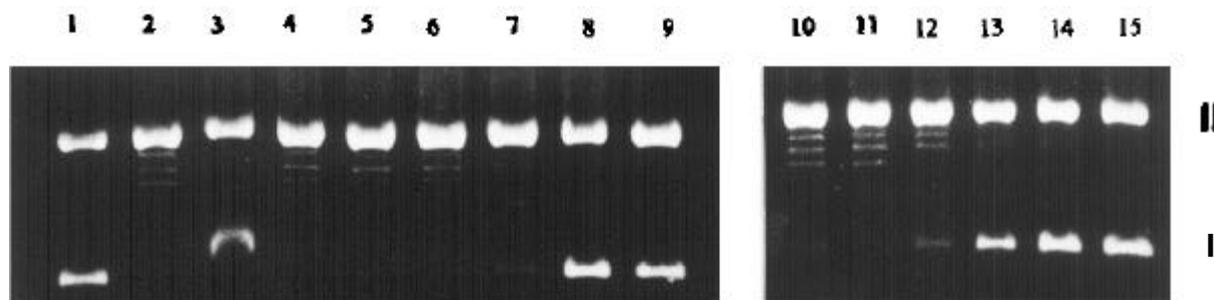
Figure 2. The DNA relaxation assay carried out in presence of the two ruthenium coordination complexes RuIm and RuInd. Supercoiled pBR322 DNA (lane 1) was incubated with topo II (lane 2) in the presence of 100 µM of m-AMSA (lane 3) and 100, 150, 200, 250, 300 and 350 µM of RuIm (lanes 4 to 9) and the same concentrations of RuInd (lanes 10 to 15). At 100 µM concentration, m-AMSA completely inhibits the relaxation activity of topo II on the DNA. RuInd completely inhibits the DNA relaxation activity of topo II at a concentration of 250 µM while RuIm inhibits the activity at 300 µM. The supercoiled and nicked circular DNA are indicated by **I** and **II**.

poisons typically interact with both DNA and topo II, allow the topo II to cleave the DNA but inhibit the enzyme's ability to religate the DNA by forming a stable complex of [cleaved DNA-drug-enzyme] called the cleavage complex. This ultimately results in double stranded nicks in DNA. The cleavage assay is a quick quantitative assay to determine if a drug is causing this cleavage complex formation by allowing excess enzyme to catalyze DNA relaxation in the presence of drug. Formation of cleavage complex is seen by the appearance of linearized DNA from supercoiled plasmid DNA due to DNA cleavage. The results of this assay show that RuInd was very potent in poisoning the enzyme activity by formation of a cleavage complex (at a concentration of 150 µM), seen by the appearance of linear DNA in the assay gels (figure 4). M-AMSA causes linear DNA formation at a concentration of 100 µM concentration. The strong DNA intercalation action of m-AMSA causes the supercoiled DNA band to migrate aberrantly (lane 3). RuIm also forms the cleavage complex, but at a higher concentration of 300 µM.

3.1d *Thymidine incorporation assay*: The [^{3}H]thymidine incorporation assays were carried out on the two human cancer cell lines, colo-205 and ZR-75-1 in the presence of the ruthenium drugs. Thymidine incorporation by cells in the absence of drugs was considered as 100% incorporation, and the incorporation of thymidine by cells in the presence of the drugs was plotted against drug concentration. Inhibition of thymidine incorporation by DNA indirectly reflects on the anticancer potential of the drugs. The results of this assay agree with the previous findings that RuInd is a stronger anticancer agent compared to RuIm (figure 5). The DNA intercalator, m-AMSA was the most effective in inhibiting the cancer cell proliferation while RuSAL was the least effective. The drugs were marginally more potent on the breast carcinoma (ZR-75-1) compared to the colon carcinoma (colo-205). In the colon
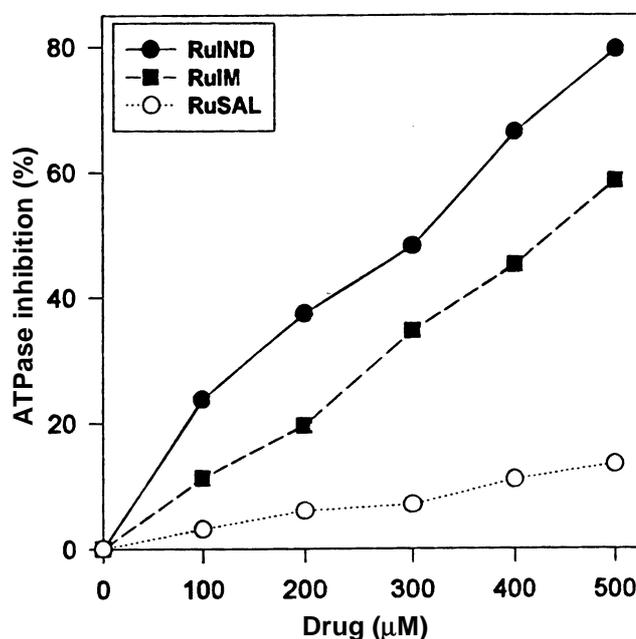

Figure 3. The ATPase assay shows that RuInd has a very potent inhibitory effect on the ATP hydrolysis reaction of topo II (80% inhbition) compared to RuIm (58% inhibiton). Both drugs show a dose dependent effect on the inhibition of ATPase activity. RuSAL does not significantly inhibit the ATPase activity of topo II (12% inhibition).

carcinoma, m-AMSA almost completely stops thymidine incorporation (2% incorporation) followed closely by RuInd (5% incorporation). The cells in the presence of RuIm show an incorporation of 17% while in the presence of RuSAL, show the highest incorporation of 28%. In the breast carcinoma, m-AMSA allows 10% thymidine incorporation while RuInd, RuIm and RuSAL show an incorporation of 13%, 19% and 42% respectively.

4. Discussion

Since the development of cisplatin, an inorganic platinum complex, numerous platinum and non-platinum metal complexes were synthesized and tested for anticancer activity. Very few match the clinical efficacy of cisplatin. In the ruthenium metal class, RuInd and RuIm are two of the few promising anticancer complexes to date. Though very effective on animal models, their clinical development was hindered due to extreme toxic effects. Histological and blood-chemical investigations show major liver and kidney damage, hyperplasia and hyperkeratosis of gastric mucosa and anemia (Keppler *et al* 1990). The present study on topoisomerase II poisoning by these two compounds suggests that they may be promising candidates for further development as topoisomerase II poisons.

The topo II antagonism studies show that these complexes poison topo II and RuInd is more potent compared to RuIm. The cleavage assay by both complexes reveals that these compounds have the ability to form the "cleavage complex" similar to other topo II poisons. This is an important feature of topo II poisons because in the presence of these drugs, the enzyme induces permanent double stranded nicks in DNA. Accumulation of sufficient double strand breaks in DNA brings about numerous adverse genetic aberrations, which ultimately force the affected cells to undergo apoptosis or necrosis (Froelich-Ammon and Osheroff 1995).
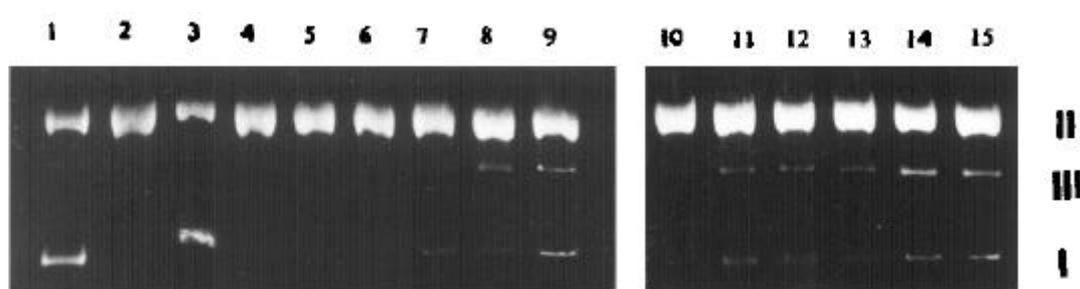

Figure 4. The cleavage assay on pBR322 DNA (lane 1) with topo II (lane 2) in the presence of 100 μM m-AMSA (lane 3) and 100, 150, 200, 250, 300 and 350 μM of RuIm (lanes 4 to 9) and the same concentrations of RuInd (lanes 10–15) shows that RuInd forms the cleavage complex at a concentration of 150 μM and RuIm at 300 μM. m-AMSA shows cleavage complex formation at a concentration of 100 μM. The supercoiled, nicked circular and linear DNA are indicated by **I**, **II** and **III**.
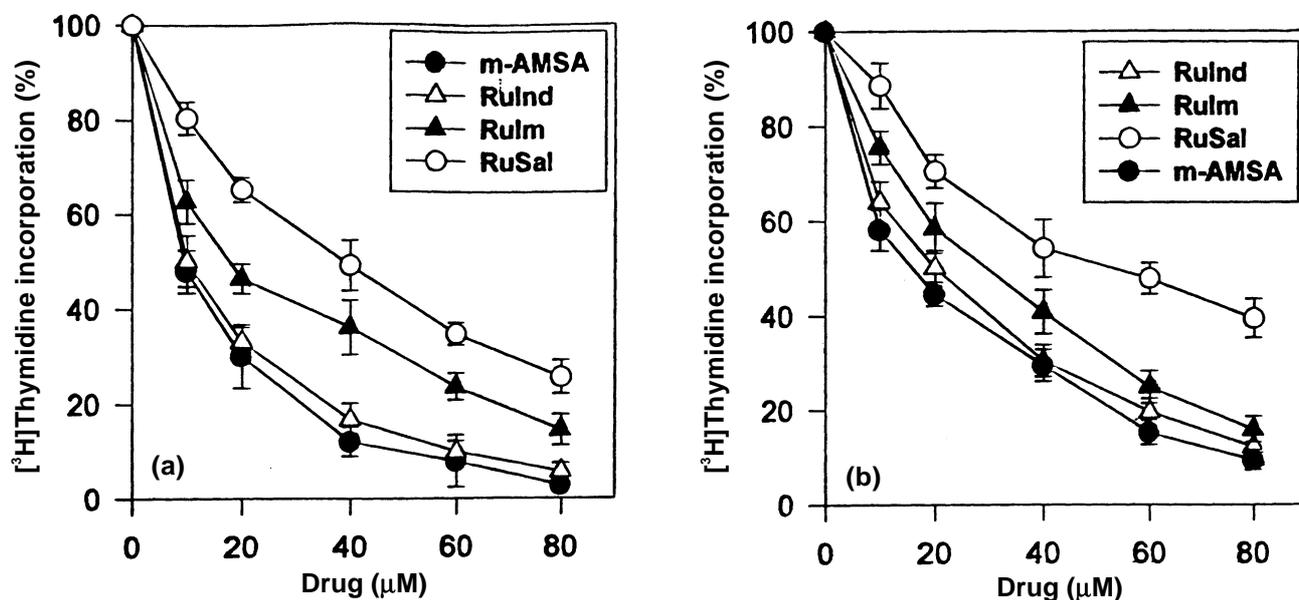

Figure 5. The thymidine incorporation assay on the two cancer cell lines shows that RuInd inhibits thymidine incorporation almost similar to m-AMSA. RuIm also showed a significant inhibition of the cancer cells. RuSAL was the least effective. The drugs were less effective on the ZR-75-1 cells (**b**) compared to the colo-205 cells (**a**). In the colo-205 cells, m-AMSA showed a thymidine incorporation of 2% followed by RuInd, RuIm and RuSAL which showed an incorporation of 5%, 17% and 28% respectively, at the highest concentration of 60 μM. In the ZR-75-1 cells, the thymidine incorporation was relatively higher than in the colo-205 cells, with m-AMSA, RuInd, RuIm and RuSAL showing incorporation of 10%, 13%, 19% and 42% respectively.

As in the case of topo II antagonism, RuInd is also more potent than RuIm in inhibiting the [³H]thymidine incorporation by the two cancer cell lines. This data also corroborates with the anticancer activity of the two drugs reported by Keppler *et al* (1989, 1990). Though the data presented here does not give any direct evidence between topo II antagonism and anticancer activity, it does suggest that topo II antagonism may partly account for the anticancer activity of these drugs, in addition to inhibition of DNA synthesis reported by Fruhauf and Zeller (1991).

Most of the work done on metal containing anticancer drugs suggests that these molecules have multiple levels of complex interactions with the cellular DNA and proteins. Many may have preferential interactions with particular DNA sequences and proteins, leading to inhibition of important cellular pathways, eventually causing anticancer activity. Identification of such interactions and the resulting anticancer properties will immensely help in the continual development of drug entities, which are high on therapeutic specificity and low on toxicity. This is particularly important because cancer cells regularly evolve mechanisms to resist the cytostatic action of anticancer drugs.

Topo II is an important target for many DNA binding anticancer drugs. Since most anticancer metal complexes of ruthenium, cobalt, platinum and titanium primarily target DNA, it would be worthwhile to search for DNA binding metal complexes that poison topo II. The present biochemical study and earlier studies on metal complexes and topo II antagonism (Jayaraju *et al* 1999; Gopal *et al* 1999, 2000) give an insight into the molecular interactions of these molecules with DNA and topo II and the subsequent effect on cancer cell proliferation. Understanding the molecular interactions leading to topo II poisoning merits a deeper investigation for the development of more potent topo II poisons of this class.

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