Chapter 3

TOPOISOMERASE II POISONING AND ANTICANCER ACTIVITY BY DNA NON-BINDING DERIVATIVES OF FERROCENE.
INTRODUCTION

Numerous inorganic and organic iron compounds and ionic ferricinium salts of the type \([(\text{CsH}_3)_2 \text{Fe}^+ \text{X}^-]\) have earlier been shown to possess anticancer activity, but they also cause typical iron toxicity (Collier and Krauss, 1981; Kopf-Maier and Kopf, 1988). The toxicity arises due to release of inorganic iron from the compounds, and its association with several biological molecules in various tissues. The toxic effects arise because of two reasons. The first is, since iron is an oxidant, it produces hydroxyl radicals which attack all classes of biological molecules and produce effects like depolymerization of polysaccharides, inactivation of enzymes and initiation of lipid peroxidation (McCord, 1996). The second reason is that iron is a nutrient for invading microbial and neoplastic cells, and these cells possess exceptionally powerful mechanisms for obtaining host iron (Weinberg, 1995).

Neutral ferrocene compounds which are less toxic compared to the above mentioned derivatives have been used in the present study. The organometallic bonds between the metal atom and the cyclopentadiene rings as well as the uncharged iron atom account for the low toxicity. Mono- and di- substituted acetyl and carboxaldoxime derivatives were synthesized for this study (described in the Experimental Procedures section). Acetyl substitutions were used because the acetyl groups are strongly interacting ligands. The carboxaldoxime substitutions were designed because an earlier study with a cobalt salicylaldoxime complex showed that the oxime group plays a critical role in topo II poisoning (Jayaraju et. al., 1999).
In the present study, mechanism of topo II interaction and the resulting antagonism of the enzyme's activity was investigated. Anticancer activity of the ferrocene derivatives has also been carried out using five different human cancer cell lines.

**Anticancer activity of the ferrocene drugs:**

The $^{3}$H thymidine incorporation assays were carried out on five different cancer cell lines. The results (Figure 1) suggest that Fecp does not appreciably inhibit proliferation of any of the five cancer cell lines tested. The mono substituted acetyl and carboxaldehyde derivatives (AcFecp and FecpOx) showed a markedly higher inhibition than the parent molecule, Fecp. The di-substitued derivatives, DacFecp and FecpDox were more effective than the mono-substituted derivatives. FecpDox showed the highest potency of proliferation inhibition, almost comparable to that of the DNA intercalating drug, m-AMSA. These drugs were most effective on the Colo-205 cell line followed by HL-60, Hut-78, ZR-75-1 and least on A-431 cell line.

**Topoisomerase II activity assays:**

*Inhibition of relaxation activity of topo II.*

The relaxation assay using ferrocene derivatives (Figure 2) shows that Fecp had no inhibitory effect on the relaxation activity of topo II. AcFecp and FecpOx could completely inhibit the enzyme activity at 500 $\mu$M concentration. DacFecp could inhibit the enzyme activity at 400 $\mu$M and FecpDox at 300 $\mu$M concentrations respectively. The
DNA intercalating topo II poison, m-AMSA showed complete inhibition of relaxation activity at 75 $\mu$M concentration (Figure 2, lane 3).

**Formation of Cleavage complex.**

The formation of enzyme-drug-DNA cleavage complex could be evidenced by the appearance of linear DNA (form III plasmid DNA) in an agarose gel after SDS treatment of the cleavage products. The linearized DNA results from dissociation of the topo II homodimer from cleaved DNA by SDS. Neither Fecp nor AcFecp and FecpOx could induce cleavage complex formation, but the di-substituted compounds do so. DacFecp induces formation of linear DNA at a concentration of 400 $\mu$M and FecpDox shows a higher potency of cleavage complex formation at 200 $\mu$M concentration (Figure 3). m-AMSA induces cleavage complex at 150 $\mu$M concentration and also causes a small shift in DNA migration through agarose gel due to DNA intercalation. No such shift was observed for the ferrocene drugs.

**Inhibition of ATPase activity of topo II.**

This assay was performed to examine the effect of the drugs on the DNA stimulated ATPase activity of topo II. Relaxation assay was performed with increasing concentrations of the drugs in presence of $^{32}$P ATP and the products were resolved on PEI Cellulose-F TLC sheets. Radioactivity of the resolved components was measured and data was plotted (Figure 4). The results show that Fecp could inhibit only 5.2% of the
DNA stimulated ATPase activity at 250 μM concentration while AcFecp, FecpOx and DacFecp could inhibit 37%, 40% and 46% of this activity. FecpDox was most effective in inhibiting the ATPase activity of topo 11 (52%). These results correlate well with the DNA relaxation assay.

Immuno-precipitation of the topoisomerase II cleavage products to determine the presence of ferrocene drugs in the cleavage complex:

Cleavage assay was conducted in the presence of 500 μM concentration of the ferrocene drugs. After incubation, the enzyme in the cleavage complex as well as the free enzyme was immuno-precipitated with anti-topo 11 antibody. The antigen-antibody complex was then trapped in protein A-agarose matrix. Samples were washed to remove unbound components and the bound enzyme was released from protein A agarose by 1% TCA and analyzed for presence of iron by atomic absorption spectroscopy. The results presented as percentage of drug bound (Figure 5, table) show that AcFecp and FecpOx associate with topo II alone and with topo II-DNA complex with the same affinity (~ 6%). DacFecp and FecpDox show a small increase in binding to topo II alone (9% and 12%) compared to the mono substituted compounds. In case of all the drugs, some amount of drug may be released from topo II in absence of DNA during the wash and TCA treatments. In presence of DNA however, DacFecp and FecpDox seem to be more strongly associated with topo II compared to AcFecp and FecpOx (15% DacFecp and 19% FecpDox is present in the topo II-DNA complex) due to which, there is a marked reduction in the release of the drug from the enzyme-DNA complex.
Melting temperature studies to determine Drug-DNA interaction:

Melting of calf thymus DNA was monitored in presence of increasing concentrations of drugs and $T_m$ was determined from the melting curves. In the absence of drugs, DNA showed a $T_m$ of 59 °C. The melting temperature curves for the highest concentration of drug used (1:1 drug to nucleotide ratio) are represented in Figure 6A, which show no change in the $T_m$ of calf thymus DNA in presence of Fecp and a very minor increase in $T_m$ in presence of FecpOx and FecpDox (~61 °C for both drugs). AcFecp and noticeably DacFecp slightly increased the $T_m$ (64 ° and 68 °C at the highest concentration). On the other hand, m-AMSA induced a very steep increase in $T_m$ (74 °C) at a drug to nucleotide ratio of only 1:10. The curve width analysis to determine the mode of DNA interaction revealed that though the acetyl groups increased the $T_m$ of calf thymus DNA, they are essentially DNA non-binders as they show no change in curve width of the melting curves plotted against drug/nucleotide (D/N) (Figure 6B). m-AMSA however showed a steep increase in the curve width, a typical feature of DNA intercalators.

Molecular Modeling Analysis:

Molecular models of the ferrocene derivatives were generated as described in the experimental section. The models are shown in Figure 7. The lateral and longitudinal views of Fecp show an iron atom in the centre bonded to two cyclopentadiene rings through organometallic bonds between the iron atom and the rc-orbitals of the ring hydrogens. In AcFecp and DacFecp, the acetyl groups orient towards the ferrocene backbone, along the longitudinal axis. This ‘closed-in’ conformation of the acetyl
derivatives may confer a weak DNA binding ability to them, but it may not provide the orientation for a strong topo II interaction. The carboxaldoxime groups in FecpOx and FecpDox, unlike the acetyl groups, extend out further from the backbone. This extended molecular conformation may allow the two oxime derivatives to interact strongly with topo II, but not with DNA, which explains their stronger topo II antagonism but not DNA binding. The double substitution in DacFecp and FecoDox may help these molecules to get a stronger hold on the enzyme. The extended conformation plus two electronegative atoms (nitrogen and oxygen) may confer on FecpDox, a stronger topo II poisoning ability. But the 'closed in' conformation and a single electronegative oxygen atom may limit a strong topo II interaction by DacFecp.
DISCUSSION

In the present study, two active chemical moieties, acetyl and carboxaldoxime, were substituted on ferrocene and tested whether the resulting compounds show anticancer action by interfering with the activity of topoisomerase II, an important molecular anticancer target.

The in vitro anticancer assay shows that while Fecp could not inhibit the proliferation of any of the cancer cell lines tested, the acetyl and carboxaldoxime substitutions on ferrocene (AcFecp and FecpOx) conferred potency to the compounds in this action. In the next step, by making a second substitution of the same groups to the singly substituted compounds, anti-proliferative action was substantially increased. The di-substituted derivatives (DacFecp and FecpDox) were clearly more effective than the mono-substituted compounds in proliferation inhibition. In both mono and di derivatives, it was observed that the carboxaldoxime group conferred a higher potency of proliferation inhibition compared to the acetyl group. Tests with more than two substitutions were not attempted as such compounds were inherently unstable.

Topo II relaxation assay with the substituted ferrocenes showed that firstly the carboxaldoxime substitution on ferrocene was more potent than the acetyl substitution in inhibiting topo II activity and secondly the di-substituted derivatives were more effective than the mono-substituted derivatives. These compounds also inhibited the ATPase activity of the enzyme which was concomitant with inhibition of DNA relaxation. These
results argue that interfering with topo II activity may be a major mechanism for anticancer action of these compounds. But a stronger correlation between anticancer action and topo II inhibition would be established if the molecular mechanism of topo II inhibition by these compounds is known, i.e., whether they inhibit the enzyme activity by interfering with the ATPase activity alone (which only inhibits catalytic activity of topo II) or by forming an enzyme-DNA cleavage complex. The cleavage assay showed that only the di-substituted derivatives were able to form the cleavage complex. FecpDox was most potent in this action.

To determine if DacFecp and FecpDox were actually present in the cleavage complex which they induce and to partly understand the mode of cleavage complex formation, an immuno-precipitation assay with anti-topo II antibody was performed. In this assay, presence of drug in the cleavage complex was verified by atomic absorption spectroscopy of the immuno-precipitated cleavage products. The results show that AcFecp and FecpOx bind to topo II alone and were also present in the topo II-DNA complex at similar levels. But since they could not form the cleavage complex (as shown by the cleavage assay), the drugs may associate weakly with the enzyme and also the enzyme-DNA complex, thus interfering with the relaxation activity and ATPase activity of the enzyme to a certain extent but without forming the cleavage complex. As expected, DacFecp and FecpDox bound more strongly to topo II, showing a definite increase of the drug in the topo II-DNA complex compared to only topo II, especially in the case of FecpDox. This shows that the di-substituted compounds interact more strongly with the enzyme-DNA complex than only with the enzyme.
Several lines of evidence suggest that there are generally three routes of ternary complex formation by topo II poisons (Hertzberg et. al., 1989; Nabiev et. al., 1994; Corbett et. al., 1993). The first route is that the drugs predominantly interact with DNA (by intercalation or otherwise) and poison topo II when the enzyme binds to DNA. The DNA thermal denaturation studies in presence of the ferrocene compounds suggested that none of them interact with DNA. Though DacFeccp slightly increases the $T_m$ of calf thymus DNA, the curve width analysis suggests that it is essentially a DNA non-binder but the slight increase in $T_m$ may be due to non specific electrostatic interaction with the phosphate backbone.

**THREE MODES OF CLEAVAGE COMPLEX FORMATION**

The second route is that, the drugs may not actually interact with the enzyme or DNA individually, but may bind specifically and exclusively to the binary complex of topo II and DNA. The third route is, the drugs may first bind to the enzyme in absence of DNA and then enter the ternary complex when the drug bound topo II interacts with DNA. The immuno-precipitation assay was helpful in interpreting which of the latter two mechanisms
are followed by the two di-substituted drugs. This assay showed that the enzyme alone does not show as much drug bound to it compared to the enzyme-DNA complex. Also, since the drugs do not bind to DNA, the conformational change induced in the enzyme after interaction with DNA may stabilize the drug association with the enzyme, which results in a stable topo II-drug-DNA ternary complex. In such an association, the drug promotes the cleavage of DNA but prevents DNA religation. These results suggest that DacFecp and FecpDox may follow the third mechanism of cleavage complex formation i.e., the drug first interacts with topo II in absence of DNA, the drug bound enzyme then interacts with DNA and cleaves it, leading to the formation of cleavage complex.

The carboxaldoxime group on ferrocene consistently conferred a higher potency compared to the acetyl group. The nitrogen and oxygen atoms of the carboxaldoxime group can form N-donor and O-donor interactions with topo II, whereas in the acetyl group, the single electronegative atom (carbonyl oxygen) may show a relatively weak enzyme interaction. The higher potency of DacFecp and FecpDox compared to AcFecp and FecpOx could be because the double substituted drugs could interact with two different regions on the enzyme, which may result in an overall stronger enzyme interaction compared to the mono-substituted drugs. Such an interaction may be responsible for the formation of the cleavage complex by these di-substituted drugs. Also, as suggested by the molecular modeling analysis, the extended orientation of the carboxaldoxime groups in FecpOx and FecpDox may also be an important determinant for a stronger interaction with topo II, leading to a stronger antagonism, compared to the acetyl derivatives.
DNA intercalating topo II poisons intercalate freely with the DNA of normal cells too and interfere with various genetic processes, posing a potential threat to the genetic material. It would be desirable to develop DNA non-intercalating topo II poisons, which should be relatively non-toxic to the genetic machinery, but specific for topo II poisoning. Though DacFecp and FecpDox are not as potent as m-AMSA in topo II poisoning and anticancer action, they are nevertheless promising candidates in their class of topo II poisons. It would be worthwhile to explore new substitutions on the ferrocene backbone to specifically poison the action of topo II and increase anticancer activity.
Figure 1: *In vitro* anti-proliferation activity of Ferrocenes was tested on five different cancer cell lines- Colo-205 (A), ZR-75-1 (B), A-431 (C), HL-60 (D) and HUT-78 (E). The cells were incubated with increasing concentrations of the ferrocene drugs for 6 h., after which the drug treatment was stopped. Following a further incubation for 48 h, $^3$H thymidine incorporation was performed for 4 h. as described in methods. The cells were harvested and radioactivity was measured. Values are presented as mean of three independent experiments. Data is graphically expressed as percentage of cell proliferation versus concentration of drug in $\mu$M. The drugs are most potent on the colon carcinoma (colo-205) followed by HUT-78, HL-60, ZR-75-1 and A-431. m-AMSA also showed a similar profile of anticancer activity in all the cell lines except in the case of ZR-75-1, on which it was more potent than on HL-60.
FIGURE 1
Figure 2: Effect of Fecp, AcFecp, FecpOx, DacFecp and FecpDox on topo II catalyzed DNA relaxation activity. Supercoiled pBR322 DNA (lane 1) was incubated with topo II in the absence (lane 2) or presence of 75 μM m-AMSA (lane 3) and 100, 200, 300, 400 & 500 μM Fecp (lanes 4-8), 100, 200, 300, 400 & 500 μM AcFecp (lanes 9-13), 100, 200, 300, 400 & 500 μM FecpOx (lanes 14-18), 100, 200, 300 & 400 μMDacFecp (lanes 19-22) and 100, 200 & 300 μM FecpDox (lanes 23-25). The positions of supercoiled (form 1) and nicked circular (form 2) DNA are indicated by I and II.
FIGURE 2
Figure 3: (A) Cleavage reaction was performed by incubating supercoiled pBR322 DNA (lane 1) with topo II (lane 2) in presence of 100 $\mu$M m-AMSA (lane 3), 300, 400 and 500 $\mu$M of Fecp (lanes 4 to 6), the same concentrations of AcFecp (lanes 7 to 9) and FecpOx (lanes 10 to 12); Lane 13 is DNA alone, lane 14 is DNA+topo, lane 15 is DNA+topo II+ 150 $\mu$M m-AMSA; DNA+topo II+ 100, 200, 300, 400 & 500 $\mu$M of DacFecp (lanes 16 to 20) and 100, 200, 300, 400 & 500 $\mu$M of FecpDox (lanes 21 to 25). The positions of supercoiled, nicked circular and linear (form 3) DNA are indicated by I, II and III. (B) Percentage of linear DNA formed with increasing concentration of DacFecp and FecpDox.
FIGURE 3

A

B

[Graph showing linear DNA as a function of drug concentration]
Figure 4: Inhibition of ATPase activity of topo II by Fecp, AcFecp, FecpOx, DacFccp and FecpDox. ATP hydrolysis in presence of increasing concentrations of the drugs are presented as mean of 3 experiments. Data is plotted as percentage inhibition of ATP hydrolysis versus concentration of drug in $\mu$M.
FIGURE 4

ATPase INHIBITION (%) vs. DRUG (μM)

- Fecp
- AcFecp
- DacFecp
- FecpOx
- FecpDox
Table: Cleavage assay was conducted with drug + topo II and drug + topo II + DNA. The cleavage products were immunoprecipitated with topo II antibody. A drug control and drug + DNA control were included in the cleavage assay to check for non specific interaction of the drugs with DNA, antibody and Protein A agarose matrix. These controls did not show any significant non specific interaction. The concentration of the bound drugs is expressed as percentage of total drug bound.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fecp</th>
<th>AcFecp</th>
<th>FecpOx</th>
<th>DacFecp</th>
<th>FecpDox</th>
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</thead>
<tbody>
<tr>
<td>Drug</td>
<td>0</td>
<td>0.8</td>
<td>0.9</td>
<td>1 +0.3</td>
<td>1</td>
</tr>
<tr>
<td>Drug + DNA</td>
<td>0</td>
<td>1+0.4</td>
<td>1 +0.3</td>
<td>1 +0.8</td>
<td>1 +0.3</td>
</tr>
<tr>
<td>Drug + Topo II</td>
<td>0.3</td>
<td>6 + 0.3</td>
<td>6 + 0.5</td>
<td>9 + 0.6</td>
<td>12+ 1.3</td>
</tr>
<tr>
<td>Drug + Topo II + DNA</td>
<td>0.5</td>
<td>5 + 0.7</td>
<td>6 + 0.2</td>
<td>15+ 13</td>
<td>19+ 1.8</td>
</tr>
</tbody>
</table>

*Data are presented as a mean of three independent experiments conducted in triplicates, with standard deviations given against each value.*
Figure 6: (A) In the thermal denaturation assay, calf thymus DNA showed a $T_m$ of 59 °C (---) in the absence of drugs. At a drug to DNA nucleotide ratio 1:1, Fccp (---) does not change the $T_m$, whereas FecpOx (--) and FecpDox (••••) induce a very small increase of ~2°, showing a $T_m$ of 61 °C. AcFecp (——) and DacFecp (——) increase the $T_m$ slightly at 64 ° and 68 °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 melting curves of the other drug to DNA ratios which have been tested, are not shown. (B) D/N plotted against curve width shows a characteristic increase in curve width by m-AMSA (•). The ferrocenes do not change the curve width of the melting temperature curves suggesting that these compounds are essentially DNA non binders.
**Figure 7:** The lateral (left) and longitudinal (right) views of the molecular model of Fecp show an iron atom in the center (shown in light yellow) bonded to two cyclopentadiene rings through organometallic bonds between the iron atom and the π-orbitals of the ring hydrogens. The organometallic bond between the aromatic ring and the iron atom is not shown (the Spartan molecular modeling software does not show the organometallic linkages in energy minimized structures of organometallic molecules). In AcFecp and DacFecp, the acetyl groups orient towards the ferrocene backbone, along the longitudinal axis. The enzyme interacting oxygen atoms are shown in green. The carboxaldoximic groups in FecpOx and FecpDox, unlike the acetyl groups, extend out away from the backbone. The nitrogen atom of the ‘N-OH’ (oxime) group is shown in white and the oxygen in green.
CONT'D. FIGURE 7

FecpOx

FecpDox