Chapter 5

DEVELOPMENT OF NOVEL RuBen DERIVATIVES TO ENHANCE POTENCY OF TOPOISOMERASE II POISONING
INTRODUCTION

RuBen(dmso) was a promising starting compound for the development of novel derivatives with potential enzyme interacting groups because it possess significant topo II poisoning and anticancer activity. In addition, association of the ruthenium atom with the benzene ring through an organometallic linkage may partly minimize its random interaction with biological molecules, thus reducing toxic side effects. Molecular modeling analysis on RuBen(dmso) suggested the involvement of ‘dmso’ in topo II interaction. Consequently, the ‘dmso’ group was replaced by other active ligands on the 'RuBen' backbone (the RuBen backbone is the ruthenium atom bonded to the benzene ring and the chloride atoms). Numerous compounds were generated and tested for topo II antagonism. Out of these, only three compounds showed efficient topo II poisoning and anticancer activity. These compounds are RuBenApy, RuBenABa and RuBenAGu, in which the 'dmso' group was replaced with 3-amino pyridine, p-amino benzoic acid and amino guanidine (synthesis described in the Experimental Procedures section). Mechanism of action of these compounds on the catalytic activity of topo II and their anticancer activity has been carried out in detail in the present study.
RESULTS

Topoisomerase II activity assays:

*Topoisomerase II poisoning by the RuBen drugs:*

Inhibition of topo II mediated relaxation of supercoiled DNA was investigated through the relaxation assay. RuBenPy does not affect the DNA relaxation activity of topo II over the concentration range employed while RuBenAPy completely inhibits topo II activity at a concentration of 200 µM. RuBenABa and RuBenAGu show complete inhibition at 350 and 250 µM. All the three complexes exhibit a dose-dependent inhibition of DNA relaxation (Figure 17).

*Inhibition of ATPase activity by the Rulien drugs:*

Drugs that inhibit the relaxation activity of topo II also interfere with the DNA-stimulated ATPase activity of the enzyme. The results of the ATPase assay indicate that RuBenAPy, RuBenAGu and RuBenABa inhibit the DNA dependent ATPase activity of topo II, with a potency of inhibition in the same order, while RuBenPy does not produce appreciable inhibition (Figure 18). The inhibition of ATPase activity by the three drugs is dose dependent and is concomitant with their action on the relaxation activity of topo II.

*Drug induced, topoisomerase II-mediated cleavage of DNA:*

The topo II mediated cleavage of DNA in presence of the RuBen drugs was monitored through the cleavage assay. Linearization of supercoiled circular DNA occurs when the
cleavage assay is carried out in the presence of increasing concentrations of RuBenAPy, RuBenABa and RuBenAGu (Figure 19A) but not RuBenPy. The linear DNA results from SDS and proteinase K treatment of the ternary cleavage complex containing topo II, drug and cleaved DNA. Hence, these RuBen drugs effectively shift the DNA cleavage/religation equilibrium of topo II towards DNA cleavage. Quantification of linear DNA shows that RuBenApy is the most potent drug, forming the cleavage complex at a concentration 150 μM. RuBenABa and RuBenAGu form the cleavage complex at 250 and 200 μM (Figure 19B). These results correlate well with the relaxation and ATPase inhibition activities of the three RuBen drugs.

DNA interaction by the RuBen drugs:

DNA thermal denaturation studies:

The DNA thermal denaturation studies show that the four RuBen drugs bind to DNA with almost similar affinity (Figure 20). Curve width analysis of the denaturation curves was carried out to determine the mode of DNA interaction by the RuBen drugs. The study shows that these drugs are essentially DNA external binders which interact with the nucleotides without disturbing the DNA helix (Figure 21). CD. spectral analysis of DNA in presence of the drugs reveals that they do not induce any conformational change in DNA, which confirms the result of curve width analysis. Where as, m-AMSA causes a very high change in curve width of the denaturation curves and also induces a steep increase in the CD. signal of DNA, which is characteristic of a DNA intercalating molecule (Figure 22).
Anticancer action of the RuBen drugs:

The \(^{3}H\) thymidine incorporation assays on the two cancer cell lines \((\text{colo-205 and ZR-75-1})\) show that the anti-proliferation activity of the RuBen drugs is concomitant with their topo II poisoning ability. RuBenAPy shows the highest anti-proliferative action followed by RuBenAGu and RuBenABa. RuBenPy also has a significant action on the proliferative response of the cancer cells (Figure 23). The DNA intercalating drug, m-AMSA shows the highest anti-proliferative action. All the drugs including m-AMSA are very active against the colo-205 cell line, while the ZR-75-1 cells are less sensitive to the anti-proliferative action of these drugs (Figure 24).

Molecular modeling analysis:

The molecular modeling analysis of the four RuBen drugs (RuBenPy, RuBenAPy, RuBenAGu and RuBenABa) suggest that these molecules have distinct spatial orientations of the ligands around the ruthenium atom, which are completely different from that of RuBen(dmso) (Figure 25). The coordinated ligands in these molecules (Py, APy, AGu and ABa) are oriented away from the chloride atoms, unlike in RuBen(dmso). The orientation of the coordinated ligands is such that they may easily be able to align into an active site of topo II and possibly interact with the electropositive sites on the protein through their free electronegative nitrogen atoms. This interpretation is supported by the observation that RuBenPy, which does not have any free nitrogen atoms on its ligand, does not poison topo II activity and also does not show very good anticancer action.
DISCUSSION

The DNA nicking function of topo II has widespread implications and has given the cancer pharmacologist, a worthy reason to develop drugs targeted at this action of topo II. These topo II drugs are popularly known as topo II poisons because, rather than inhibiting the catalytic activity of the enzyme, they promote one part of the reaction mechanism, namely, DNA cleavage and block the second part, which is religation of the cleaved DNA (Robinson and Osheroff, 1990). Our earlier study with RuBen(dmso) introduced this molecule as a potential anticancer drug whose molecular target is topo II. Though a rather high concentration of this drug is required for topo II poisoning and anticancer activity, it nevertheless served as a good lead molecule for the development of potent derivatives, in which the topo II interacting ‘dmso’ group was replaced with pyridine, 3-amino pyridine, p-amino benzoic acid or amino guanidine. All the four molecules bind to DNA with a similar affinity indicating that a common entity in these molecules, the ruthenium atom, may be the exclusive DNA binding group. The chlorides on the ruthenium atom may also serve this function, but such an interaction, similar to that of cisplatin with DNA, would lead to drug-DNA adduct formation. That seems improbable because the DNA binding studies do not suggest such a drug-DNA adduct formation by any of these RuBen drugs. The results of the relaxation assay, ATPase assay and the cleavage assay indicate that RuBenAPy, RuBenAGu and RuBenABa poison topo II by cleavage complex formation. RuBenAPy is the most potent followed by RuBenAGu and RuBenABa. Surprisingly, RuBenPy, which has a structure and DNA binding affinity similar to RuBenAPy, does not
poison topo II. This profound difference between the two similar molecules may be due to the amino group on the pyridine ring of RuBenAPy, which may solely interact with topo II. Similarly, in RuBenAGu and RuBenABa, the amino groups may be responsible for topo II interaction leading to effective poisoning of the enzyme. Though the higher potency of RuBenAGu could be due to multiple interactions by the three amino groups of the AGu ligand with topo II, as compared to that of a single amino group in RuBenABa, the drug conformation during topo II interaction may also be an important determinant for topo II poisoning. This is because RuBenApy has a single amino group, but is the most potent topo II poison among the RuBen drugs. The conformation and spatial orientation of the ligands on the drugs may be appropriate for effectively freezing topo II in the cleavage complex. The molecular modeling analysis of the RuBen drugs argues in this direction. The spatial orientation of the amino pyridine ligand may help the drug to secure a strong hold on the enzyme, which could explain the highest potency of topo II poisoning by this drug.

Our studies reveal that the three RuBen drugs interact bi-directionally with DNA and topo II, similar to RuBen(dmso). The ruthenium atom binds to DNA and the ligand (APy, ABa or AGu) interacts with topo II. It is not clear if the chloride atoms and the organometallic bonded benzene ring involve in topo II interaction, because these groups are oriented away from the coordinated ligand. Even if they do, they may interact with a region on the enzyme which is away from the ligand interaction site, unlike in the case of RuBen(dmso), where all the groups may interact with one region of the enzyme.
The thymidine incorporation assay on the two human cancer cell lines shows that the RuBen drugs are effective anticancer agents and merit a detailed analysis. RuBenPy does not poison topo II but still shows significant anti-proliferation activity, indicating that this drug as well as the other RuBen drugs may act on other cellular constituents as well. Though m-AMSA is more effective than the RuBen drugs in topo 11 poisoning and anticancer activity, it is a DNA damaging agent and a possible mutagen, which limits its therapeutic potential, while the RuBen drugs interact externally with DNA without destabilizing the DNA helix. This is an important factor to be considered for the development of anticancer drugs because most of the DNA destabilizing anticancer drugs like cisplatin, m-AMSA and adriamycin cause permanent genetic damage to the host, often resulting in the development of neoplasticity as a long term effect.
Figure 17: Supercoiled pBR322 DNA (lane 1) was incubated with topo II in the absence (lane 2) or presence of 75 μM m-AMSA (lane 3), 100, 150, 200, 250, 300 and 350 μM of RuBenAPy (lanes 4 to 9), RuBenAGu (lanes 10 to 15), RuBenABa (lanes 16 to 21) and 100, 200, 300, 400 and 500 μM of RuBenPy (lanes 22 to 26) The positions of supercoiled (form 1) DNA and relaxed (nicked circular or form 2) DNA are indicated by I and II.
**Figure 18:** Inhibition of the ATPase activity of topo II by RuBenPy, RuBenAPy, RuBcnAGu and RuBenABa ATP hydrolysis in the presence of increasing concentrations of the drugs are presented as mean of 3 experiments. Data is plotted as percent inhibition of ATP hydrolysis versus concentration of drug in $\mu$M. RuBenAPy showed the highest inhibition of ATP hydrolysis (~80%) followed by RuBenAGu and RuBenABa. RuBenPy does not show significant inhibition of ATP hydrolysis.
FIGURE 18

ATPase INHIBITION (%) vs. DRUG (µM)

- RuBenPy
- RuBenApy
- RuBenAGu
- RuBenABA
Figure 19: (A) Cleavage assay was performed by incubating supercoiled pBR322 DNA (lane 1) with topo II (lane 2) in presence of 100 μM m-AMSA (lane 3), 100, 150, 200, 250, 300 and 350μM RuBenPy (lanes 4 to 9), the same concentrations of RuBenAPy (lanes 10 to 15), RuBenAGu (16 to 21) and RuBenABa (22 to 27). The positions of supercoiled, nicked circular and linear (form 3) DNA arc indicated by I, II and III. The formation of the cleavage complex is evidenced by the appearance of the linear DNA (III). (B) Quantification of the linear DNA shows that RuBenAPy is the most potent in cleavage complex formation followed by RuBenAGu and RuBenABa. RuBenPy docs not show any visible linear DNA formation even at the highest concentration of 350 μM. The lower concentration of 50 μM is not shown in the gel photographs.
Figure 20: In the absence of any drug, the melting temperature curve of calf thymus DNA shows a $T_m$ of 57 °C (—). At a drug to DNA nucleotide ratio 1:5, RuBenAGu (—) increases the $T_m$, to 68 °C, whereas RuBenPy (--) and RuBenAPy (—) and RuBenABa (—) increase $T_m$ to 69.5, 70 and 70.5 °C. The DNA intercalator, m-AMSA induces a strong increase in $T_m$ (74 °C) at a drug to nucleotide ratio only 1:10 (••••). The $T_m$ curves for the ratios lower than 1:5 (1:10, 1:20, 1:40) are not shown. Drug to DNA ratios of more than 1:5 were not attempted because these complexes show intense visible spectra which interferes with the uv spectroscopic studies.
**Figure 21:** D/N (drug/nucleotide) ratio plotted against curve width of the $T_m$ curves (4 curves for drug to DNA nucleotide ratios of 1:40, 1:20, 1:10 and 1:5 for all the RuBen drugs and 3 for m-AMSA) shows a characteristic increase in curve width by m-AMSA (•). The RuBen drugs however do not greatly affect the curve width of the melting temperature curves suggesting that they are essentially DNA non binders.
**Figure 22:** The Circular Dichroism spectra of pBR322 DNA (—) in the presence of RuBenAGu (—), RuBenPy (—–), RuBenAPy (•—) and RuBenABa (-•-) shows that these drugs induce very minute changes in the molar ellipticity of DNA, while m-AMSA(-••-) shows a very prominent change at a concentration of ~3.5 times less than that of the RuBen drugs. This large increase in the C.D. signal indicates a conformational change induced in the DNA due to intercalation by m-AMSA.
**Figure 23:** *In vitro* anti-proliferation activity of the RuBcn drugs was tested on two fast growing cancer cells- colo-205 (top panel graph) and ZR-75-1 (lower panel graph). The cells were incubated with increasing concentrations of the RuBen drugs and proliferation was quantified by [H] thymidine incorporation, as described in the experimental section. Cell proliferation at a drug concentration of 80 μM is shown in the figure m-AMSA is the most effective in anti-proliferative action, followed by RuBenAPy, RuBenAGu, RuBenABa and RuBenPy. The data presented is a mean of three independent experiments conducted in triplicates.
FIGURE 23

Graph showing the effect of different drugs on proliferation. The x-axis represents DRUG concentration (μM), and the y-axis represents PROLIFERATION (%) for each drug (m-AMSA, RuBenAPy, RuBenAGu, RuBenABa, RuBenPy). The graph illustrates the varying degrees of proliferation inhibition across different drug concentrations.
**Figure 24:** The colon carcinoma cells (black bars) are more sensitive to the action of the RuBen drugs compared to the ZR-75-1 cells. The control, m-AMSA also shows a similar effect. This indicates that the ZR-75-1 cells may have an inherent resistance mechanism which makes them less susceptible to the action of topo II poisons compared to the colo-205 cells.
Figure 25: The molecular models of RuBenPy, RuBenAPy, RuBcnAGu and RuBcnABa reveal that these molecules do not possess the typical 'chloride' and 'coordinated ligand' interacting domain of RuBen(dmso). The coordinated ligands in these molecules are oriented away from the chloride groups. The angular orientation of the ligands may allow them to interact with the enzyme by aligning themselves into an active site in the enzyme. In RuBenPy, the ruthenium atom is shown in green, chlorides in yellow and the nitrogen atom in the pyridine ring, which interacts with ruthenium, is in white. In RuBcnAPy, ruthenium is shown in light yellow, chlorides in dark yellow, the pyridine ring nitrogen (which interacts with ruthenium) as well as the free NH$_2$ nitrogen are shown in white. In RuBcnABa, the oxygen atoms are shown in green and NH$_2$ nitrogen in white. In RuBenAGu, all the nitrogen atoms are shown in white. The ruthenium-benzene organometallic bonds are not shown in all the molecules.