CHAPTER IV

Anticancer Drugs and Biosensors
It has already been mentioned in the first chapter of the thesis that cancer is a dreaded and insidiously spread disease. It is caused by cells which divide in an unregulated manner. It does not mean that they are dividing any faster than the normal cells, merely there is no control mechanism in place. As such, the differentiation between healthy and cancerous DNA is difficult. Cancer cells are invasive whereas, normal cells stay within a certain organ.

**ANTICANCER DRUGS**

Anticancer drugs irreparably damage the DNA of cancerous cells or prevent the replication and synthesis of DNA. Both will prevent the affected cells from replicating and spreading the cancer. Due to the fact that they interact directly with DNA, most chemotherapy drugs are extremely potent. Since these drugs have a tendency to interact with a specific section of DNA they are also called as sequence specific. The main problem associated with anticancer drugs is their side effects and toxicity associated with them. Because, in addition to effecting the cancerous cells the drugs also damage the normal cells. Most chemotherapy treatments have well known side effects, such as hair loss etc.
Anticancer Drug - DNA Interaction

We know that both RNA and DNA molecules consist of a ribose (RNA) or a deoxyribose (DNA) sugar backbone linked together by 3', 5' phosphodiester bonds. [Fig. 4.1]

![Sugar Backbone of the DNA Molecule Connected by Phosphodiester Linkage](image)

**Fig. 4.1**: Sugar Backbone of the DNA Molecule Connected by Phosphodiester Linkage

The bases shown in the Fig. 4.1 are aromatic ring structures and these are five, which are found in nucleic acids, Cytosine (C), Adenine (A) and Guanine (G) are common in both DNA and RNA. Thymine (T) is only found in DNA and Uracil (U) in RNA (only). Cytosine and adenine are based on structures called pyrimidines and the other three on purines. RNA existing in single strands, where as DNA forms the famous alpha helix structure discovered by Watson and Crick. In the alpha helix two strands of DNA run in opposite direction twisting about
themselves, held together by sets of complimentary hydrogen bonds before either adenine and thymine or cytosine and guanine. [Fig. 4.2] DNA replication involves unwrapping of the helix which is an important feature exploited by certain anti cancer treatments.

![Base Pairing of DNA](image)

**Fig. 4.2 : Base Pairing of DNA**

While considering the Drug-DNA interaction it is important to consider same structural features of alpha helix. The double helical DNA is not a uniform structure. There are places where the strands are further a part and where they are closer together, known as major and minor grooves.(2) In B-DNA, the major groove is wider than minor grooves, making it accessible to interacting molecule. The base pair arrangement for each groove is specific, each containing certain hydrogen bond donors and acceptors. Besides, the major grooves also contain the methyl group of thymine. The drug molecules and proteins have tendency to bind at these sites. The difference between the donor and acceptor groups in each groove is of great importance, which
makes it possible for drug molecules to distinguish selectively between the different bases and sequences of bases.

**Mechanism of Drug Action**

A molecule can bind with DNA in two different ways. Some drugs like doxorubicin can form non-covalent complexes through hydrogen bonding, either by intercalating between the stacked base pairs of the helix or binding to the major or minor groove. Whereas, other drugs like alteran and cis-platin form covalent bonds with DNA.

**Intercalating Drugs**

The first type i.e. intercalating drugs are in use for many years. The drug after intercalation in the double helix causes lengthening of DNA helix and perturbation of the phosphate back bone. To accommodate the intercalating molecule the DNA chain must lengthen and unwind slightly, which leads to long range deformation of DNA helix. Thus altering the structure and functionality of the molecule.\(^{3,4}\) This prevents RNA synthesis and replication of DNA, lending to cell death.

**Covalent Binding Drugs**

Some drugs which bind covalently to DNA are used to either add substituents on to base residues or to form cross links between different sections of DNA.
The first mechanism results in a base pairing mismatch during DNA replication and the DNA is fragmented by the enzymes which try to repair it. The second mechanism binds together the two strands of DNA helix preventing separation during the replication process.

Alkylating agents such as Alkeran, Mitomycin-C etc, can form strong electrophilicities. These can then react with nucleophilic sites on the DNA, normally the N7 in guanine. [Fig. 4.3] The N7 in guanine causes a conformational change in the base. But the drug may also deactivate DNA in any other tissue as well as the cancerous cells, because it may react non-selectively with any guanine residue, causing several side effects. These types of drug are used to treat bone marrow and advanced breast cancer.

Fig. 4.3 : Alkylation of DNA

Thus pharmaceuticals that interact with nucleic acids are a major target drug for research and development in the field of chemotherapy. In particular, most anticancer and chemotherapy
treatments involve molecules that interact directly with DNA and it is this important class of drugs which are in use in the present times as for as the treatment of different by types of cancers is concerned.

Chabner(6) et al. classified some important categories of chemotherapeutic agents as under:-

1. Alkylating agents
2. Antimetabolites
3. Antibiotics
4. Antimitotics.
CHAPTER IV

Adriamycin Analysis and Biosensor
INTRODUCTION

The anthracyclines antibiotic and its derivatives are among the most important antitumor agents. They are produced by the fungus Streptomycin, Peucetius Varcaesius. A number of important biochemical effects have been discovered for the anthracyclines, which could have a role in the therapeutic and toxic effect of the drug. These compounds can intercalates with DNA.

DNA Intercalation

It has already been said earlier that among the nucleic acids RNA exists as a strands, whereas DNA forms a double helical structure, also known as alpha helix structure. In this alpha helix two strands of DNA run in opposite directions twisting about themselves. The two strands are held together and A (Adenine) and T (Thymine) or C (Cytosine) and G (Guanine) bases. For Drug DNA-interaction certain structural features of the alpha helix are important.

The anticancer drug has to destroy the DNA replication i.e. DNA cross linking. The drug can do it in two different ways. Firstly drugs like doxorubicin can form non covalent complexes through hydrogen bonding in two ways:

(i) By intercalation between the stacked base pairs of the helix or binding.

(ii) By forming a covalent bond with the DNA.
As regards to DNA intercalating drugs, they are mainly antibiotic and are products obtained from microorganisms and have prominent antitumor activity. They mainly interaction between DNA strands and interfere with its template function. Actinomycin-D, Daunorubicin (Rubidomycin) Doxorubicin, Mitoxantrone, Bleomycin etc are the important DNA intercalating anticancer drugs, presently being widely used.

**ADRIAMYCIN**

<table>
<thead>
<tr>
<th>Commercial Name</th>
<th>Doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Name</td>
<td>[5,12-naphthacenedione,10-[(3-amino-2,3,6-tri-deoxy-alpha-L-lyxo-hexopyranosyl)-oxy]-,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxylacetyl)-1-methoxy-hydrochloride (8-cis)]</td>
</tr>
<tr>
<td>Molecular Formula</td>
<td>C_{27}H_{29}NO_{11}</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>579.99</td>
</tr>
<tr>
<td>Chemical Structure</td>
<td>![Chemical Structure Image]</td>
</tr>
<tr>
<td>Family</td>
<td>Anthracycline</td>
</tr>
</tbody>
</table>
It is an antibiotic anticancer drug, belongs to anthracyclenes family with a wide spectrum of chemotherapeutic applications\textsuperscript{[6]} and antineoplastic action. It causes very high cardio-toxicity that ranges from a delayed and insidious cardiomyopathy to irreversible heart failure.\textsuperscript{[7]}

The anticancer property of Adriamycin is known for the past thirty five years, but its pharmacokinetics and biochemical studies to establish its mechanism of action and improve its administration and anticancer activity are still an important goal to achieve.

Adriamycin was initially isolated from Streptomycin Paucities Varcaesitue\textsuperscript{[8]} and is water soluble. There is experimental evidence that the drug promotes oxidative damage to double strands DNA (ds-DNA) in cancerous cells through the generation of the reactive oxygen species and that it interacts with DNA through intercalation.\textsuperscript{[9,10]}

**Adriamycin-DNA Interaction**

Doxorubicin, also known as Adriamycin is an antibiotic used as a drug for the treatment of wide range of solid tumors. When the drug intercalates with DNA, the cyclohexane "A" ring resides in the minor groove acting as an anchor, hydrogen bonding to base pairs above and below. The planar 'D' ring residues in the major grooves. The drug is held in place by the formation of favourable hydrogen bonds to the bases with DNA.
However, these drugs are not specific to base pair sequences and show high level toxicity and may interact with DNA in many other tissues. It also inhibits the ability of enzymes such as topoisomerase-II to interact with DNA.

**Groove Binders**

Minor groove binding drugs bind to the exterior of the minor groove of the DNA rather than intercalating within the molecule which has the tendency to inhibit DNA and preventing the replication and growth of cancer cells. It also prevents molecules such as enzymes and proteins from being able to bind with and act upon DNA. In contrast intercalating drugs tend to contain fused aromatic hetero-cycles, minor groove binder tend to be un-fused aromatic heterocycles.

**SURVEY OF LITRATURE**

The antitumor activity of compounds is based on their ability to interact with DNA, causing changes in its structure and base sequence, which inhibits the DNA cross linking reaction. As such, in the field of medical science, the drug-DNA, interaction can be highly useful for evaluating the damage caused to DNA. Some carcinogens and oxidising substances cause oxidative damage to DNA structure and thus the study on the caused oxidative damage can prove highly useful in preventing perspective.
In the recent years the uses of modified electrodes have proved to be highly important for the study of interaction mechanism between substances of medicinal importance.\textsuperscript{(14)} They also work as electrochemical biosensors\textsuperscript{(15)} for a simple and inexpensive technique for the diagnosis of genetic diseases and the detection of pathogenic biological species.\textsuperscript{(16,17)} We know that the sequence specific hybridization of nucleic acids can be detected directly or by DNA intercalators,\textsuperscript{(18-22)} which generally work as anticancer drugs.

Adriamycin, an antibiotic drug of anthracyclines family, possesses a wide spectrum chemotherapeutic applications and antineoplastic action. Antitumor properties of Adriamycin are known for more than three decades but, the pharmacokinetic and biochemical studies to establish its \textit{in-vivo} mechanism of action and to improve its administration and anticancer activity are still an important goals to achieve. Literature records that Adriamycin and other analogous anthracyclines behave as DNA intercalators and their activity accumulates in nuclear genome.\textsuperscript{(23-26)}

The present work reported in this chapter is aimed that the fabrication of voltammetric DNA-biosensor on a GCFE and study the interaction of Adriamycin in situ with ds-DNA at a charged surface i.e. DNA modified GCFE and to propose a suitable mechanism for the DNA-Adriamycin interaction.
EXPERIMENTAL

Instrumentation:

An Elico (Hyderabad, India) μp-polarographic analyser model CL-362 was used for voltammetric studies. Glassy Carbon Fibers (NF-12, Sigti Eletitiogiafit, U.K.) were used for the fabrication of GCFE, and the pH-measurements were made on a Systronics (India) digital μ-pH meter system-361.

Chemicals:

The chemicals, used were of Anal-R/BDH grade. Calf thymus DNA, Himedia Lab. Pvt. Ltd., Mumbai and Adriamycin hydrochloride gift sample supplied by MS. Dabur Pharma. Ltd. Baddi (distt. Solan H.P.) were used for the present study. Solutions of the other chemicals were prepared in conductivity water.

Preparation of stock solution:

The stock solutions of DNA and Adriamycin were prepared in conductivity water using different concentrations of DNA (800μg/ml) and Adriamycin (200μg/ml). The solutions of the other reagent required for the experiment by dissolving a calculated amount in required volume of conductivity water. Experimental details recording voltammogram has already been discussed in previous chapters.

Supporting electrolyte:

1M acetate buffer as a supporting electrolyte for Adriamycin and DNA analysis was used.
RESULTS AND DISCUSSION

Adriamycin Analysis at Bare GCFE

When a solution of Adriamycin (4µg/ml) in a 0.1M acetate buffer of pH 4.5±0.1, was electrolysed using bare GCFE as working electrode, it produced two reduction peaks Ep= -0.45V and -0.60V vs SCE, on scanning the potential in cathodic direction. [Fig. 4.4] This peak is due to the reduction of 5,12-diquinone groups to produce a highly reactive semiquinone radical.\(^{27}\)

However, if the solution is electrolysed performing positive potential scanning of the working electrode, the resulting differential pulse voltammogram produced a well defined oxidation peak at +0.56V, [Fig. 4.5] which is due to the oxidation of 6,11-dihydroquinone of Adriamycin.\(^{28}\)

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**Fig. 4.4** : Differential Pulse Voltammogram for 80µg/ml Adriamycin (reduction) in 0.1M acetate buffer at pH 4.5±0.1 at bare GCFE
Fig. 4.5: Differential Pulse Voltammogram for 80μg/ml Adriamycin (oxidation) in 0.1M acetate buffer at pH 4.5±0.1 at bare GCFE

DNA Analysis at Bare GCFE

DNA at lower concentration did not produce any voltammetric response. However, at increased DNA concentration (800μg/ml) or more, very well defined peaks for its oxidation at the electrode surface was observed i.e. when a solution of DNA (800μg/ml) in 0.1M acetate buffer of pH 4.5±0.1 was electrolysed using bare GCFE, it produced three oxidation peaks at +0.40V, +0.75V and +1.09V [Fig. 4.6] respectively. The diffusion current/peak (s) current was found to be proportional to DNA concentration in analyte.

The first peak at +0.40V and the second peak at +0.75V may be due to oxidation of purine bases (guanine and cytosine). Whereas, the third peak at +1.09V is due to oxidation of adenine
site of DNA molecule. Significantly, ds-DNA did not produce any reduction peak under the given conditions.

![Graph showing differential pulse polarography](image)

**Fig. 4.6:** Differential Pulse Voltammogram for 800μg/ml ds-DNA in 0.1M acetate buffer at pH 4.5 ±0.1 at bare GCFE

**Study of Adriamycin-ds-DNA Interaction at Bare GCFE**

Looking, at the possibility of oxidation/reduction of Adriamycin at the bare GCFE, attempts have been made to study the possible interaction of the anticancer drug with ds-DNA. For the said purpose experimental sets of solutions were prepared by taking a fixed concentration of Adriamycin (20μg/ml) in 0.1M acetate buffer of pH 4.5±0.1, and varying the concentration of DNA from 80μg to 400μg/ml. The oxidation of Adriamycin was investigated for each set.

The first set i.e. without ds-DNA, produced a DP Voltammetric oxidation peak with peak potential at +0.56V vs SCE, which shifted to more electro positive potential with
increasing DNA concentration and the peak current shortened. The shift in Ep value and shortening of peak current may be explained on the basis of change of species that is oxidized at the GCFE surface, i.e. due to the formation of drug-DNA complex.\textsuperscript{[29]}

Although, the above findings approve the formation of Adriamycin-DNA complex, but to have a clear-cut understanding on the mechanism of the Drug-DNA interaction at charged surfaces, the GCFE has been modified in three different ways:

(I) **Adriamycin Modified GCFE**

Adriamycin is irreversibly adsorbed at glassy carbon fiber surface with or without applying deposition potential. As such, to prepare Adriamycin adsorbed (modified) GCFE, the electrode was dipped in a solution of Adriamycin 4\(\mu\)g/ml for 300 sec. at the deposition potential of +0.4V. The electrode was thoroughly rinsed with water and then used for voltammetric measurements.

To study the Adriamycin-DNA interaction the modified electrode was dipped in a voltammetric cell containing 80\(\mu\)g/ml ds-DNA and 0.1M acetate buffer of pH 4.5±0.1, and the DP Voltammogram was recorded. The voltammetric result clearly shows a big peak at +0.50V due to oxidation of adsorbed Adriamycin and the other peak may be due to oxidation of purine bases of DNA. The argument is based on the presumption that DNA diffuses from bulk of the solution on to electrode
surface and the chemisorbed Adriamycin intercalated into double helix of DNA. As such, the distance of double strand takes place, which allow the oxidation of purine bases.

Besides, if after the first scan, a potential of -0.6V was applied for 60 sec, and then the voltammogram was recorded [Fig. 4.7], it produced a peak at +0.40V. The appearance of this peak may be due to the interaction of Adriamycin with ds-DNA at guanine rich regions.

Fig. 4.7: Differential Pulse Voltammogram for a 80μg/ml ds-DNA solution in 0.1M acetate buffer at pH 4.5 ±0.1, after applying a potential of -0.60V during 60 sec. at Adriamycin modified GCFE

(II) Thin Layer ds-DNA Modified GCFE

A thin layer of ds-DNA was prepared by immersing the GCFE in 80μg/ml ds-DNA solution at +0.40V applied potential for 600 sec. This modified ds-DNA GCFE was dipped in 20μg/ml
Adriamycin solution for 180 sec, then rinsed with water and transferred to a polarographic cell containing acetate buffer solution of pH 4.5±0.1 and the differential pulse voltammogram was recorded. The DP Voltammogram for the oxidation of Adriamycin shows a well defined peak with peak potential +0.50V vs SCE. [Fig. 4.8] This peak may be attributed to the oxidation of 6,11-dihydroquinone group of the Adriamycin molecule.

However, after recording the oxidation peak, a negative potential of -0.60V was applied on the modified electrode for 60 sec followed by positive potential scanning. The voltammogram now showed two new peaks in addition to the Adriamycin oxidation peak. [Fig. 4.9]

![Differential Pulse Voltammogram](image)

**Fig. 4.8 :** Differential Pulse Voltammogram in 0.1M acetate buffer at pH 4.5±0.1, obtained with a thin layer ds-DNA modified GCFE after being immersed in 20μg/ml Adriamycin solution during 180 sec.
Fig. 4.9: Differential Pulse Voltammogram 0.1M acetate buffer at pH 4.5±0.1, obtained with a thin layer dsDNA modified GCFE after being immersed in 20μg/ml Adriamycin solution during 180 sec, after applying a potential -0.60V during 60 sec.

The peak at +0.90V may be attributed as due to 8-oxo-G (8-oxo-Guanine) oxidation. A clear separation of the peak due to 8-oxo-G and Adriamycin can be explained on the basis of non-uniform coverage of the GCFE surface by DNA and adsorption of Adriamycin at these uncovered surfaces. [The results are in good agreement with those observed using thick layer DNA modified GCFE]. This shift of 8-oxo-G peak to less positive potential informs about the DNA-Adriamycin interaction (damage to DNA). Authors have attempted to study the said interaction at bare GCFE, also as discussed earlier.
(III) Thick Layer ds-DNA Modified GCFE

Thick layer ds-DNA modified GCFE was fabricated by dipping GCFE in 25mg/ml solution of ds-DNA for 600 sec. It was then taken out and allowed to dry. The electrode was then dipped in a solution of Adriamycin (20µg/ml) for varying time intervals. The electrode was then taken out of Adriamycin solution, washed with distilled water and allowed to dry. The dried electrode was dipped in a solution of acetate buffer (0.1M) of pH 4.5±0.1 and the voltammogram was recorded.

Adriamycin produced a well-defined voltammetric oxidation peak with Ep value +0.50V. The height of the Adriamycin oxidation peak with respect to the time of immersion of the thick layer ds DNA modified GCFE in Adriamycin solution was investigated. [Fig. 4.10] The results showed a linear relationship between the peak height and time of immersion of the electrode in Adriamycin solution i.e.0.00 to 60 min, and then it attained a constant value. Thus, indicating the preconcentration of Adriamycin at the thick layer ds-DNA modified electrode surface, which was not possible with bare electrode.

Significantly, reproducible peak currents were observed for the similar time of immersion of the thick layer ds-DNA modified GCFE in Adriamycin solution for the first scan only. However, if the differential pulse voltammogram is recorded using the same modified electrode, an abrupt decrease in the peak current was observed. This suggests a fast consumption of the neoplastic drug at the modified electrode surface.
Fig. 4.10: Differential Pulse Voltammogram in 0.1M acetate buffer at pH 4.5±0.1, obtained with time of immersion of the thick layer ds-DNA modified GCFE in a 20μg/ml Adriamycin solution during 5 min - 60 min.

However, if the voltammograms are recorded separately using bare GCFE and thick layer ds-DNA modified GCFE and scanning the potential from -0.70V to -0.00V, the bare GCFE produced only one peak Ep= -0.56V. Whereas, at thick layer ds-DNA modified GCFE two peaks [Fig. 4.11] were observed at -0.60V and -0.45V respectively. The observed new peak at -0.45V speaks of a different interaction mechanism of Adriamycin-DNA, at the modified GCFE surface.

Since, Adriamycin is irreversibly adsorbed at the bare GCFE surface, it becomes necessary to clean the electrode each time before use. Whereas, the thick layer ds-DNA modified GCFE did not require cleaning of the GCFE. This clearly reveals that all
the Adriamycin is intercalated inside the ds-DNA film and could not reach the electrode surface. As such, it could be concluded that the voltammetric peaks, that are observed due to Adriamycin which was intercalated into thick layer of ds-DNA. Since, the voltammograms were recorded in the acetate buffer as supporting electrolyte solution only, the possibility of any contribution to the voltammetric peaks from Adriamycin present in solution is ruled out.

As such, a critical comparison of the [Fig. 4.5] & [Fig.4.11], the observed new peak at -0.45V in [Fig.4.11] may be attributed as due to the Adriamycin-guanine site (in DNA) interaction leading to a charge transfer reaction to from Adriamycin semiquinone and guanine radical cation. However, as regards to the peak at -0.60V in [Fig. 4.11] may be attributed to the reduction of the Adriamycin. Reduction of Adriamycin at bare GCPE produces a peak at -0.56V the shift in the peak potential for the Adriamycin reduction at the two different electrode surfaces may be explained as due to the change in the electrode surfaces.

However, if the ds-DNA modified GCPE after being dipped in Adriamycin for ten min., rinsed and immersed in a buffer of pH 4.5±0.1, was subjected to a potential of -0.60V for about 60 sec and then the voltammogram was recorded by positive scanning of the modified electrode potential, the voltammogram produced two new peaks [Fig. 4.12], one at +0.80V and other at +1.1V vs SCE. The former peak may be due to guanine oxidation and the later due to adenine oxidation\(^{(30)}\).
Fig. 4.11: Differential Pulse Voltammogram in 0.1M acetate buffer at pH 4.5±0.1, obtained with a thick layer ds-DNA modified GCFE after being immersed in 20μg/ml Adriamycin solution during 180 sec. with applying negative potential from -0.70V to -0.00V during 60 sec.

Fig. 4.12: Differential Pulse Voltammogram in 0.1M acetate buffer at pH 4.5 ±0.1, obtained with a thick later ds-DNA modified GCFE after being immersed in a 20μg/ml Adriamycin solution for 60 sec. at potential -0.60V.
PROPOSED MECHANISM

Adriamycin generates a free radical by way of transfer of an electron to its quinone portion. The highly reactive Adriamycin radical formed at -0.60V may oxidise the guanine site of ds-DNA in which it is intercalated within double helix. As such Adriamycin-DNA complex is formed; this stops DNA cross linking reaction. Adriamycin therefore works as an anticancer drug.

This argument is based on the basis of results obtained by other methods. \(^{12,31}\) In addition to this, peak at +0.56V as observed in case of pure Adriamycin oxidation, at bare GCFE shifts to less positive side i.e. +0.45V, which may be explained on the basis of interaction between Adriamycin and 8-oxo-G which is formed as a result of interaction of Adriamycin in guanine rich region of ds-DNA. As such, one electron transfer from guanine moiety to quinine leading to guanine cation formation appears to be the probable reaction. However, due to the tendency of guanine cation to undergo hydrolysis, and finally the semiquinone is further reduced to form Adriamycin and 8-oxo-G is formed. It’s **Mechanism Model-A** as follow :-
Mechanism Model-A : Mechanism of Electrochemical in-situ

Adriamycin Oxidative Damage to DNA

CONCLUSION

Voltammetric in-situ sensing of DNA oxidative damage caused by reduced Adriamycin intercalated into DNA is possible using ds-DNA modified GCFE. The results show that Adriamycin intercalated in double helix DNA can undergo oxidation or
reduction and react specifically with the guanine moiety and thus form mutagenic 8-oxo-G residue. A mechanism model for the reaction may be proposed.

The developed biosensor is of utmost relevance because the mechanism of interaction of DNA-Adriamycin at charged interfaces is parallel to \textit{in-vivo} DNA-Drug (Adriamycin) complex reaction, where DNA is in close contact with charged phospholipids membranes and proteins rather than when intercalation is in solution. It also promises the use of voltammetric techniques for in-situ generation of reaction intermediates. As such, is a complementary tool for the study of biomolecular interaction mechanism.
CHAPTER IVB

Mitomycin-C Analysis and Biosensor
INTRODUCTION

During world war-II the chemical warfare possibility of the use of nitrogen mustards was studied and scientists found that these alkylating agents suppressed lymphosarcoma, both in mice and in human beings. Since then these compounds have been used in treating a variety of human cancers. The alkylating agents replace the hydrogen in the guanine part of DNA and thus make it prone to breakage while replicating.

The study of interaction of DNA with legands of differing chiralities provides a rich ground for applications to mechanisms of molecular recognition, probing of polymorphism of DNA, and structure biological activity correlations. A comparison between the DNA interaction chemistry of individual members of enantiomeric or diastereomeric pairs of chiral metal complexes, antitumor agents and carcinogens have provided sharp insights into such phenomena.\textsuperscript{(32)} Some examples are the alkylation of DNA by the topoisomers of the carcinogenic benzo (a) pyrendiol epoxides.\textsuperscript{(33,34)} The natural and unnatural enantiomers of CC-1065 and enantiomeric pairs of CC-1065 functional derivatives.\textsuperscript{(35)}

DNA Alkylation

Despite the major advances that have been made in cancer research during 1990’s, the major mechanism by which most of
the clinically relevant DNA anticancer agent kill cells involves interfering with DNA replication. One of the simplest methods of bringing this about is via alkylation of DNA. The alkylation is brought about as the alkylating agent acts as an electrophilic trap for the neophilic sites in DNA.

Alkylation normally occurs on guanines or adenines as these are the most easily oxidized bases and are best nucleophiles. For a simple alkylating agent, the N7 position of guanine in the major groove is the most preferred site of alkylation. It is the most negative site anywhere within the bases of DNA. In the cases with more complex agents, the sites of alkylation can be controlled by non-covalent DNA interactions.

Simple alkylation of DNA results in many types of damages, including mismatching of DNA base pairs. The production of a purine sites and the formation of single strand breaks.

Bis-alkylation of DNA helix can lead to inter strand, intra strand and DNA protein cross links. It is generally recognized that the inter strands cross links are the most toxic as they can prevent strand separation during DNA replication and are difficult to repair due to involvement of both DNA strands. Some of these concepts can be illustrated with the clinically relevant quinone antitumor agents, like Mitomycin-C.
MITOMYCIN-C

Chemical Name: [1as-(1αα, 8β, 8bα)-6-amino-8-[(dninocarbonyl oxy)methyl]-1, 1a, 2, 8, 8a, 8b, hexahydro-8a-methoxy-5-methylazirinal-(2', 3': 34) pyrrolo (1, 2, - a] idole-4, 7; dione]

Molecular Formula: C₁₅H₁₈N₄O₅

Molecular Weight: 334

Chemical Structure:

```
\begin{align*}
\text{O} & \\
\text{C} & \\
\text{H₂N} & \\
\text{CH₂O} & \\
\text{NH₂} & \\
\text{H₃C} & \\
\text{O-CH₃} & \\
\text{N-H} & \\
\end{align*}
```

Family: Mitomycin – C

It is an antitumor antibiotic used clinically against cancer. It acts as a DNA alkylation agent forming mono adducts and bisadducts with guanine residues at their N² position, in minor groove of DNA.\(^{36}\)

Both DNA interstrand and intrastrand cross links are formed as the consequence of bisadducts of Mitomycin-C with two guanines in opposite strands and with two adjacent guanines in the same strand, respectively. The reactions with
DNA are dependent on reductive activation of Mitomycin, hence; it is regarded as the prototype bioreductive alkylating agent. The DNA-damage activities of Mitomycin represent the primary basis of its cytotoxicity.\textsuperscript{37,38}

\textbf{Mitomycin-C Inactivation}

Inactivation of antitumor drugs by Glutathione (GSH) is believed to be the general mechanism of resistance to drugs, which often develop in tumor cell populations. Resistance to Mitomycin-C has been correlated with elevated cellular GSH level.

\textbf{Mitomycin-DNA Interaction}

Mitomycin-C and other Mitomycines are isolated from various strains of Streptomyces and in particular, Streptomyces Caepitosus. Mitomycin-C is the best known of all aziridinylquinones and at the present time, is the most clinically relevant. The drug has three potentially active constituents viz. quinone, an unusual aziridine are utilized in its cytotoxic action. However, the mechanism of activation is yet to be investigated.

Under physiological pH Mitomycin-C is very stable but, becomes unstable on reduction by chemical reducing agents or reductive enzymes. Although, it is an accepted fact that Mitomycin-C has to undergo reduction in order to interact with DNA, but there has been some controversy as to which enzymes
are responsible for this process. Until recently, it was believed that Mitomycin-C toxicity was solely due to the formation of reactive semiquinone produced by the one electron reducing enzymes, but some research groups also produced evidence to show that Mitomycin-C is not a substrate for the obligatory two electron reducing enzyme, DT-diaphorase. It was therefore proposed that the hydroquinone plays no role in the activation of the quinone.\textsuperscript{(39-41)} Some other earlier studies had shown that reactive spices can be formed by selective one electron reduction by chemical agents or different one electron reducing enzymes indicating that the hydroquinone played no role in the cytotoxic mechanism.\textsuperscript{(42,43)}

However, the precise roles of the one or two electron reducing enzymes in the activation of Mitomycin-C becomes more confused when it was shown that the hydroquinone, formed from the dismutation of the semiquinones yields similar products to those produced from the different one electron reducing systems.\textsuperscript{(44)} It has now been proved that the Mitomycin-C is a substrate for UT-diaphorases\textsuperscript{(45)} and therefore the cytotoxicity of Mitomycin-C in different cell lines can be correlated with the intracellular levels of enzymes.

Some mitomycin-base adducts have been isolated and the main site of attack is at guanines with about 90% of the adducts at N-2 position.
SURVEY OF LITRATURE

Bioreductive chemotherapy is the most successful treatment for certain types of cancer. It is based on two major factors; the first to develop drugs that are activated by specific proteins and the second is to identify tumor types that are rich in these proteins. Besides bioreductive drug development is based on difference in oxygen content and cellular pH between normal and tumor tissues.\textsuperscript{46-52} Several natural and synthetic quinones including Mitomycin-C,\textsuperscript{48} indioquinone EO9, diaziquinone, methyl diaziquinone etc, have found their application in bioreductive therapy because of their potential to undergo reduction by different cellular enzymes in aerobic and hypoxic conditions.

Mitomycin-C is a prototypical bioreductive alkylating antitumor drug that is effective against several tumor tissues including colon, breast, lung, head and neck.\textsuperscript{53} It is activated metabolically to cause DNA alkylating, monofunctional adducts formation and DNA cross linking that leads to cytotoxicity and cell death.\textsuperscript{54,55} Some enzymes like cytosolic (NRO) and other unknown cytosolic proteins and microsomal (P-450) reductase etc, have been shown to catalyse Mitomycin-C activation, leading to DNA cross linking and cytotoxicity.\textsuperscript{56-62}

Enzymatic reductive activation of Mitomycin-C by NADPH has been studied by D. Gargiulo et al.\textsuperscript{63} They have used different analytical methods for their study.
The formation of molecular complex between Mitomycin-C and native DNA was examined by means of various experimental methods by G. Rodighiero et al.,\(^{(64)}\) P. Andrew and his co-workers\(^{(65)}\) have investigated the electrochemical reductive activation of Mitomycin-C and have assigned a mechanism for the interaction of Mitomycin-C with DNA. Besides, many other workers have reported the DNA alkylation by Mitomycin-C.

However, the uses of modified electrodes has not exclusively used for the study of Mitomycin-DNA interaction. The results of author's observation on the said interaction using modified GCFE have been reported in this chapter.

**EXPERIMENTAL**

**Instrumentation:**

An Elico (Hyderabad, India) µp polarographic analyser model CL-362 was used for voltammetric studies. Glassy Carbon Fibers (NF-12, Sigtí Eleititiofit, U.K.) were used for the fabrication of Glassy Carbon Fiber Electrode GCFE, and the pH-measurements were made on a Systronics (India) digital µ-pH meter system-361.

**Chemicals:**

Calf thymus DNA (Himedia Lab. Pvt. Ltd., Mumbai) and Mitomycin-C (gift sample from Ms. Chandra Bhagat Pharma Pvt. Ltd., Mumbai) were used for the present work. All other reagents
were of Anal-R/BDH grade. Conductivity water was used for the preparation of the other solutions.

**Preparation of stock solution:**

The stock solutions of DNA and Mitomycin-C were prepared in conductivity water using different concentrations of DNA (800µg/ml) and Mitomycin-C (200µg/ml). The solution of the other reagent required for the experiment by dissolving a calculated amount in required volume of conductivity water. Experimental details recording voltammogram has already been discussed in previous chapters.

**Supporting electrolyte:**

1M acetate buffer as a supporting electrolyte for Mitomycin-C, DNA analysis and its interaction mechanism was used.

**RESULTS AND DISCUSSION**

**Mitomycin-C Analysis at Bare GCFE**

On electrolysis a solution of Mitomycin-C (5µg/ml) in a 0.1M acetate buffer (pH= 5.6±0.1) using a bare GCFE as working electrode, two well defined differential pulse voltammetric (DPV) peaks with peak potentials Ep = -0.94V and -1.41V vs SCE were obtained, [Fig.4.14] which are due to the reduction of diquinone group to form hydroaninone.

However, if the solution is electrolyzed performing positive scanning unlike Adriamycin it did not produce any DPV response.
Fig. 4.13: Differential Pulse Voltammogram for Mitomycin-C (blank) in 0.1M acetate buffer at pH 5.6±0.1 at bare GCFE

Fig. 4.14: Differential Pulse Voltammogram for 5μg/ml Mitomycin-C in 0.1M acetate buffer at pH 5.6±0.1 at bare GCFE
Study of Mitomycin-DNA Interaction at Bare GCFE

Looking at the possibility of the reduction of Mitomycin-C at the GCFE, the author has attempted to study the possible interaction of the anticancer drug with ds-DNA. For which experimental sets were prepared by taking a fixed concentration of Mitomycin-C (15μg/ml) in 0.1M acetate butter of pH 5.6±0.1 and the DNA concentration was varied from 10μg/ml to 150μg/ml, and the DPV response for each set was studied at the GCFE.

The differential pulse voltammograms showed gradual negative shift in Ep values for both the Mitomycin-C peaks. Thus indicating complex formation between Mitomycin-C and ds-DNA. Similar results were observed if the potential was scanned upto -1.20V only. Thus, indicating that only one electron reduction of Mitomycin-C is sufficient to electrochemically activate Mitomycin to form ds-DNA-Mitomycin complex.

However, to ascertain the binding sites for complex formation between Mitomycin-C and ds-DNA, the test solutions were prepared by taking 15μg/ml DNA in 0.1M acetate butter of pH 5.6±0.1, and the Mitomycin-C concentration was varied from 00μg/ml to 250μg/ml and DPV response of each set at the GCFE was recorded with positive scanning of the potential. The first set without Mitomycin-C showed a well defined peak Ep = +0.75V vs SCE in addition two other peaks [Fig. 4.3] which shifted to less
electropositive value with increasing Mitomycin-C concentration and the peak height gradually decreased. [Fig. 4.15]

Since the peak at +0.75V is due to the oxidation of guanine of DNA. The shift in Ep value and decrease in peak height clearly speaks the Mitomycin-C combination with DNA through guanine.

It was also observed that the shift in peak potential attains a constant value after a definite Mitomycin-C concentration and the peak height also remains constant. Thus indicating a combination of Mitomycin-C with guanine sites of the DNA, without any further product formation.

**Fig. 4.15: Differential Pulse Voltammogram for complex between 15µg/ml ds-DNA & 15µg/ml Mitomycin-C in 0.1M acetate buffer at pH 5.6±0.1 at bare GCFE**
Modification of GCFE as Biosensor

However, to throw deep insight for assigning DNA-Mitomycin-C interaction mechanism the GCFE was modified in the following ways to use it as a biosensor.

[I] Mitomycin-C Modified GCEF:

Like Adriamycin, Mitomycin-C is also irreversibly adsorbed at the Glassy Carbon Fiber surfaces with or without applying deposition potential.

To prepare Mitomycin-C modified (adsorbed) GCFE, the electrode was dipped in 20μg/ml solution of Mitomycin-C for 300 sec. at a deposition potential of -1.20V. It was taken out of the solution, rinsed with conductivity water, dried and used for DPV studies of Mitomycin-ds-DNA interaction. The modified electrode was dipped in voltammetric cell containing 0.1M acetate buffer of pH 5.8±0.1 and 50μg/ml DNA.

The DP voltammogram was then recorded using the anodic potential scanning. The resulting voltammogram produced a big peak at +0.65V vs SCE [Fig. 4.16] due to the guanine oxidation of Mitomycin-ds-DNA complex which clearly speaks that the distortion of double strand has taken place allowing the oxidation of guanine base, whose Ep value has undergone a shift from +0.75V for pure guanine to +0.65V for guanine present in Mitomycin-C-DNA complex.
Fig. 4.16 : Differential Pulse Voltammogram in 0.1M acetate buffer at pH 5.6±0.1, obtained with Mitomycin-C modified GCFE after being immersed in 50μg/ml ds-DNA solution during 300 sec

(II) Thin Layer ds-DNA Modified GCFE:

For the fabrication of thin layer ds-DNA modified GCFE, the electrode was dipped in a 80μg/ml ds-DNA solution at +0.4V applied potential for 600 sec. It was then taken out from the solution, washed, dried and dipped in a solution of Mitomycin-C (500μg/ml) for 180 sec. It was rinsed with water and placed in a voltammetric cell containing 0.1M acetate buffer solution of pH 5.6±0.1 and the DPV was recorded. The voltammogram produced two peaks at -0.95V and -1.56V vs SCE, [Fig. 4.17] due to the reduction of diquinone of Mitomycin-C.

However, after recording the above peaks the potential of the electrode was fixed to -1.20V for about 60 sec. followed by positive potential scanning.
Fig. 4.17: Differential Pulse Voltammogram in 0.1M acetate buffer at pH 5.6±0.1, obtained with thin layer ds-DNA modified GCFE after being immersed in 50μg/ml Mitomycin-C solution during 180 sec

Fig. 4.18: Differential Pulse Voltammogram in 0.1M acetate buffer at pH 5.6±0.1, obtained with thin layer ds-DNA modified GCFE after being immersed in 50μg/ml Mitomycin-C solution during 3 min, after applying a potential -1.20V during 180 sec
The differential pulse voltammogram clearly shows a peak at +0.60V vs SCE, [Fig. 4.18] due to the oxidation of Mitomycin-DNA complex through the guanine site. This shift in Ep value to less positive potential confirms the Mitomycin-DNA complex formation through guanine site of DNA, which is in support to authors observation for the said Mitomycin-DNA interaction at bare electrode.

(III) Thick Layer ds-DNA Modified GCFE

A thick layer ds-DNA modified GCFE was fabricated by dipping the electrode in 25mg/ml ds-DNA solution for 500 sec. It was taken out of this solution and allowed to dry. The electrode was then dipped in a solution of Mitomycin-C (50μg/ml) for varying time intervals. Each time the electrode was taken out of the solution, rinsed with conductivity water and dried. The dried electrode was dipped in a solution of 0.1M acetate buffer pH 5.6±0.1 and DP Voltammogram was recorded. The DP Voltammogram showed two peaks with Ep = -0.95V and -1.56V vs SCE, [Fig. 4.19] for the two electron reduction of Mitomycin-C at quinone site. The ip for each peak of the Mitomycin-C was found to be proportional to the Mitomycin-C concentration form 0.0 to 60 min, and then it attained a constant value. Thus, indicating preconcentration of Mitomycin-C at DNA modified GCFE surface, which was not possible with bare GCFE.
Fig. 4.19: Differential Pulse Voltammogram in 0.1M acetate buffer at pH 5.6±0.1, obtained with thick layer ds-DNA modified GCFE after being immersed in 50μg/ml Mitomycin-C solution during 0 min – 60 min.

Significant reproducible peak currents were observed for the similar time of immersion of the thick layer ds-DNA modified GCEF in Mitomycin solution for the first scan only. However, if the same modified electrode is used to record DPV for repeating the experiment, an abrupt decrease in the peak current has observed. Thus, indicating a fast consumption of Mitomycin-C at this modified electrode surface.

Mitomycin-C is irreversibly adsorbed at GCFE surface, it therefore becomes necessary to clean the electrode each time before use, whereas, the thick layers ds-DNA modified GCFE does not require pre cleaning before use. It clearly explains that
Mitomycin-C which is covalently bonded to DNA does not reach the electrode surface. It may also be concluded that voltammetric peaks that are observed due to Mitomycin-C bonded to DNA, since the voltammogram was recorded in a solution of acetate buffer of pH 5.6±0.1 only. As such, any contribution to the voltammetric peaks due to Mitomycin-C present in solution is ruled out. As such the peak observed at +0.65V may be attributed due to Mitomycin-guanine site (in DNA) interaction due to possible covalent bonding.

**PROPOSED MECHANISM**

On the basis of the above voltammetric studies and relevant literature a reaction mechanism model for the electrochemical activation of Mitomycin-C and its interaction with the anticancer DNA alkylating agent Mitomycin-C may be proposed as under **Mechanism Model-B**.

The Mitomycin-C at the electrode surface undergoes two electron reductions to form hydroquinone.

This hydroquinone forms reaction intermediates (C) and (D). The quinone methide (D) reacts with DNA to produce mainly adducts at the N-2 position of guanine in the minor groove. The formation of guanine N₂/N-2 cross linked adducts has been confirmed by isolation of the adducts from mouse mammary tumors following Mitomycin-C treatment by Tomaz et al.(43) and Bizanek et al.(46)
Mechanism Model-B: Proposed Mechanism for the Electrochemical Activation and DNA Cross Linking of Mitomycin-C

CONCLUSION

Electrochemical activation of Mitomycin-C caused by the reduction of the anticancer alkylating agent Mitomycin-C has been studied, the results show that Mitomycin is covalently linked with DNA to form guanine N$_2$/N-2 cross linked adducts.
Thus inhibiting the multiplication of DNA. As such the alkylating agent Mitomycin-C works as an anticancer drug.

The fabricated biosensor is of utmost relevance because the *in-vivo* mechanism of interaction of ds-DNA Mitomycin-C which is caused due activation of Mitomycin-C by some enzyme activators which reduce the quinone of the compound forming hydroquinone which forms reactive intermediates as shown in **Mechanism Model-B** is parallel to the electrochemical reduction of Mitomycin-C to form reaction intermediates. Hence, is a complementary tool for the study of biomolecular interaction mechanism.
REFERENCES


