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Ruthenium Complexes as DNA Photonucleases in Treatment of Malignant Skin Carcinoma

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RUTHENIUM COMPLEXES AS DNA PHOTONUCLEASES IN TREATMENT OF MALIGNANT SKIN CARCINOMA

BY

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ABSTRACT:

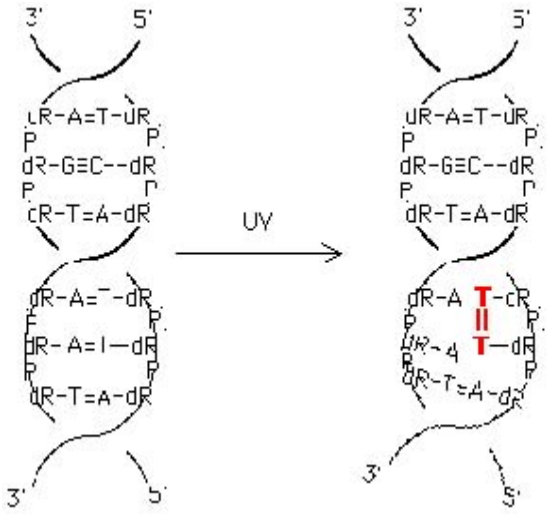
A variety of Ruthenium complexes possessing terpyridine complexes as ligands in their coordination sphere have been investigated extensively as DNA photo nucleases in vitro[1,2]. This work has led to the realization that compounds of Ruthenium bind to DNA purines and that the interactions are of an unprecedented bridging variety. Owing to the molar absorptivity of these complexes in the visible region, direct cleavage of duplex DNA by these complexes requires irradiation in the range of 400-500 nm.

Although the observation of DNA photo cleavage *in vitro* makes these systems good candidates for further investigation, it does not provide any information on whether the compounds would enter cells in vivo, cause cellular damage or death, or be able to penetrate the nucleus to affect DNA photo cleavage. In the present study, we explore the DNA photo cleavage using gel electrophoresis, cytotoxicity, photocytotoxicity and nuclear DNA damage by the Ruthenium compounds, which were recently shown to possess an emissive, pH- dependent ligand centered excited state.

INTRODUCTION:

Skin cancer is the most common form of cancer in the United States. More than 3.5 million cases in two million people are diagnosed annually[3]. The incidence of many common cancers is falling, but the incidence of melanoma continues to rise significantly, at a rate faster than that of any of the seven most common cancers[4]. According to the U.S. Department of Health and Human Services, Ultraviolet radiation is a proven carcinogenic to humans. Frequent tanners using new high-pressure sunlamps may receive as much as 12 times the annual UVA dose compared to the dose they receive from sun exposure[5]. UV light (200 – 300 nm) causes

exogenous, irreversible damage to the DNA. UV-B radiation causes cross-linking of adjacent cytosine and thymine bases creating pyrimidine dimers. This is called direct DNA damage. UV-A light results in the formation of free radicals. The damage caused by free radicals is called indirect DNA damage.



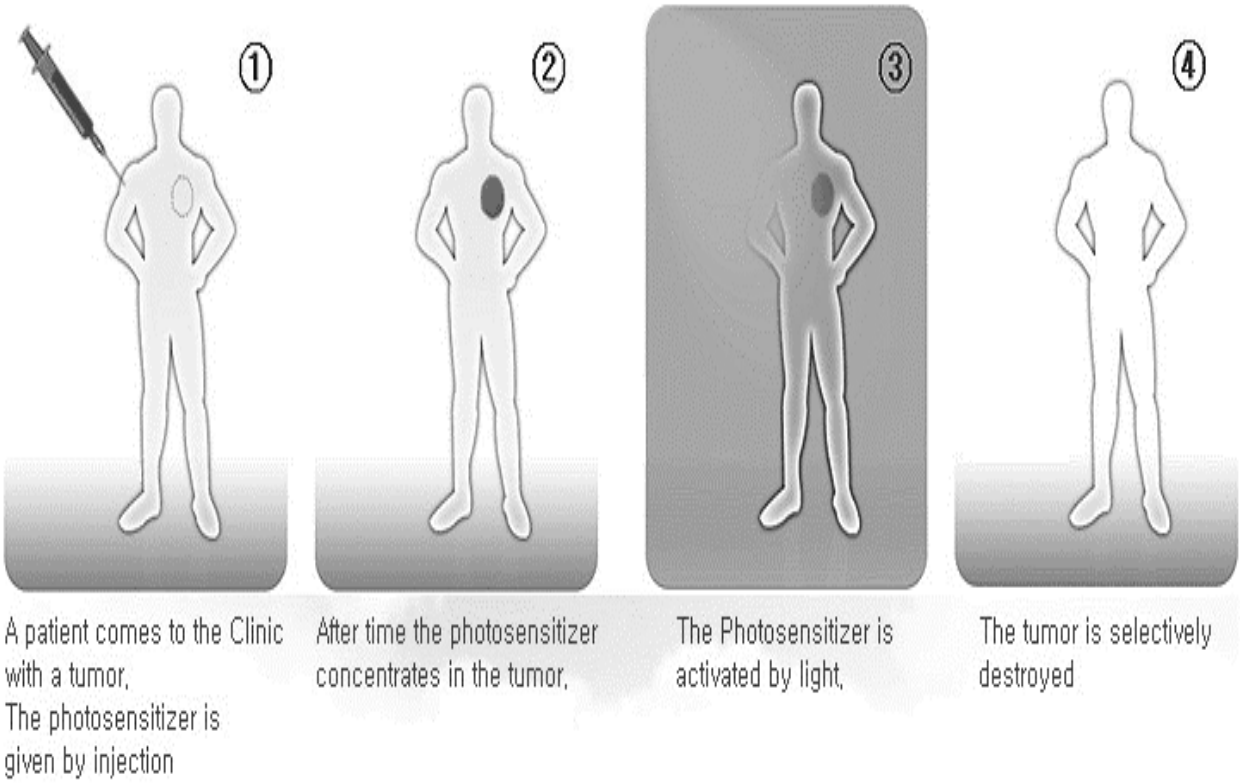
If the doctor suspects that a spot on the skin is melanoma, the patient will need to have a biopsy. In this procedure, the doctor tries to remove all of the suspicious-looking growth. This is an excisional biopsy. If the growth is too large to be removed entirely, the doctor removes a sample of the tissue. The doctor will never "shave off" or cauterize a growth that might be melanoma. This might cause metastasis of the cancer. Some cancer cells acquire the ability to penetrate the walls of lymphatic and/or blood vessels, after which they are able to circulate through the bloodstream (circulating tumor cells) to other sites and tissues in the body. After the tumor cells come to rest at another site, they re-penetrate through the vessel or walls, continue to multiply, and eventually another clinically detectable tumor is formed. This new tumor is known as a metastatic or secondary tumor. If the cancer spreads to other tissues and organs, it may decrease a patient's likelihood of survival. At present, the treatments available for skin cancer are Surgery,

Chemotherapy, Biological therapy, Radiation therapy and Photodynamic therapy. Of these, surgery and chemotherapy are conventional, painful and have a large number of side effects like diarrhoea, anorexia, fatigue and hair loss. Biological therapy includes use of interferons, interleukins, colony-stimulating factors, monoclonal antibodies, vaccines, gene therapy, and nonspecific immunomodulating agents and it is associated with flu-like symptoms including fever, chills, nausea, vomiting, appetite loss and increase in blood pressure. Radiation therapy can cause many side effects. Some are minor and diminish after therapy is stopped. The side effects include fatigue, skin inflammation in the treated areas, frequent or uncomfortable urination and rectal bleeding or irritation. Some side effects, however, are permanent. Bowel function may never become normal even after treatment is stopped. Impotence can occur up to 2 years post-treatment in some patients.

PHOTODYNAMIC THERAPY (PDT):

Photodynamic therapy (PDT) is a treatment that uses a drug, called a photosensitizer or photosensitizing agent, and a particular type of light. When photosensitizers are exposed to a specific wavelength of light, they produce a form of oxygen that kills nearby cells[7,8,9].Each photosensitizer is activated by light of a specific wavelength[8,9]. This wavelength determines how far the light can travel into the body[9,10]. Thus, doctors use specific photosensitizers and wavelengths of light to treat different areas of the body with PDT. In the first step of PDT for cancer treatment, a photosensitizing agent is injected into the bloodstream. The agent is absorbed by cells all over the body but stays in cancer cells longer than it does in normal cells. Approximately 24 to 72 hours after injection[7], when most of the agent has left normal cells but

remains in cancer cells, the tumor is exposed to light. The photosensitizer in the tumor absorbs the light and produces an active form of oxygen that destroys nearby cancer cells[7,8,9].



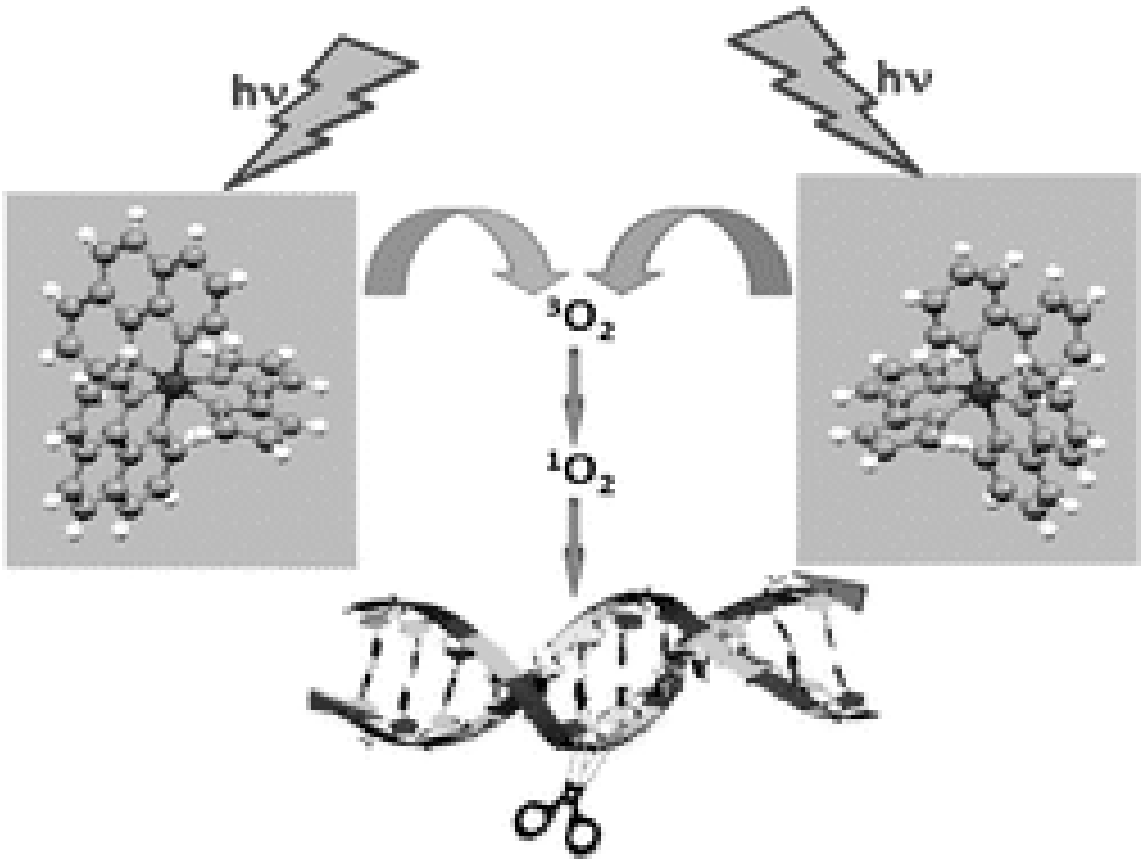
In addition to directly killing cancer cells, PDT appears to shrink or destroy tumors in two other ways[1,7,8,9]. The photosensitizer can damage blood vessels in the tumor, thereby preventing the cancer from receiving necessary nutrients. In addition, PDT may activate the immune system to attack the tumor cells. The light used for PDT can come from a laser or light-emitting diodes (LEDs). Photosensitizers tend to build up in tumors and the activating light is focused on the tumor. As a result, damage to healthy tissue is minimal. However, PDT can cause burns, swelling, pain, and scarring in nearby healthy tissue[9]. Other side effects of PDT are related to the area that is treated. They can include coughing, trouble swallowing, stomach pain, painful breathing, or shortness of breath but these side effects are usually temporary.

DNA PHOTONUCLEASES:

DNA photonucleases or Photosensitizers (PS) are one of the key elements in PDT. After the approval of Photofrin® for PDT treatment, researchers from all over the world became actively involved in developing efficient compounds that can be used as photosensitizers. An ideal PS should fulfill the following requirements –

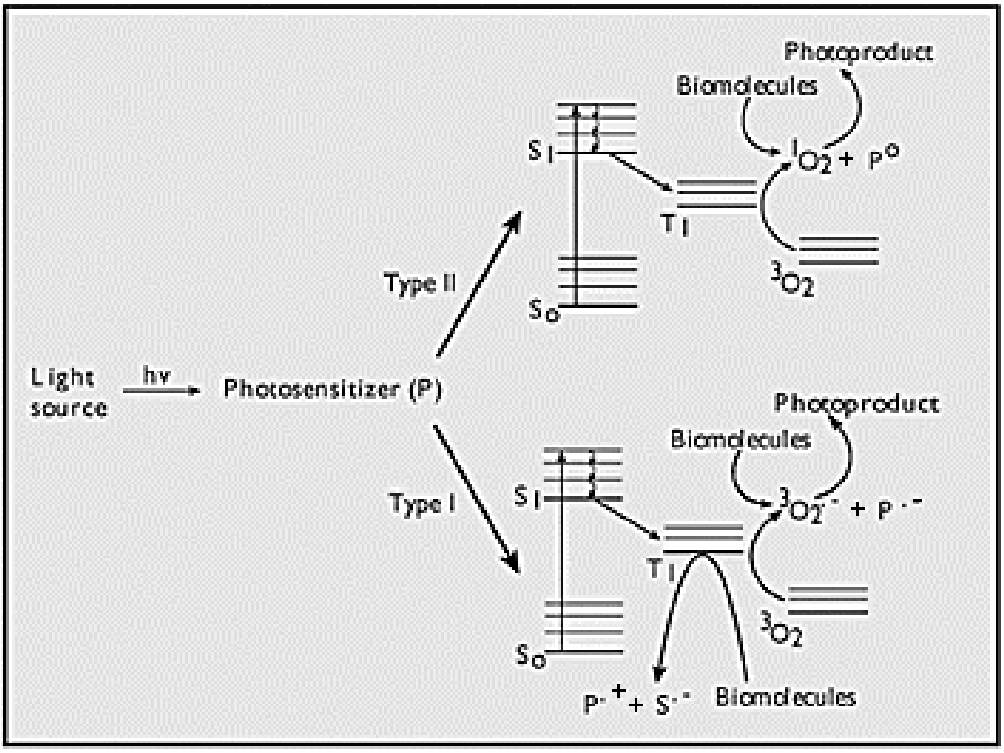
a) It should be able to produce singlet oxygen efficiently because singlet oxygen and Norrish type II photochemical reaction is responsible for the majority of lesions generated during PDT.
b) It should have high absorption coefficient.
c) It should have no dark toxicity, minimal or absent skin photosensitivity, and should selectively accumulate in tumor tissue, in order to minimize skin sensitivity.
d) The distribution of PS is important in PDT processes and is influenced by its chemical structure. It is particularly useful if PS is amphiphilic, which should facilitate the crossing of cell membranes.
e) It should be stable and easy to dissolve in the injectable solvents. However, after administration, the compounds should show high tumor accumulation and rapid clearance from the system.
f) It should be chemically pure and can be obtained in a short and high yielding synthetic route.

Unfortunately, till date, no PS with all these ideal characteristics has been developed.



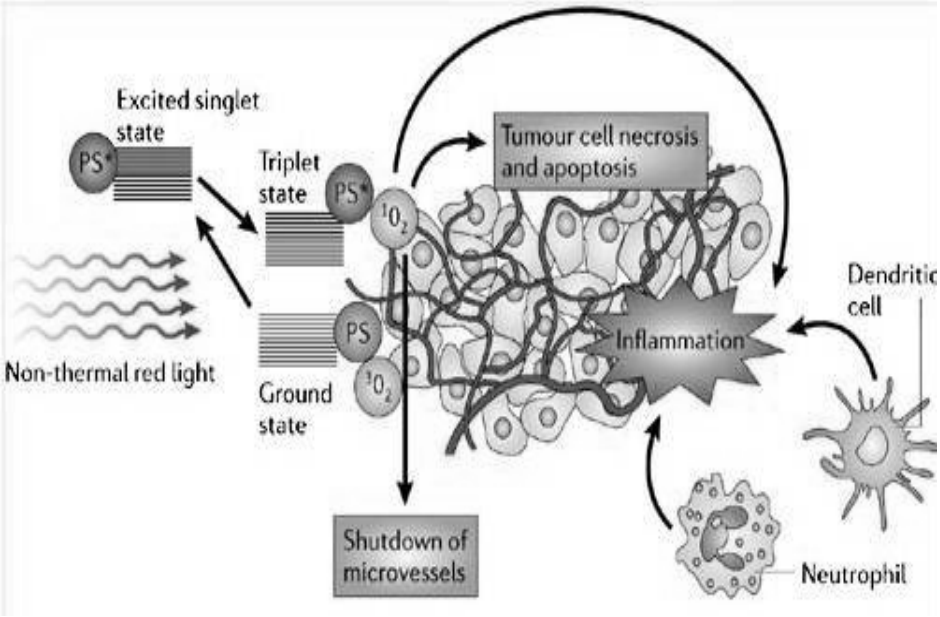
The DNA photonucleases get excited by light of a specific wavelength. This excitation uses either visible or near-infrared light. In photodynamic therapy, either the photo sensitizer molecule or the metabolic precursor of one is administered to the patient. The tissue to be treated is exposed to light suitable for exciting the photo sensitizer. Usually, the photo sensitizer is excited from a ground triplet state to an excited singlet state. It then undergoes intersystem crossing to a longer-lived excited triplet state. One of the few chemical species present in tissue with a ground triplet state is molecular oxygen. When the photo sensitizer and an oxygen molecule are in proximity, an energy transfer can take place that allows the photo sensitizer to relax to its ground singlet state, and create an excited singlet state oxygen molecule. The ROS, generated in PDT, damage the biomolecules, such as proteins and lipids, generating photoproducts. The mechanisms of

ROS production can be type I, type II or both. The superoxide and other radicals species are generated with the type I mechanism by electron transfer from photo sensitizer, in the excited triplet state (T_1), to ground-state oxygen ($^3O_2$). Singlet oxygen is generated with the type II mechanism by energy transfer from photo sensitizer T_1 to $^3O_2$[12,13].



Type I radical mechanisms may work to a limited extent in the absence of oxygen based on the oxygen independent photosensitized cross-linking of DNA. However, this does not lead to cell photoinactivation[11]. Singlet oxygen is a very aggressive chemical species and will very rapidly react with any nearby biomolecules. Ultimately, these destructive reactions will kill cells through apoptosis or necrosis.

MECHANISM OF CELL APOPTOSIS:



The lifetime of photo sensitizer molecule in the triplet state in tissues is about 10μs[11], which is long enough or the triplet state oxygen to form singlet oxygen, therefore is dependent on the oxygen concentration. It is therefore, realized that the oxygen dependency of PDT in cells is a crucial factor. Most normal tissues contain about 5% oxygen, which is supplied by blood circulation. The reason why they contain less than 20% is simply respiration. But, the skin cells are often inflamed, well vascularized, and have slightly higher temperatures. Hence, the photo sensitizers has good selectivity for skin cancer cells[11]. PDT is unique in its ability to induce extensive apoptosis of a cancer cell. PDT causes acute inflammation, expression of heat-shock proteins, invasion and infiltration of the tumor by leukocytes, and might increase the presentation of tumor-derived antigens to T cells. The hallmarks of apoptosis in animal cells include chromatin condensation, inter-nucleosomal DNA cleavage, cell fragmentation, and formation of apoptotic bodies. These bodies are removed by scavenging macrophages[14]. DNA

fragmentation in particular has been used as an indication of apoptosis, and several simple assays have been used to assess the extent of DNA fragmentation in apoptotic cells. For example, agarose gel electrophoresis[15] is used to demonstrate the ladder pattern of DNA which is generated by endonucleolytic cleavage of genomic DNA into nucleosomal size DNA of approximately 180 bases long (monomers) or oligonucleotides, which are multiples of 180 bases (oligomers)[16].

TYPES OF INORGANIC PHOTOSENSITIZERS BEING TESTED:

Various metal compounds have been tested as possible photosensitizers for PDT[17]. Some of them are –

A) Transition metal complexes: Metal complexes can act as prospective photosensitizers through both energy and electron transfer to oxygen molecules. Most of the studies on photogeneration of singlet oxygen involve polypyrrolic dyes and their metalloderivatives, but there is a significant number of other metal complexes capable of photosensitized singlet oxygen generation. A low energy triplet excited state of long lifetime is a prerequisite for this phenomenon. Mono and dinuclear Ru^{II}, Cr^{III}, Os^{II}, Ir^{III} and Pd^{II} complexes with polypyridines, can be used as singlet oxygen photogenerators.
B) Macrocyclic Photosensitizers modified by metal ions: Insertion of a metal ion to a polypyrrolic photo sensitizer can modify properties of its ground and excited state in such a way that not only photophysical and spectroscopic properties of the photo sensitizer will be modulated, but also its hydrophobicity, degree of aggregation, stability and consequently the route of the photo sensitizer transport into the cell and its further distribution.

C) Semicondutors: Redox properties of excited semiconductor particles, especially TiO_2 are responsible for high efficiency of ROS generation. TiO_2 itself shows a very weak or no toxicity in vitro and in vivo. A significant cytotoxicity of TiO_2 particles irradiated with UV light has been reported in the context of PDT applications.

RUTHENIUM COMPLEXES AS PHOTOSENSITIZERS[17]:

The drastic growth in research and development of DNA photonucleases based on Ruthenium complexes has been seen over the last decade. Various strategies have been used to improve the sequence selectivity of cleavage, which can arise from the preferential binding or activity at a certain site. Several mechanisms can be engaged in photoactivated DNA cleavage by Ruthenium complexes:

a) Oxidation of the base by singlet oxygen formed via energy transfer from the triplet excited state of the photocleaver
b) Direct electron transfer from the base to the excited state of the photocleaver
c) Oxidation of the base by Ru^{III} complex formed *in situ* by oxidative quenching of the triplet excited state of the corresponding complex

There are three main properties that make ruthenium compounds well suited to target the cancer cells:

(i) rate of ligand exchange

(ii) the range of accessible oxidation states and

(iii) the ability of ruthenium to mimic iron in binding to certain biological molecules.

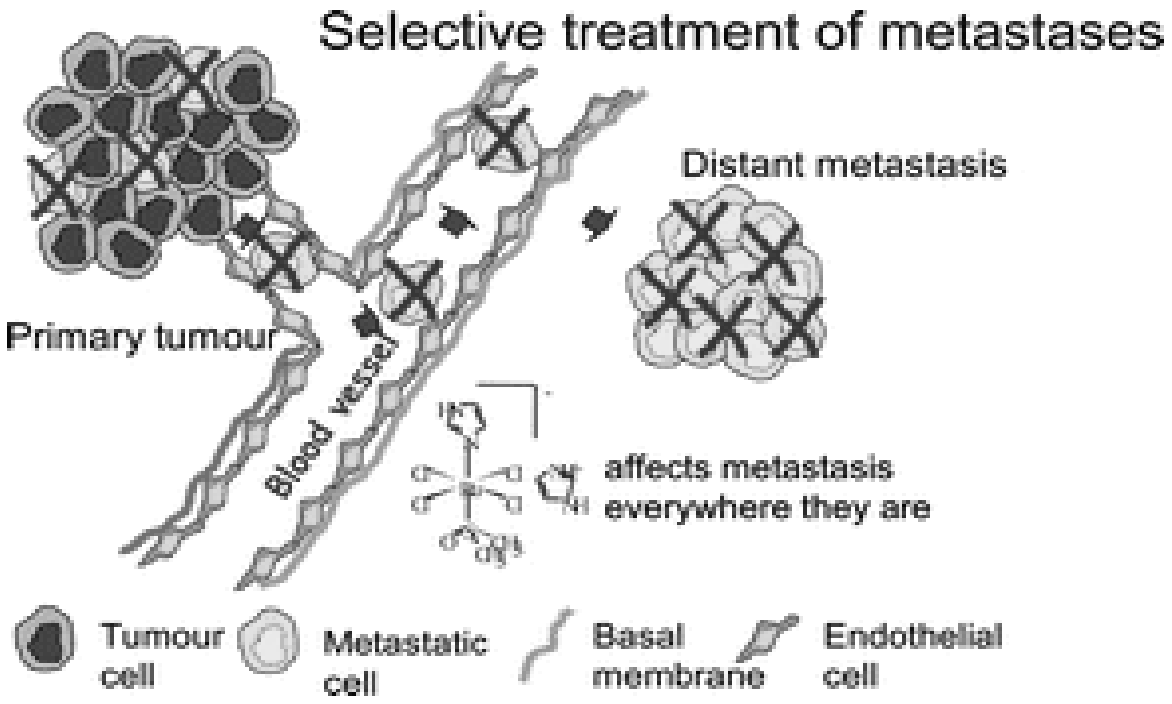
(i) Ligand Exchange[18]

Many ruthenium complexes have been evaluated for clinical applications, particularly in the treatment of cancer, due in part, to Ru(II) and Ru(I1I) complexes having similar ligand exchange kinetics to those of Pt(II) complexes. Ligand exchange is an important determinant of biological activity, as very few metal drugs reach the biological target without being modified. Most undergo interactions with macromolecules, such as proteins, or small S-donor compounds and/or water. Some interactions are essential for inducing the desired therapeutic properties of the complexes. As the rate of ligand exchange is dependent on the concentration of the exchanging ligands in the surrounding solution, diseases that alter these concentrations in cells or in the surrounding tissues can have an effect on the activity of the drug.

(ii) Oxidation State[18]

Ruthenium is unique amongst the platinum group in that the oxidation states Ru(Il), Ru(III) and Ru (IV) are all accessible under physiological conditions. In these oxidation states the ruthenium centre is predominantly hexacoordinate with essentially octahedral geometry, and Ru (III) complexes tend to be more biologically inert than related Ru(II) and (IV) complexes. The redox potential of a complex can be modified by varying the ligands. In biological systems glutathione, ascorbate and single electron transfer proteins are able to reduce Ru(III) and Ru(IV), while molecular oxygen and cytochrome oxidase readily oxidize Ru(II). The redox potential of ruthenium compounds can be exploited to improve the effectiveness of drugs in the clinic. Cancer cells are known to have higher levels of glutathione and a lower pH than healthy tissues, creating a strongly reducing environment. If the active Ru(II) complex leaves the low oxygen environment, it may be converted back to Ru(III) by a variety of biological oxidants. Proteins

that can catalyse the reduction of Ru(III) to Ru(II) include mitochondrial and microsomal single electron transfer proteins. The mitochondrial proteins are of particular interest in drug design as apoptosis, the desired mechanism for cell death, can be initiated in the mitochondria, as well as by other pathways, for instance, by the Fas/FasL pathway. Transmembrane electron transport systems can also reduce Ru(III) complexes outside of the cell and this is highly relevant to the mechanism of action of a ruthenium based drug in clinical use which has anticancer activity independent of cell entry.



(iii) Iron Mimicking

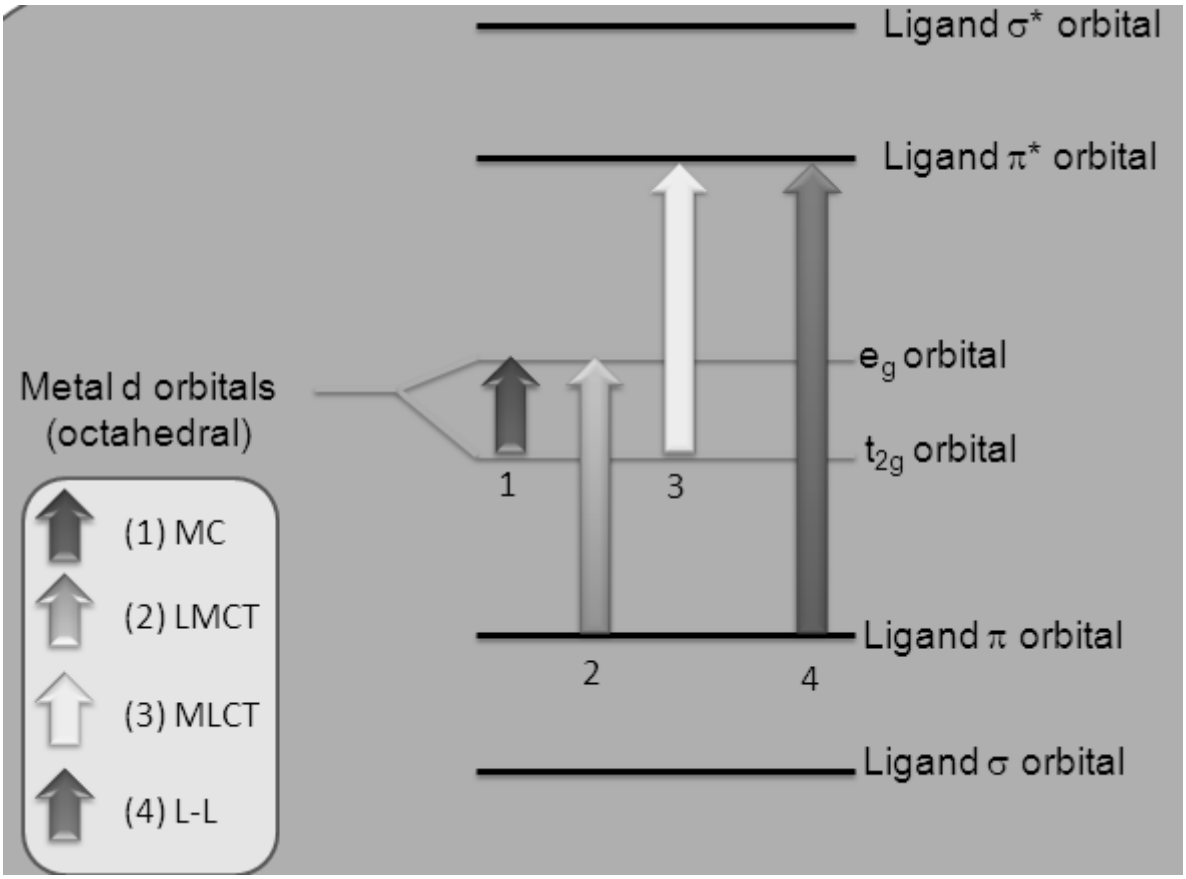
The low toxicity of ruthenium drugs is also believed to be due to the ability of ruthenium to mimic iron in binding to many biomolecules, including serum transferrin and albumin. These two proteins are used by mammals to solubilise and transport iron, thereby reducing its toxicity.

Since rapidly dividing cells, for example microbially infected cells or cancer cells, have a greater requirement for iron, they increase the number of transferrin receptors located on their cell surfaces, thereby sequestering more of the circulating metal loaded transferrin. As a result, ruthenium drugs bind more selectively to the cancer cells and hence are less toxic to the surrounding normal cells.

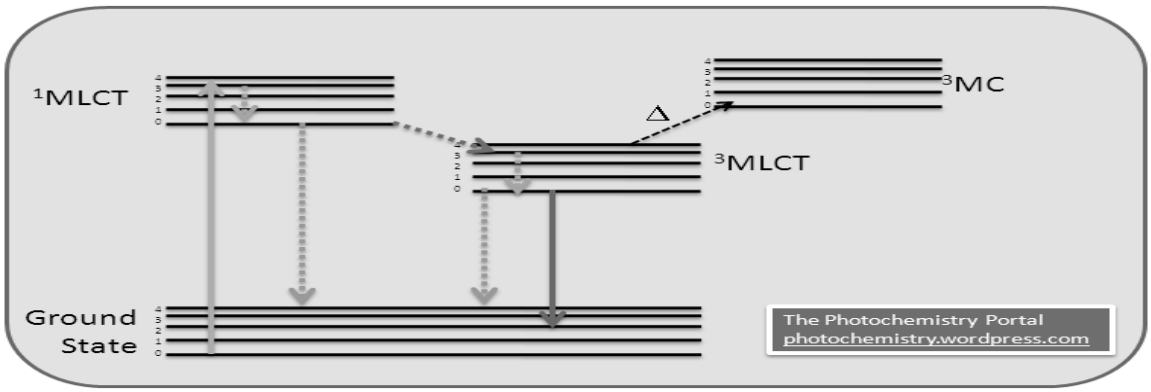
PHOTOCHEMISTRY OF RUTHENIUM COMPOUNDS[19,20]:

Ruthenium compounds have been selected as photosensitizers because of their unique combination of chemical stability, redox properties, excited state reactivity, luminescence emission and excited state lifetime. Ru^{2+} is a d^6 system and the polypyridine ligands are usually colourless molecules possessing the σ donor orbitals localized on the nitrogen atoms and π donor and π^* acceptor orbitals more or less delocalized on aromatic rings. This opens up a range of new transitions, aside from the HOMO-LUMO transition observed in organic chromophores. This transition in inorganic photochemistry is called a ligand-field or ligand-ligand transition, as the excited state electron is located on the ligand. Apart from this, because of the presence of the metal's molecular orbitals, 3 other transitions are available:

a) d-d transition, where an electron is excited from a metal orbital to an unoccupied metal orbital. This is usually referred to as a metal centred (MC) transition
b) Transitions between the metal and the ligand. These can be either an electron excited from the ligand to the metal, called Ligand to Metal Charge Transfer (LMCT) or from the metal to the ligand (MLCT).
c) Ligand – ligand transitions



Because of the energy differences between the various types of transitions, ligand field transitions are usually in the near-UV region, charge transfer transitions are in the visible region. The resulting emission from charge-transfer states is often highly coloured.

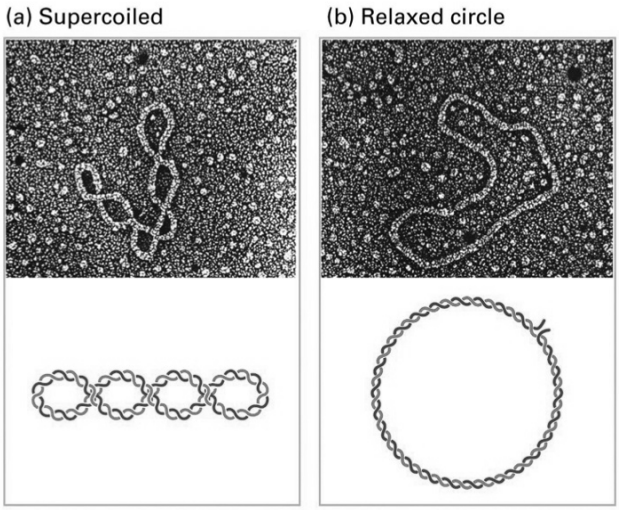


Ruthenium in oxidation state II is d^6, and so as an octahedral complex its electrons are in the low-spin t_{2g}^6 configuration. Incident light at about 450 nm promotes one of these electrons to a ligand anti-bonding orbital, a metal to ligand charge transfer. Therefore, the $S_0 - S_1$ notation used in the Jablonski diagrams for excited state of Ruthenium compounds can be represented as ^{1}MLCT. Transfer to ^{3}MLCT is efficient and so ruthenium complex's photochemistry generally happens from here. From the Jablosnki diagram[21], we can note that promotion of an electron from π_M metal orbital to π_L^* ligand orbitals gives rise to metal to ligand charge transfer (MLCT) excited states whereas promotion of an electron from π_M to σ_M^* orbitals gives rise to metal centered (MC) excited states. Ligand centered (LC) excited states can be obtained by promoting an electron from π_L to π_L^*.

DNA BINDING:

Deoxyribonucleic acid, DNA, is a molecule of great biological significance. The total DNA content of a cell is termed the 'Genome'. The 'Genome' is unique to an organism, and is the information bank governing all life processes of the organism, DNA being the form in which this information is stored. Stretches of DNA called 'genes' have the extremely important function of coding for proteins. DNA is present in the body in the form of a double helix, where each strand is composed of a combination of four nucleotides, adenine (A), thymine (T), guanine (G) and cytosine (C) [22]. The structure of DNA does not only exist as secondary structures such as double helices, but it can fold up on itself to form tertiary structures by supercoiling. Supercoiling allows for the compact packing of circular DNA. Circular DNA still exists as a double helix, but is considered a closed molecule because it is connected in a circular form. A superhelix is formed when the double helix is further coiled around an axis and crosses itself. Supercoiling not

only allows for a compact form of DNA, but the extent of coiling also affects the DNA's interactions with other molecules by determining the ability of the double helix to unwind.



Supercoiling changes the shape of DNA. The benefit of a supercoiled DNA molecule is its compatibility. A nick is a discontinuity in a double stranded DNA molecule where there is no phosphodiester bond between adjacent nucleotides of one strand typically through damage or enzyme action. In comparison to a relaxed DNA molecule of the same length, a supercoiled DNA is more compact. This is reflected in experimentation as the supercoiled DNA moves faster than relaxed DNA. Therefore, the structural differences can be analyzed in techniques such as electrophoresis and centrifugation.

DNA starts transcribing or replicating only when it receives a signal, which is often in the form of a regulatory protein binding to a particular region of the DNA. Thus, if the binding specificity and strength of this regulatory protein can be mimicked by a small molecule, then DNA function can be artificially modulated, inhibited or activated by binding this molecule instead of the protein. Thus, this synthetic/natural small molecule can act as a drug when activation or

inhibition of DNA function is required to cure or control a disease. DNA inhibition would restrict protein synthesis, or replication, and could induce cell death. Though both these actions are possible, mostly DNA is targeted in an inhibitory mode, to destroy cells for antitumor and antibiotic action.

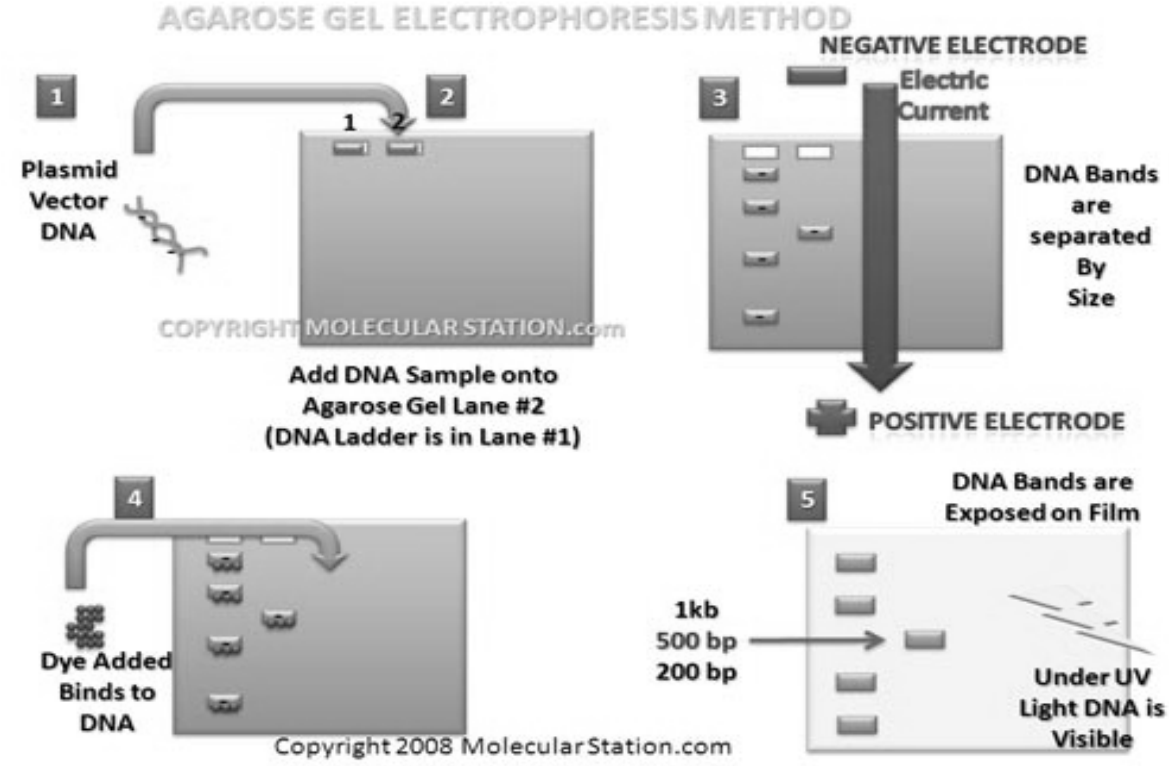
Drugs bind to DNA both covalently as well as non-covalently. Covalent binding in DNA is irreversible and invariably leads to complete inhibition of DNA processes and subsequent cell death. Non-covalently bound drugs mostly fall under the following two classes:

1. Minor groove binders- Minor groove binding drugs are usually crescent shaped, which complements the shape of the groove and facilitates binding by promoting Van der Waals interactions. Additionally, these drugs can form hydrogen bonds to bases, typically to N of adenine and O of thymine. Most minor groove binding drugs bind to A/T rich sequences.
2. Intercalators- These contain planar heterocyclic groups which stack between adjacent DNA base pairs. The complex, among other factors, is thought to be stabilized by π-π stacking interactions between the drug and DNA bases. Intercalators introduce strong structural perturbations in DNA.

AGAROSE GEL ELECTROPHORESIS:

Agarose gel electrophoresis is the method used to acknowledge the damage done by the compound to the DNA. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving[23]. The most important factor is the length of the DNA molecule, smaller molecules travel faster. In this case, the supercoiled

DNA moves faster than the nicked DNA due to its compact size and hence moves faster and to a longer distance. Increasing the agarose concentration of a gel reduces the migration speed and enables separation of smaller DNA molecules. The higher the voltage, the faster the DNA moves. But voltage is limited by the fact that it heats and ultimately causes the gel to melt. High voltages also decrease the resolution. The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide. It fluoresces under UV light when intercalated into DNA (or RNA). By running DNA through an EtBr-treated gel and visualizing it with UV light, any band containing more than ~20 ng DNA becomes distinctly visible. EtBr is a known mutagen, however, so safer alternatives are available.



EXPERIMENTAL SECTION:

The metal complexes under study should be nontoxic in the dark, absorb in the phototherapeutic window (600-1000 nm), have good DNA binding ability, able to cause photo-destruction of the cell. Ruthenium complexes are developed to imitate the action of cisplatin and show better activity, particularly on secondary tumors, and to reduce the host toxicity at active doses. In this research, we have tested the possibility of 3 ruthenium complexes showing good photodynamic activity. The 3 compounds tested were:

a) Ru (tpy) (Br-pic) (dmso) (PF_6)
b) Ru (tpy) (mal) (dmso)
c) Ru (tpy) (bpy) (CH_3CN) ($OTf)_2$

Where, bpy- 2,2$^{'}$- bipyridine

tpy- 2,2$^{'}$,6,2$^{''}$- terpyridine

Otf- trifluoromethylsulfonate

Br-pic- 6- bromopicolinate

mal- malonate

These 3 compounds were tested for photophysical, photochemical and photobiological activity in order to understand their photo anti-cancer activity.

A) SPECTROSCOPIC STUDIES:

1) UV-VIS studies- All the three complexes under study are colored. It is evident that, they absorb in the visible region. UV-Visible absorption studies were performed on the solution of complexes in DMSO using ocean optics spectrophotometer. The results of wavelength maxima and molar extinction co-efficient were noted.
2) Fluorescence studies: We performed the fluorescence studies using Ocean optics instrument USB 2000 for each compound. We did not observe any fluorescence in these compounds.

B) DNA BINDING STUDIES:

DNA binding constant (K_b) is a proportionate measure of the binding ability of the complex with the DNA. Binding constant, K_b is measured by the optical titration method as both the complexes tested are showing an increasing hypochromicity as calf thymus DNA is added[24].Binding constant, K_b, is determined from the absorption changes during the DNA titration using the equation given below:

$$(\varepsilon_a-\varepsilon_b)/(\varepsilon_b-\varepsilon_f) = (1/ K_b) \times (1/[DNA]) + 1$$

ε_a = molar extinction coefficients for the absorption values at a given DNA concentration.

ε_b = molar extinction coefficient for the absorption value of the complex fully bound to DNA.

ε_f = molar extinction coefficient for the absorption value of the complex free in solution

The absorption and ε_b of the bound complexes was determined from the titration where further addition of DNA did not result in changes to the spectrum. The binding constant for each molecule was determined by plotting $(\varepsilon_a-\varepsilon_b)/(\varepsilon_b-\varepsilon_f)$ vs 1/[DNA], and K_b can be calculated as the reciprocal value of the slope.

C) DNA PHOTOCLEAVAGE STUDIES:

Gel electrophoresis is a technique used to separate the DNA fragments based on their size. First, the gel is prepared. Gels are made of Agarose, a sea weed extract similar to gelatin. The finished gel has a consistent appearance. This consistency offers resistance to the pieces of DNA as they try to move through the gel. Once the DNA samples are loaded onto the gel, an electric current is applied to the gel. The gel electrophoresis is based on the fact that the supercoiled DNA (uncut plasmid) will travel more rapidly than nicked DNA. DNA is negatively charged due to the phosphate back bone. Thus DNA will move towards the positive electrode.

1.5% Agarose gel was prepared by dissolving 0.75 g of Agarose in 50 ml of 2.5 X Tris-EDTA buffer, with the application of heat using a hot plate and a magnetic stirrer until the morbid solution became clear. Ethidium bromide was added to the lukewarm solution in order to minimise the vapours of ethidium bromide. Ethidium bromide is mutagenic and should be handled with extreme care. The DNA is visualised in the gel by the addition of ethidium bromide, which strongly binds to the DNA by intercalating between the bases .Buffers not only prevent ionic changes in the surroundings, but also provide ions to support conductivity. The use of high concentration of buffers (10X), may result in the melting of the gel due to the heat generated. Slowly, the gel solution was poured into the gel plate without

any air bubbles. Pouring the gel solution slowly into the gel plate reduces the air bubbles in the gel. Insert the gel comb at one end. It can be left to solidify for 15-20 minutes.

The gel plate is then placed in the gel box, containing the buffer, and is connected to the power supply. Slowly remove the comb, so that the wells formed are well resolved. Samples are prepared by adding 5µl of plamid, 5µl of the complex and 2µl of the loading dye (bromophenol blue). Loading dye provides the density required for the sample to be easily loaded in to the well. Loading dyes are negatively charged in the neutral buffers and thus move in the same direction as the DNA, allowing us to monitor the progress of the gel. Bromophenol blue migrates at a rate equivalent to 200-400 base pair DNA. So, to look at fragments near this size (ie., anything smaller than 600 bp), a different dye has to be used. Samples are then loaded in to the wells. The lid of the gel box is closed and voltage is applied. Stop the current supply when the loading dye has run ¾ the length of the gel. Carry the gel to a dark room to look under the UV light, which is carcinogenic. So, protective glasses, gloves and long sleeves should be used to avoid the contact of UV light with the skin.

D) SINGLET OXYGEN STUDIES[11]:

Singlet oxygen is the only electronically excited state of molecular oxygen. Used intentionally as a deleterious species in photodynamic therapy, its role as a biological messenger is being increasingly recognized. $^1O_2$ is mainly a product of photochemical reactions and its synthesis is eukaryotes is very limited confined to specific type of cells like eosinophils and macrophages. It exists in excited state for only a short time before losing its reactivity by transferring excess energy to other molecules or by returning to ground state.

There are no anti-oxidant enzymes for elimination of singlet oxygen and chemical scavengers that intercept this oxygen metabolite must be present at high concentrations to be effective.

The lifetime of $^1O_2$ in water, which is only up to 4μs, limits the distance it can diffuse in cells and therefore, restricts its reactivity to the so-called "spatially resolved" reactions[29]. It readily reacts with DNA, lipids and proteins. Among amino acids residues, the most reactive with singlet oxygen are histidine, tryptophan,methionine, cysteine and tyrosine which form short lived endo- or hydroxy peroxides. In the presence of redox-reactive metal ions, these peroxides undergo decomposition with the formation of reactive radicals that can propagate chain reactions and oxidative damage to other biomolecules[30,31]. In the presence of Fe^{2+} or Cu^{2+}, lipid peroxides can participate in Fenton reactions to produce oxy- (LO) and peroxy- (LOO) radicals capable of inducing DNA damage.

Methods for $^1O_2$ detection include EPR spectroscopy using spin traps[32], phosphorescence at 1270 nm[33] and chemical trapping[34]. The current spread of fluorescence imaging techniques has lead to the development of a number of $^1O_2$ fluorescent probes, such as *trans*-1-(2′-methoxyvinyl)pyrene (MVP)[35], dansyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole (DanePy)[36],or fluorescein-based probes such as DMAX or DPAX[37]. Invitrogen/Molecular Probes has recently marketed a highly selective sensor for $^1O_2$ without any appreciable response to hydroxyl radicals or superoxide, under the trade name Singlet Oxygen Sensor Green (SOSG) reagent®[38]. While the exact structure of SOSG has not been disclosed, its absorption spectrum resembles that of DMAX and it may therefore be assumed to contain a fluorescein bound to a dimethylanthracene derivative.

The Singlet Oxygen Sensor Green reagent[38] is highly selective for $^1O_2$. Unlike other available fluorescent and chemiluminescent singlet oxygen detection reagents, it does not show any appreciable response to hydroxyl radical (.OH) or superoxide ($.O_2$). This new singlet oxygen indicator initially exhibits weak blue fluorescence, with excitation peaks at 372 and 393 nm and emission peaks at 395 and 416 nm. In the presence of singlet oxygen, it emits a green fluorescence similar to that of fluorescein (excitation/emission maxima ~504/525 nm). The Singlet Oxygen Sensor Green reagent is supplied as a cell impermeant derivative.

Coming to the method, 100 µg vial in 330 µL of methanol was used to make a stock solution of ~500 uM which produced green fluorescence at 525 nm. Then, compound solutions were irradiated for their time of irradiation respectively, then 40 uL of sensor green (10 uM) was added and immediately a fluorescent reading was taken. The fluorescent reading of only sensor green (no compound) and only compound without irradiation with sensor green were also taken to compare the results.

E) CELL STUDIES:

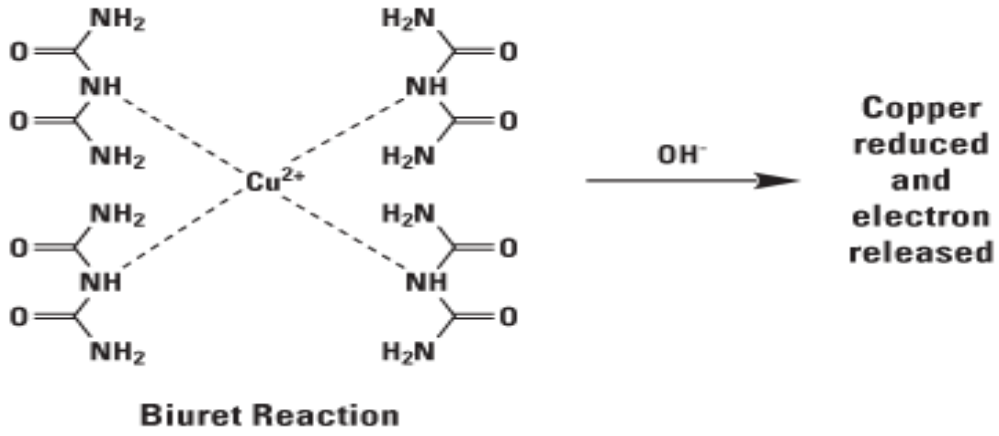
The human skin fibroblast cells obtained from the ATCC are cultured in 600 ml culture flasks using DMEM. The media is previously inoculated with Amphotericin B and Gentamycin. Media is changed at regular intervals (24,48,72 hours) until the cells are fully grown. Once the cells have fully grown, they are replated. The media is drained off. The flasks are washed with PBS, at least thrice. Trypsin is added to the flasks, which suspends the cells struck to the walls of the flask. The flask are tapped and placed in the incubator for not more than 1 minute, as trypsin can digest the cells if placed in contact with them for a long time. To ensure that most of the cells remain suspended, observe under the microscope. The

media is added immediately to inactivate the trypsin. The cells are then transferred to 60mm^2 petri dishes and allowed to grow until the plate is full.

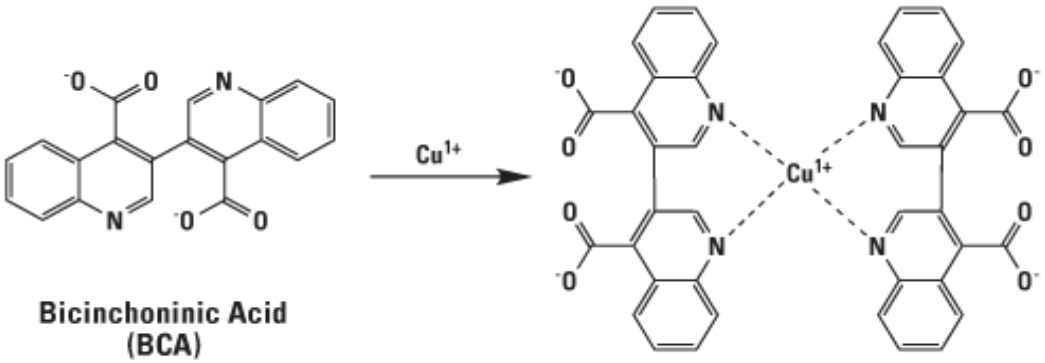
After the cells are fully grown in the petri dishes, they are transferred into 24 well plates and then, different concentrations of the drug are added. Two such plates are made for each compound tested – Dark and Light. One plate is not exposed to any light and immediately placed in the incubator after wrapping with sterilized aluminium foil which is labelled as Dark. The other plate is irradiated for a definite period of time for the drug to be photoactivated and hence show the activity; this plate is labelled as Light. Both the plates are incubated for 24 hrs and then the cell growth is observed. The cell growth in the wells is quantified by BCA protein assay.

F) BCA PROTEIN ASSAY[25,26,27]:

The Pierce BCA protein assay is a detergent compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well known reduction of Cu^{2+} to Cu^{1+} by protein in ana alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation using a unique reagent containing bicinchoninic acid[25]. The purple colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over broad working range (20-2000 µg/ml). The BCA method is not a true end-point method, i.e, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.



Biuret Reaction



Bicinchoninic Acid (BCA)

BCA-Copper Reaction

The macromolecular structure o protein, the number of peptide bonds and the presence o four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA[28].Coming to the procedure for BCA protein assay, the cells are incubated for 24 hrs are they are exposed to the drug. Then the media is drained off and the cells are washed with PBS (twice). 100µl of surfactant (Tween 80) is added to each well, and incubated for 15 to 30 minutes. The cells are destructed by the surfactant. Then, BCA (bicinchoninic acid) assay reagent is added to each well and incubated (30 minutes-1 hour), and then the absorbance reading can be noted using a plate reader at 562 nm.

RESULTS AND DISCUSSION

A) SPECTROSCOPIC STUDIES:

When UV- Visible studies were performed using the 75μM concentration of the 3 compounds, following results were obtained:

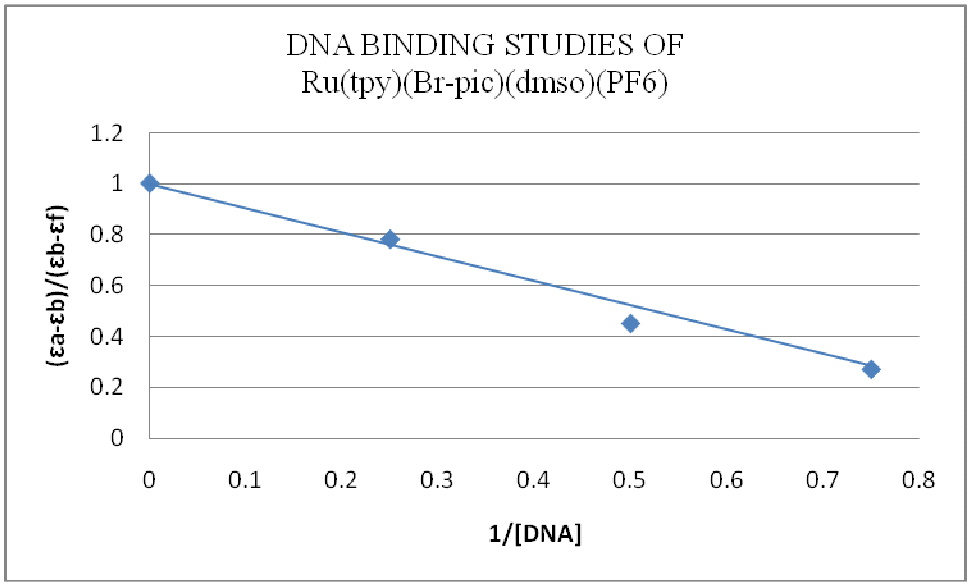
S.NO	Compound	λmax in nm	Molar absorptivity co-efficient (ε) in $M^{-1}cm^{-1}$
1)	Ru (tpy) (Br-pic) (dmso) (PF_6)	446	3529
2)	Ru (tpy) (mal) (dmso)	502	5134
3)	Ru (tpy) (bpy) (CH_3CN) ($OTf)_2$	455	10414

From the above data, it can be elucidated that all the three compounds likely absorb light in the visible region. Molar extinction coefficient, □**,** is the measure of how strongly a chemical absorbs particular wavelength of light. We can also observe that molar absorptivity co-efficient has considerably increased with the increase in the number of organic ligands. In case of compound 3, the solvent intended was CH_3CN which showed an ε of 10,200 $M^{-1}cm^{-1}$. But, owing to the systemic toxicity of CH_3CN, dmso was used as the solvent and the ε was slightly increased to 10414 $M^{-1}cm^{-1}$. Compounds 1, 2 and 3 show maximum absorbance at a wavelength of 446, 502 and 455 nm respectively. The compounds have to be irradiated at that particular wavelength to observe their photodynamic activity.

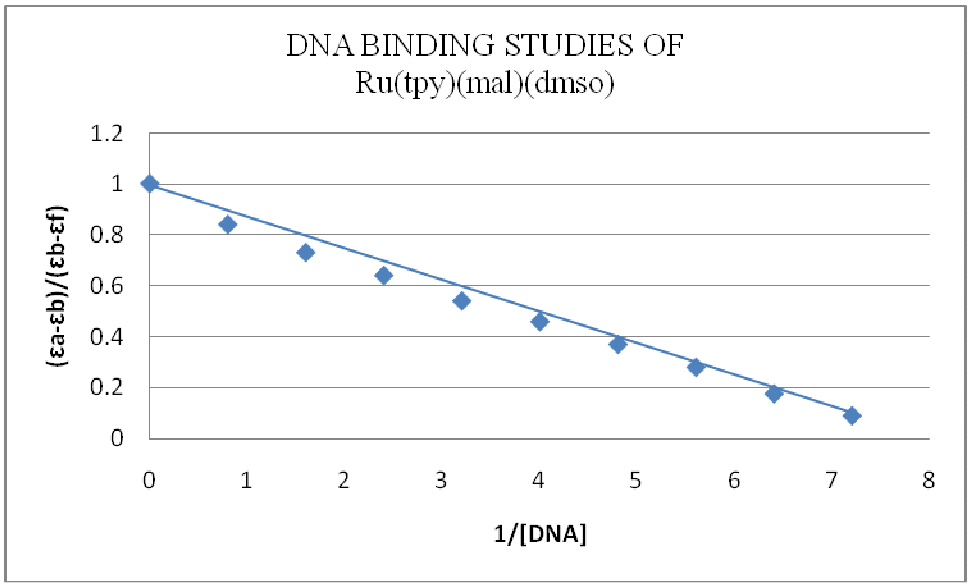
B) **DNA BINDING STUDIES:**

Binding constant, K_b was measured by the optical titration method as all the complexes tested were showing an increasing hypochromicity as calf thymus DNA is added.

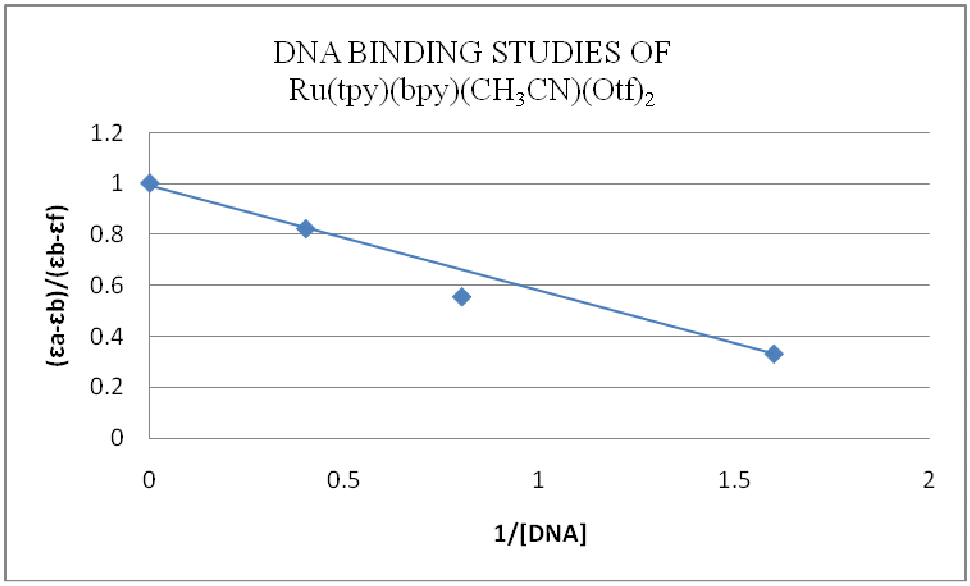
1) Ru(tpy)(Br-pic)(dmso)(PF_6) was titrated with CT-DNA of concentration 800 µM until the absorbance became steady which meant saturation or fully bound DNA. From these values, a graph was plotted using ($\square_a$-$\square_b$)/($\square_b$-$\square_f$) on Y-axis and 1/[DNA] on X-axis. The slope obtained was the value of $1/K_b$ which was found to be 9.4×10^{-6} and hence the DNA binding constant for this compound is 1.06×10^5/ M.



2) Ru(tpy)(mal)(dmso) solution in DMSO was titrated with calf thymus DNA of concentration 250 µM until the absorbance starting at 0.789 steadily decreased and became steady at 0.715. From this data, a graph was plotted and the slope was calculated to be 1.98×10^{-6}. Therefore, the drug DNA intrinsic binding constant was found to be 5.05×10^5 /M.



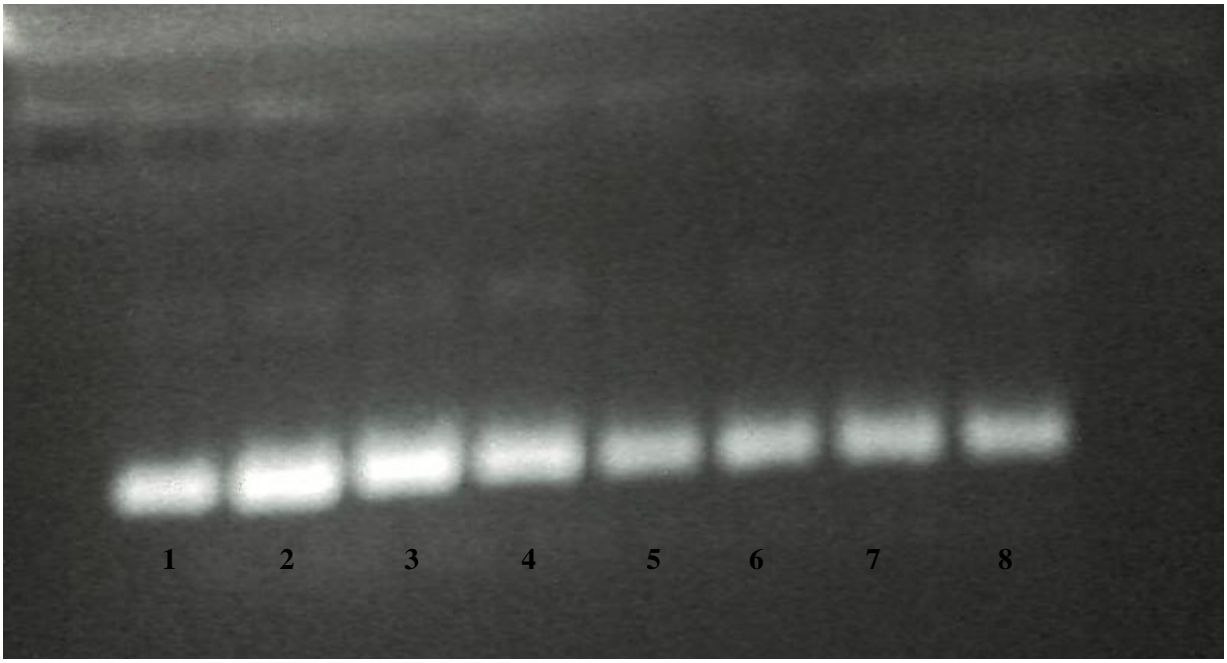
3) Ru(tpy)(bpy)(CH_3CN)$(Otf)_2$ which was found to have the highest molar absorptivity co-efficient among the three compounds showed better DNA binding when titrated with 800 µM concentration of CT-DNA. From the graph, the slope value was calculated to be 0.8 x 10^{-6} and hence the intrinsic binding constant was found to be 1.16 x 10^{6} /M.



The results of this experiment showed that all the 3 compounds bind very tightly to the DNA when compared to the standard ethidium bromide.

C) DNA PHOTOCLEAVAGE STUDIES

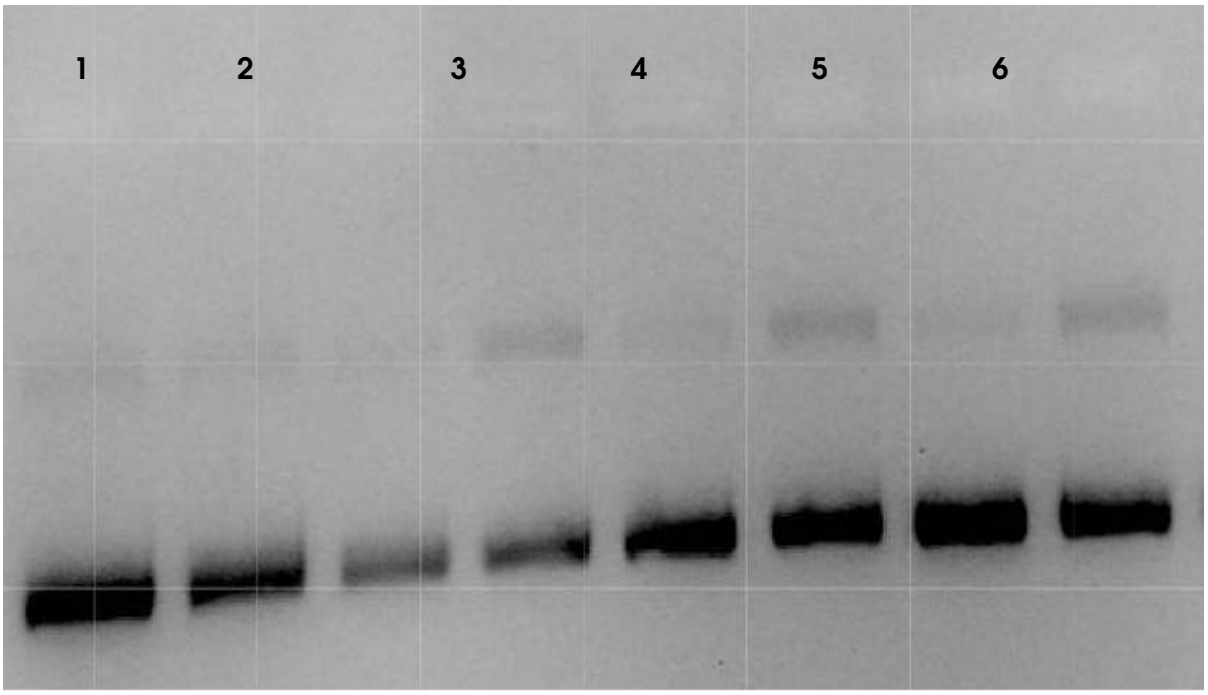
All the above results cumulatively indicate that these Ruthenium terpyridine complexes bind to CT-DNA in the groove mode. In order to show that these complexes could cause damage to DNA when photoactivated, agarose gel electrophoresis using pBR 322 DNA was performed using various concentrations of the compound in both dark and photo-activated forms. Photocleavage studies using the compound Ru(tpy)(Br-pic)(dmso)(PF_6) produced the following results:



Here, Lane 1& 2- Control dark & irradiated; Lane 3 & 4- 300µM dark & irradiated; Lane 5 & 6- 350µM dark & irradiated; Lane 7 & 8 – 400µM dark & irradiated. From these results, we can see that there is defintely a decrease in the distance travelled by the DNA as the concentration of the drug increases. This is a proof for the high DNA binding constant of the compound. Now coming to the photocleavage results, no photocleavage was observed and this only proves the singlet

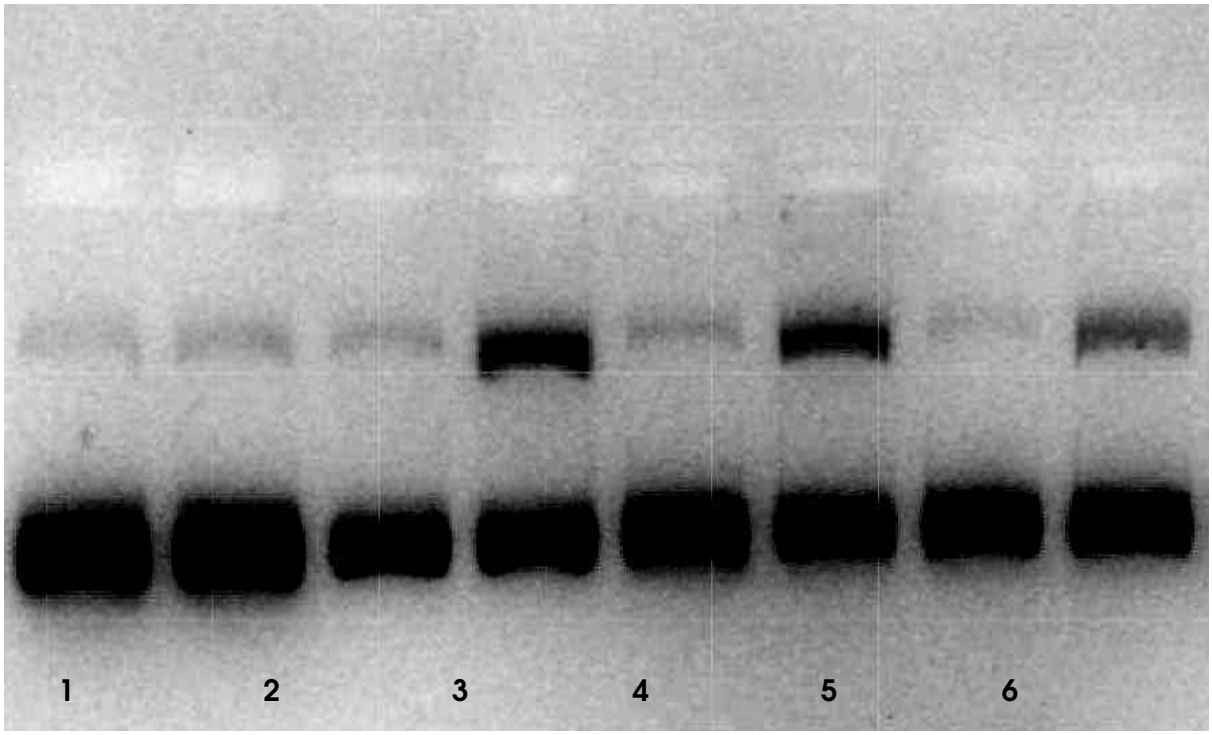
oxygen studies that this compound might not be showing any photodynamic activity or the pathway by which it acts is not by the destruction of DNA.

When we performed the DNA photocleavage studies of Ru(tpy)(mal)(dmso) after irradiating for 1.5 hrs at 502 nm, we could observe the photocleavage as below-



In the photograph of the gel, Lane 1& 2- Control dark & irradiated; Lane 3 & 4- 250µM dark & irradiated; Lane 5 & 6- 300µM dark & irradiated and Lane 7 & 8 – 350µM dark & irradiated.No DNA cleavage was observed for controls in which complex was absent (lane 1 and 2), With increasing concentration of the Ru (II) complex (lanes 3–8), the amount of Form I (supercoiled) of pBR322 DNA diminish gradually, whereas Form II (nicked) increases. The mechanism of the photocleavage activity can be tested by singlet oxygen studies. From this data, we can also observe the difference in the distance travelled as we increase the concentration and hence DNA migration studies to prove the good intrinsic binding of the compound.

Because of its very high intrinsic binding constant, $Ru(tpy)(bpy)(CH_3CN)(Otf)_2$ was of considerable interest. DNA cleavage was monitored by reaction of supercoiled circular pBR 322 at 455 nm after irradiating for 1 hr. The following results were obtained –

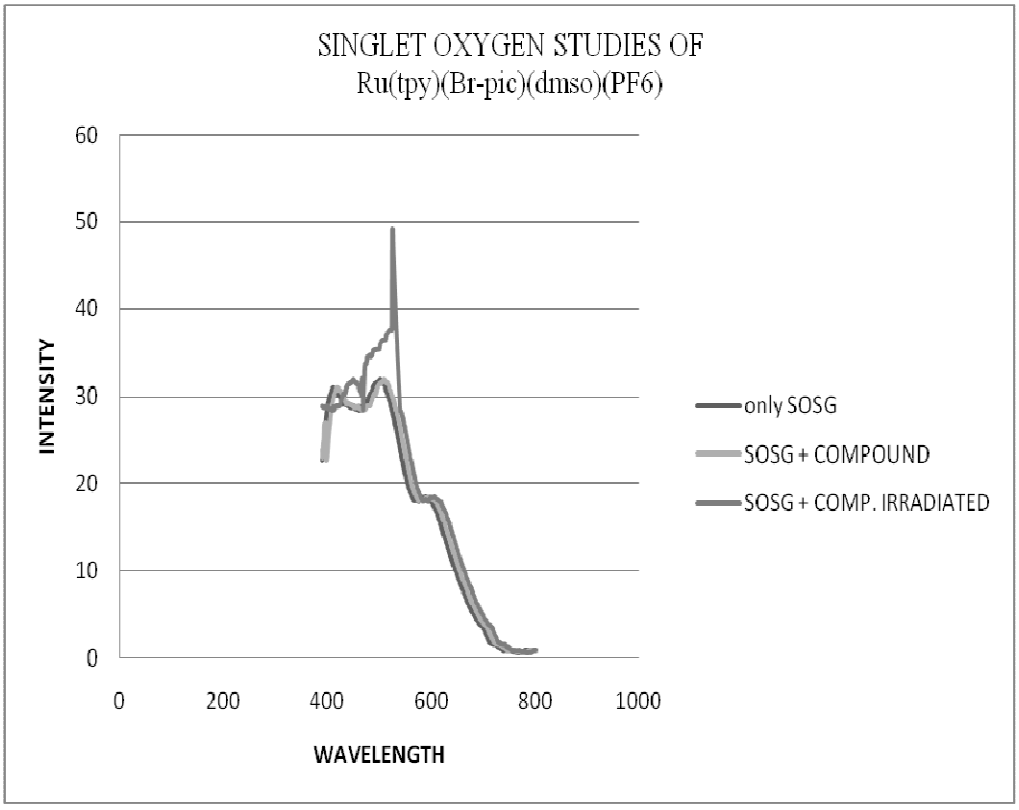


When circular plasmid DNA was subjected to electrophoresis, relatively fast migration was observed for the supercoiled (form I) as in Lanes 1 and 2. But, when the concentration of the compound was gradually increased, the supercoils were relaxed to generate a slower-moving open circular form or nicked DNA (form II). On comparing the photographs obtained for the 3 compounds tested, we can see that the size of the plasmid varies though we used the same amount of plasmid for all the experiments. This shows that the compound has good intercalation with DNA. The difference in the distance travelled by the bands also proves the very high binding constant of this compound.

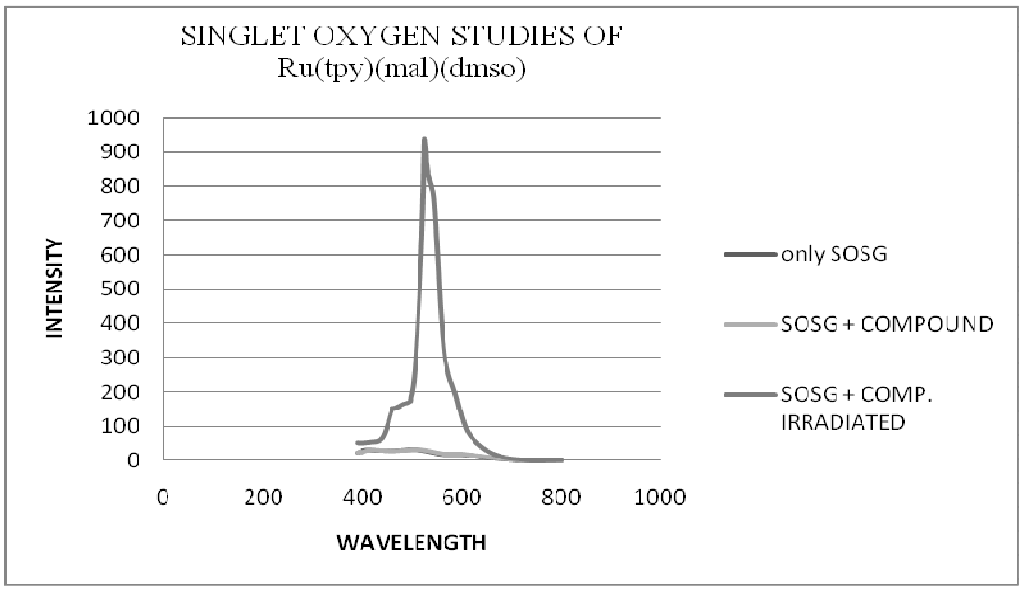
D) SINGLET OXYGEN STUDIES:

The formation of $^1O_2$ has been assessed by fluorescence detection by using Singlet oxygen sensor green (SOSG) as fluorescent probe. SOSG emits green fluorescence with an excitation and emission maxima at 504 and 525 nm respectively. In the course of our study, we have observed that the probe doesn't have any fluorescence by itself even when irradiated. We already know that none of the compounds exhibit any fluorescence. We have also observed that all the 3 compounds when added to the probe did not produce any fluorescence without irradiation. Finally, when the mixture was irradiated, we observed the following results –

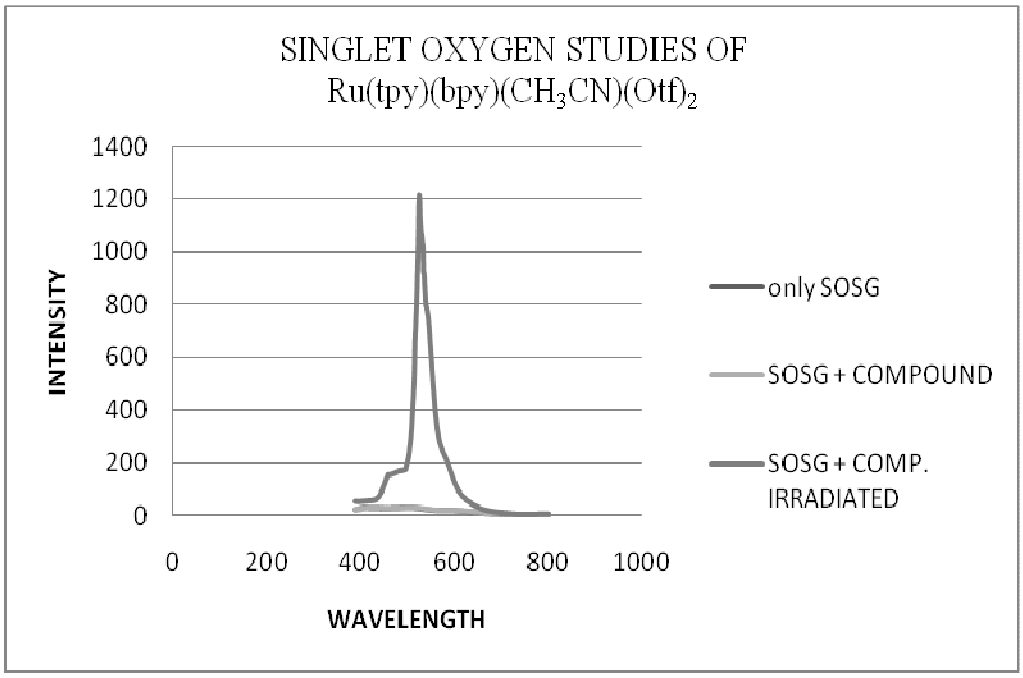
a) Ru(tpy)(Br-pic)(dmso)(PF_6) did not show any fluorescence even when irradiated. This proves that the compound might not be having photodynamic activity. If it has any activity, it might not be due to the production of singlet oxygen.



b) Ru(tpy)(mal)(dmso) showed an intensity of 938.99 at 525nm when irradiated together which was high compared to the readings probe (28.18) and their mixture (29.96).



c) Ru(tpy)(bpy)(CH_3CN)($Otf)_2$ on irradiation in the presence of SOSG produced a fluorescence intensity of 1213.

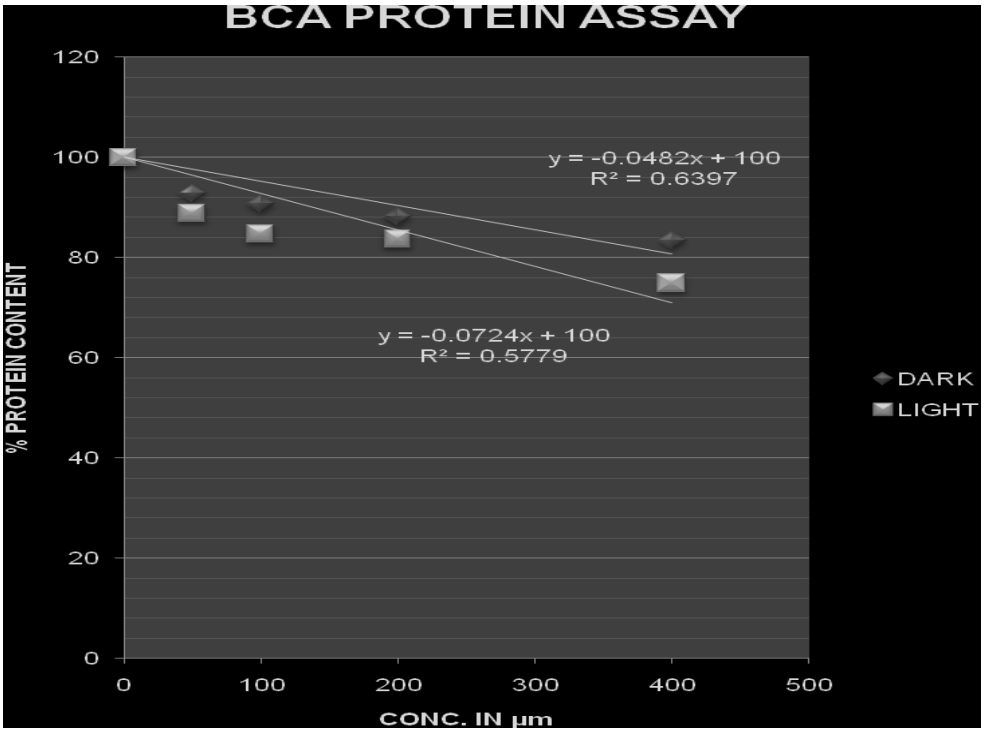


The results above show that Ru(tpy)(mal)(dmso) and Ru(tpy)(bpy)(CH_3CN)($Otf)_2$ can act as good $^1O_2$ photosensitizers.

E) CELL STUDIES AND BCA PROTEIN ASSAY:

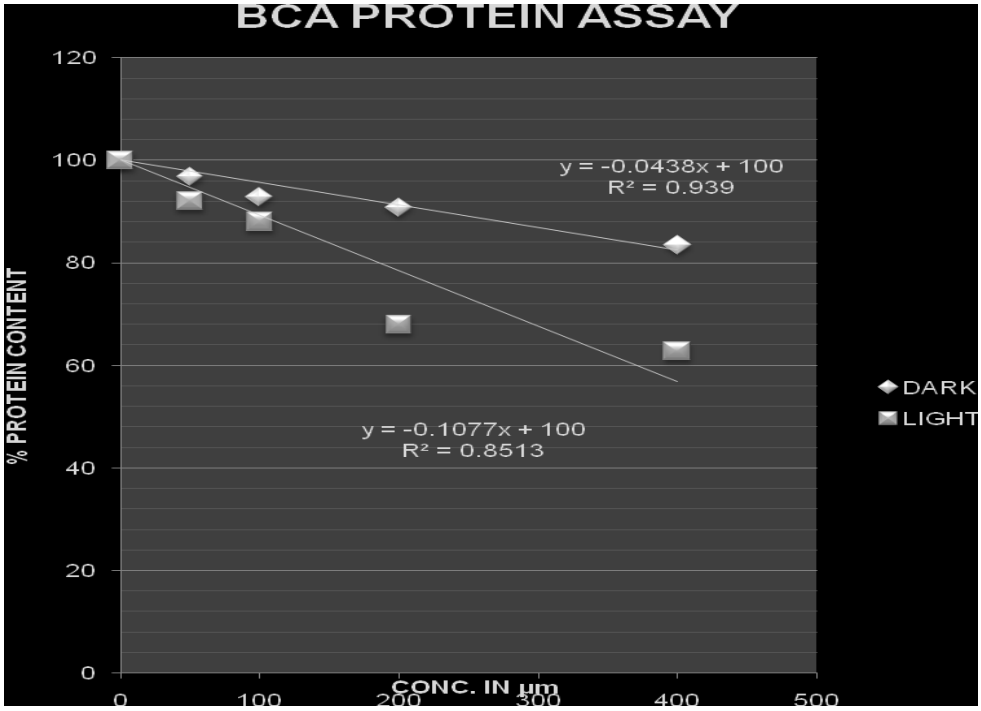
After the cell studies were performed, BCA protein assay was performed to quantify the cell viability. Based on the results obtained, LC 50 value was calculated for the compounds. This experiment was performed using Ru(tpy)(mal)(dmso) and Ru(tpy)(bpy)(CH_3CN)($Otf)_2$. The results of BCA protein assay, plotted as a graph to calculate the LC 50 are as follows –

a) Ru(tpy)(mal)(dmso)



From the graph, LC 50 value in the dark and light were found to be 1307 µM and 848 µM respectively. These results show that this compound is a good photo sensitizer and shows activity when irradiated with visible light. The main mechanism behind its action as we have seen can be attributed to the production of singlet oxygen. Compared to the concentrations we used, the lethal concentration is very high almost 5 fold and hence can be considered to have a very good potential. Its effect on cancer cells has to be tested to confirm its clinical use.

b) $Ru(tpy)(bpy)(CH_3CN)(Otf)_2$



As we can see, the results were convincing that this compound has good DNA photocleavage activity at a low concentration. The LC 50 value in the dark and light were found to be 1244 µM and 488 µM respectively. This shows that though the compound might have good DNA binding and photocleavage activity, it might still be toxic when used clinically. The result of its effect on cancer cells has yet to be tested.

METAL COMPLEX	LC 50 (DARK)	LC 50 (LIGHT)
Ru(tpy)(mal)(dmso)	1307 µM	848 µM
$Ru(tpy)(bpy)(CH_3CN)(Otf)_2$	1244 µM	488 µM

CONCLUSION:

Octahedral Ruthenium- terpyridine complexes were employed to improve the sequence selectivity of cleavage which can arise from preferential binding at a certain site for treatment of cancer. All the three compounds showed good absorbance in the visible region and good DNA binding capacity. But, only Ru(tpy)(mal)(dmso) and Ru(tpy)(bpy)(CH_3CN)($Otf)_2$ could be proven to be good potential photosensitizers owing to the consistent results observed in DNA photocleavage studies, Singlet oxygen studies and the Cell studies using Human skin Fibroblasts.

FUTURE STUDIES:

In the future, we would like to continue the research for testing the compounds on cancer cells for cytotoxicity and photocytotoxicity.

REFERENCES:

1) Dougherty TJ, Gomer CJ, Henderson BW, et al. Photodynamic therapy. Journal of the National Cancer Institute 1998; 90(12):889–905.

2) Dickson EFG, Goyan RL, Pottier RH. New directions in photodynamic therapy. Cellular and Molecular Biology 2003;48(8):939–954.

3) Howard W. Rogers, MD, PhD, Martin A. Weinstock, MD, PhD, et al. Incidence Estimate of Nonmelanoma Skin Cancer in the United States, 2006. Archives of Dermatology 2010.

4) SEER Cancer Statistics Review, 1975-2004 (NCI)

5) "11th ROC: Ultraviolet Radiation Related Exposures." 27 January 2005. U.S. Department of Health & Human Services. 15 April 2008.

6) Xu, C.; Green, Adele; Parisi, Alfio; Parsons, Peter G (2001). "Photosensitization of the Sunscreen Octyl p-Dimethylaminobenzoate b UVA in Human Melanocytes but not in Keratinocytes.". Photochemistry and Photobiology 73 (6): 600–604. doi:10.1562/0031-8655(2001)073<0600:POTSOP>2.0.CO;2. PMID 11421064.

7) Dolmans DEJGJ, Fukumura D, Jain RK. Photodynamic therapy for cancer. Nature Reviews Cancer 2003; 3(5):380–387.

8) Wilson BC. Photodynamic therapy for cancer: Principles. Canadian Journal of Gastroenterology 2002; 16(6):393–396.

9) Vrouenraets MB, Visser GWM, Snow GB, van Dongen GAMS. Basic principles, applications in oncology and improved selectivity of photodynamic therapy. Anticancer Research 2003; 23:505–522.

10) Dickson EFG, Goyan RL, Pottier RH. New directions in photodynamic therapy. Cellular and Molecular Biology 2003; 48(8):939–954.

11) Michael R. Hamblin, Pawel Mroz, editors. Advances in Photodynamic therapy- Basic, Transitional and Clinical 2008; 29: 14 – 16, 137,

12) FOOTE, C. S. Definition of type I and type II photosensitized oxidation. Photochem Photobiol, v. 54, n. 5, p. 659, 1991.

13) SIBATA, C. H. et al. Photodynamic therapy: a new concept in medical treatment. Brazilian J Med Biol Res, v. 33, p. 869-80, 2000.

14) FATH, A. et al. Barley aleurone cell death is not apoptotic: characterization of nuclease activities and DNA fragmentation. The Plant Journal, v. 20, n. 3, p. 305-15, 1999.

15) NARENDRA, P. S. A simple method for accurate estimation of apoptotic cells. Experiment Cell Res, v. 256, p. 328-37, 2000.

16) WYLLIE, A. H. et al. Cell death: the significance of apoptosis. Int Rev Cytol, v. 68, p. 251-306, 1980.

17) Grazyna Stochel, Malgorzata Brindell, Wojciech Macyk, Zofia Stasicka and Konrad Szacilowski . Bioinorganic Chemistry, 2009, p-305-313

18) Claire **S.** Allardyce **and** Paul J. Dyson, Ruthenium in Medicine: Current Clinical-Uses and Future Prospects; Platinum Metals Rev.,2001 ,45, (2), 62-69

19) Photochemistry of polypyridine and porphyrin complexes, K. Kalyanasundaram, Academic, London: 2002.

20) Vos, J. G. and Kelly, J. M., Ruthenium polypyridyl chemistry: from basic research to applications and back again, Dalton. Trans., 2006, 4869 – 4883.

21) Vincenzo Balzani, Sebastiano Campagna, Gianluca Accorsi, Photochemistry and photophysics of coordination compounds, Volume 1, Pg. 118 – 123

22) Saher Afshan Shaikh and B. Jayaram, DNA drug interaction, Supercomputing Facility for Bioinformatics and Computational Biology

23) Sambrook J, Russel DW (2001). Molecular Cloning: A Laboratory Manual 3rd Ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

24) (a) Fu, K.-L.P. Dissertation thesis, 2001, pp. 48-50. The Ohio State University, Columbus, OH. (b) Fu, K.-L P., P.M. Bradley and C. Turro, Inorg. Chem,. 2003. 42, 878-884. (c) Sorasaenee, K.,P.K.L-Fu, A.M. Angeles-Boza, K.R. Dunbar and C. Turro Inorg.chem., 2003, 42, 1267-1271.

25) Smith, P.K., et al. (1985). Anal. Biochem. **150**, 76-85.

26) Sorensen, K. (1992). BioTechniques **12(2)**, 235-236.

27) Thermoscientific Pierce BCA Protein Assay Kit Manual

28) Wiechelman,K.,et al.(1988). Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. Anal Biochem. 175:231-7.

29) Redmond, R.W., and Kochevar, I.E., "Spatially resolved cellular responses to singlet oxygen," Photochem Photobiol, Vol.82,2006, pp. 1178-1186.

30) Wright, A., Hawkins, C.L., and Davies, M.J., "Singlet oxygen- mediated protein oxidation: evidence for formation of reactive peroxides," Redox Rep, Vol. 5, 2000, pp. 159-161

31) Wright, A., et al., "Singlet oxygen- mediated protein oxidation: evidence for the formation of reactive side chain peroxides on tyrosine residues," Photochem Photobiol, Vol. 76, 2002, pp. 35-46.

32) E. Hideg, C. Spetea and I. Vass, Singlet oxygen production in thylakoid membranes during photoinhibition as detected by EPR spectroscopy, Photosynth. Res., 1994, 39, 191–199

33) A. Jimenez-Banzo, S. Nonell, J. Hofkens and C. Flors, Singlet oxygen photosensitization by EGFP and its chromophore HBDI, Biophys. J., 2008, 94, 168–172

34) A. Telfer, S. M. Bishop, D. Phillips and J. Barber, Isolated photosynthetic reaction-center of photosystem-II as a sensitizer for the formation of singlet oxygen-detection and quantum yield determination using a chemical trapping technique, J. Biol. Chem., 1994, 269, 13244–13253

35) A. Thompson, H. H. Seliger and G. H. Posner, Chemiluminescent probes for singlet oxygen in biological reactions, Methods Enzymol., 1986, 133, 569–584

36) T. Kalai, E. Hideg, I. Vass and K. Hideg, Double (fluorescent and spin) sensors for detection of reactive oxygen species in the thylakoid membrane, Free Radical Biol. Med., 1998, 24, 649–652

37) K. Tanaka, T. Miura, N. Umezawa, Y. Urano, K. Kikuchi, T. Higuchi and T. Nagano, Rational design of fluorescein-based fluorescence probes. Mechanism-based design of a maximum fluorescence probe for singlet oxygen, J. Am. Chem. Soc., 2001, 123, 2530–2536

38) Molecular Probes Product Information, 2004.