Light Mediated Drug Delivery Using Photocaged Molecules and Photoswitchable Peptides

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Light Mediated Drug Delivery Using Photocaged Molecules and Photoswitchable Peptides

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Abstract

LIGHT MEDIATED DRUG DELIVERY USING PHOTOCAGED MOLECULES AND PHOTOSWITCHABLE PEPTIDES

Deboleena Mitra

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Advisor: Matthew C.T. Hartman, Associate Professor, Department of Chemistry

There are many different types of cancer and various methods have been developed to treat them. Some of the methods of treatment are not highly specific and often patients suffer from various side effects. To make the treatment more specific the method of targeted therapy has evolved. One of the methods of targeted therapy that is highly selective as well as specific is the light mediated treatment of cancer. Photodynamic therapy and photochemical internalization fall under this category. Photodynamic therapy utilizes photosensitizers which operate only in the presence of light to form cytotoxic singlet oxygen. Light of a particular wavelength activates the photosensitizers and initiates the formation of singlet oxygen which eventually leads to cell death. However, this approach also has some drawbacks. There are problems associated with the light to drug administration time due to the slow uptake of the photosensitizers and the amount of molecular oxygen in the cancer cells is in low concentration for the generation of singlet oxygen.
The method of light mediated targeted therapy that we have developed uses photocaged molecules and photoswitchable peptides that is devoid of the above drawbacks and is highly specific.

In photocaging, a biologically active molecule is made inactive by the attachment of a photocleavable blocking group. On exposure to UV radiation the photocleavable entity is removed and the biologically active molecule is released. Using this concept we have designed a prodrug that consists of a cell impermeable hydrophilic molecule attached to a photocaged doxorubicin. The hydrophilic moiety makes the entire prodrug cell impermeable in the dark. Upon irradiation with UV light the photosensitive group is removed and cytotoxic doxorubicin is released at the tumor site. This concept has been further modified by attaching receptor binding molecules to the photocaged entity to increase its specificity. Folic acid has been attached to the photocaged doxorubicin that would interact with the folate receptors which are overexpressed in cancer cells. These targeting groups enable cancer cell selective drug delivery with light.

A photoswitchable peptide has been used which, in the dark state is randomly coiled and cell impermeable but upon illumination becomes helical and cell permeable and can be used to deliver drugs into the cells. It has been established that the conformation of α helical peptides can be controlled by azobenzene photoswitches. So azobenzene linker with iodoacetamide group has been synthesized to link to the cysteine units of the synthesized peptides. It has also been established that an arginine patch developed on the surface of an impermeable protein will make it cell permeable as the positively charged arginines will interact with the heparin sulfate proteoglycans of the cell membrane. Upon illumination with UV light of suitable wavelength the azobenzene linker will change from a trans to a cis form and this will convert the randomly coiled cell impermeable peptide into an α helical permeable form. Thus a series of peptides have been
designed with different arginine mutations which develop an arginine patch in the helical form. The change in their secondary structure has been studied with circular dichroism. Cell studies would be done in future with these peptides to verify their cell permeability. The method could potentially be used to deliver drugs into cells in presence of light.
CHAPTER 1

INTRODUCTION
1.1 **Cancer and its Treatment**

Cancer is a disease of uncontrolled cell growth which eventually spreads to the different parts of the body. It is a leading cause of death worldwide. According to the data sheet published by International Agency for Research on Cancer 8.2 million people died of cancer in 2012 worldwide. There are many reasons for the cause of cancer. The external causes include chemicals, radiation, tobacco and infectious organisms while the internal causes are hormones, metabolic mutations, the immune system and the mutations that have been inherited genetically. There are many ways to treat cancer such as chemotherapy, radiation, surgery and targeted therapy.

1.1.1 **Chemotherapy**

Chemotherapy\(^1,2,3\) is the treatment of cancer with a drug or a combination of drugs to kill the rapidly growing cells. Chemotherapy is often used in combination with radiation or surgery. Chemotherapy used to shrink the tumor before radiation or surgery is called neo-adjuvant chemotherapy. Adjuvant chemotherapy involves treatment to destroy cancer cells that remain after surgery or radiation.

1.1.1.1 **Side Effects of Chemotherapy**

Chemotherapy destroys the healthy tissues also and that leads to the development of many side effects in patients\(^4,5,6\). It affects the healthy cells of the mouth and intestine, bone marrow and cells that are responsible for hair growth. Once these healthy cells get affected it leads to the emergence of a series of side effects that include nausea, fatigue, hair loss and reduced blood cell count.

1.1.2 **Targeted Therapy**

To overcome the side effects of chemotherapy and make the treatment more specific the method of targeted therapy\(^7,8\) has evolved. As the name suggests in targeted therapy the growth and spread of cancer is stopped by using drugs that specifically bind to a target cancerous cells. In this method
there are many ways of delivering cytotoxic agents into cancer cells. Small molecular drugs are used in targeted therapy which can cross the plasma membrane and are often used to target proteins to affect their enzymatic activity or interaction with other substances. Imatinib mesylate (gleevec) is one such example of a small molecule that is used in targeted therapy and it is believed to affect the signaling pathways by targeting tyrosine kinase enzyme BCR-Abl that participates in signal transduction. Monoclonal antibodies are also used as a vehicle to deliver radioactive molecules or toxins inside the cancer cells. Tositumomab (Bexxar) is a radioactive immunotoxin currently approved for therapy against certain types of non-Hodgkin lymphoma. Antibodies also can be designed to interact with the cell surface receptors to have a direct therapeutic effect. For example, Rituximab (rituxan) interacts with CD20 on the surface of mature B lymphocytes and triggers an immune response against the cancer cells. Vaccines are another means of targeted therapy which influence the immune system of the body to attach the cancer cells. Another method of targeted therapy that is highly selective as well as specific is the light mediated treatment of cancer. Photodynamic therapy and photochemical internalization fall under this category.
1.1.2.1 Photodynamic Therapy

In Photodynamic therapy (PDT) the treatment consists of two components—a photosensitizer and a light of a particular wavelength that activates that photosensitizer. The photosensitizer is administered to the body using intravenous injection or by topical application on the affected region and then it gets absorbed by the cells and distributed all over the body. These photosensitizers stay in the cancer cells much longer than in the normal healthy cells. Usually after a time span of 24-72 hrs the normal healthy cells no longer retain the photosensitizer. After this time frame the tumor is exposed to irradiation of a particular wavelength that is specific for the photosensitizer. On absorption of the radiation the photosensitizer gets excited from the ground state (singlet state) to a triplet state. When it relaxes back to the singlet state it releases energy to the neighboring oxygen molecule. The oxygen absorbs the energy to form singlet oxygen that is toxic to the cells and finally leads to apoptosis. The process has been shown in Figure 1.1.1. It operates only in presence of light so it is highly selective. Photofrin is a FDA approved photosensitizing agent that is used for the treatment of esophageal cancer and non-small cell lung cancer.
1.1.2.2 Disadvantages of Photodynamic Therapy

In photodynamic therapy the light that is used to activate the photosensitizers usually has moderate ability to penetrate into tissues. Thus the use of PDT is usually limited to regions that are easily penetrated by light such as the esophagus or tissues immediately below the skin. This limits the use of the technique to certain regions of the body. It cannot be used if cancer has metastasized. The effectiveness of this process also depends on the generation of singlet oxygen\textsuperscript{14}. The amount of molecular oxygen available in cancer cells is usually not very high and hence the generation of singlet oxygen is also low. This slows down the process of apoptosis. Lastly there is much disparity in the light to drug administration time\textsuperscript{13} probably due to the slow uptake of the photosensitizers.
1.1.2.3 Photochemical Internalization

Photochemical internalization (PCI)\textsuperscript{15,16,17} works on the same principle as photodynamic therapy with the only difference that an additional toxin is present. Certain macromolecular toxins are degraded in the endosomes before their release into the cells and this hinders the use of these molecules in cancer therapy. To improve their therapeutic use they are used with photosentizers that adhere to the endosomal membrane. Upon shining light of a particular wavelength these photosensitizers generate singlet oxygen that rupture the endosomal membrane and the toxin is released from the endosomes. This released toxin then causes cell death. The process has been shown in Figure 1.1.2.

![Photochemical Internalization Diagram](image)

**Figure 1.1.2** Photochemical Internalization
1.1.2.4 Disadvantages of Photochemical Internalization

One of the drawbacks of photochemical internalization is that a toxin molecule has to be internalized via endosome first. This reduces the efficiency of this process. The low level of molecular oxygen in cancer cells also affects the process as the generation of singlet oxygen is dependent on the amount of molecular oxygen available. Lastly even in photochemical internalization there is much disparity in the light to drug administration time\textsuperscript{13}.

Thus to improve the light mediated targeted therapy some new drug delivery system needs to be developed that is devoid of the drawbacks present in PDT and PCI. This system should not depend on the availability of molecular oxygen in cancer cells and at the same time the disparity between light to drug administration time should not exist.
1.2 Photocaged Drug Delivery System

1.2.1 Origin of Photocaged Molecules

In photocaging\textsuperscript{18} a biologically active molecule is made inactive by the attachment of a photocleavable group. On exposure to UV radiation the photocleavable entity is removed and the biologically active molecule is released as shown in Figure 1.2.1. The term “caged”\textsuperscript{19} was first introduced by Joseph F. Hoffman in 1978 where he reported the development of “caged ATP”. His work showed for the first time how the caged molecules could be used to have a spatiotemporal control over biochemical experiments. The terminal phosphate group of ATP was attached to photolabile groups such as 2-Nitrobenzyl and 1-(2-)-nitrophenylethyl. The caged molecule in absence of radiation was not hydrolyzed by an enzyme as it was not a substrate for that enzyme. Upon irradiation at 340nm the photolabile group was removed and ATP was released. This released ATP was a substrate for the enzyme and was quickly hydrolyzed by it. Since then this idea of photocages has been used extensively to control the activity of many biological molecules. It is an irreversible turning on of the activity of a molecule.
1.2.1 Types of Photocages

There are many photo-labile or photocaging groups that are available\textsuperscript{18}. The most commonly used photocage molecule is o-nitrobenzyl group. The reason behind its frequent use is the fact that it is easily synthesized and can be easily incorporated in any molecule. Typically the compound used is the nitroveratryl cage which has an $m$- and $p$- ($\text{-OCH}_3$) groups in its structure (Figure 1.2.2). These electron donating groups shift the absorption maximum to a longer wavelength such as 365 nm. One nice application of this nitroveratryl group has been used by Lawrence and coworkers in the photocaging of ecdysone\textsuperscript{20}. Caged ecdysone would help to control gene expression with light. A luciferase reporter controlled by ecdysone system was introduced in mammalian cells and the
expression of luciferase was studied in the presence and absence of light. Upon irradiation when
the natural ecdysone molecule was released it led to about 60% of luciferase expression.

Figure 1.2.2 Nitroveratryl group: Typically used nitroveratryl group in photocaging with (-
OCH₃) groups at meta and para positions

The other type of photocaged molecules that are commonly used are the derivatives of coumarin
(Figure 1.2.3). Coumarin has been used to develop a caged mRNA that has been used by Roger
Tsien and Okamato to control the gene expression in zebrafish embryos²¹,²². Wen-Hong Li and
coworkers have developed caged coumarins that showed 200 fold more fluorescence enhancement
after being photolysed by UV²³.
Figure 1.2.3 Coumarin Derivative

The photocaging molecules provide an irreversible spatiotemporal control over some biological systems. It would be good to have a reversible control over a biological system using light.
1.3 Photoswitches in Drug Delivery

Photoswitches\textsuperscript{24,25} are molecules that switch reversibly between two different conformations when exposed to light of a particular wavelength. They help to attain several cycles of active and inactive state and it is usually seen in ring-opening/closing and isomerization reactions. This behavior of the photoswitches is effectively used in controlling the spatiotemporal behavior of many biomolecules. Some of the most commonly used photoswitches are azobenzenes, stilbenes and spiropyrans\textsuperscript{26,27,28,29}.

1.3.1 Azobenzene Molecule as a Photoswitch

Azobenzene is one of the most commonly used photoswitches\textsuperscript{30,31,32,33,34,35,36} in biological studies. It exists in two photoisomeric forms—the trans form and the cis form. The trans form of azobenzene switches to the cis form when exposed to a radiation of 340 nm. The cis form thermally relaxes back to the trans form or upon irradiation with light of 450 nm. This has been shown in Figure 1.3.1.

![Figure 1.3.1 Azobenzene Photoswitch](image-url)
The trans form of the azobenzene is planar\textsuperscript{37} and has been found to be more stable than the bent conformation adopted by the cis form by 10-12 kcal/mol. The trans form is also the dominant isomer in the dark state.

The time needed for the unmodified cis azobenzene to thermally relax to the trans form is a few days at room temperature. So if this azobenzene photoswitch is to be incorporated in any biological system and a faster photoswitch is needed then this molecule needs to be modified. Much work has been done to modify the azobenzene with different substituents.

The wavelength used to photoswitch the azobenzene molecule is in the UV range at 340 nm. At this wavelength light does not have a good tissue penetration ability. To improve the tissue penetration ability various substituents have been introduced in the azobenzene ring that would red shift the absorption maximum of the compound. This shift towards higher wavelength would improve the tissue penetration.

\textbf{1.3.2 Modification of Wavelength of Azobenzene Photoswitch}

The unmodified azobenzene photoswitch undergoes photoisomerization\textsuperscript{38,39} in the UV region. To make it less harmful for the tissues during in vivo studies, substituents have to be incorporated in the azobenzene rings to red shift the absorption maximum. This red shift of wavelength would also improve the tissue penetration ability that was a drawback of the UV light used to photoswitch unmodified azobenzene. The nature and position of the substituents\textsuperscript{40} on the benzene rings affect the red shift. The presence of electron donating group at the para position of one phenyl ring and the presence of electron accepting group at the para position of the other phenyl ring red shifts the absorption maximum of the azobenzene ring to a great extent. The excited state for the
photoisomerization is charged and hence more dipolar than the ground state. When electron donating and electron accepting groups are present in these molecules at para positions it helps in the charge distribution of the excited state by resonance and this reduces the activation energy barrier. So a higher wavelength of radiation is required to switch the molecule from trans isomeric form to the cis isomeric form. An example of such azobenzene derivative is shown in Figure 1.3.2 where there is an electron donating –OCH\(_3\) group at the para position of one ring and an electron accepting –NO\(_2\) group in the other ring.

![Figure 1.3.2 4'-Methoxy-4-nitro-azobenzene](image)

1.3.3 Modification of Azobenzene Relaxation Rate

The relaxation rates from the cis to the trans form of azobenzene molecule is of prime importance as that would decide the type of study these azobenzene photoswitches would help to monitor. If a fast biochemical reaction needs to be monitored that involves a pulsed conformational change then a short lived cis isomer with a fast relaxation time is desired. Kamei and group have developed the azobenzene photoswitch 4-(dimethylamino)-4’amidoazobenzene that has a relaxation time of around 0.1 ms. They have introduced this photoswitch into a single stranded
DNA. On the other hand if a reaction needs to be sustained over several hours then an azobenzene derivative with a longer relaxation time needs to be used.

There are many factors that affect the cis to trans relaxation rate of azobenzene. Some of these factors are the solvents\textsuperscript{41} used, pH of the solvent\textsuperscript{42} and the nature of substituents on the phenyl rings. The thermal transition state is dipolar in nature. Polar solvents would stabilize the dipolar transition state and reduce its activation barrier thus making the thermal transition faster. At a lower pH the azobenzene nitrogens would be protonated thus stabilizing the transition state and accelerating the reaction.

The nature of the substituents present in the phenyl ring also influences the transition rate. The presence of groups that resonance stabilize the thermal transition state reduces the activation energy barrier and increases the rate of thermal relaxation. The dipolar nature of the transition state for the excited state is similar to that of the thermal relaxation. So substituents that red shift the absorption maximum also accelerate the thermal relaxation. Woolley and coworkers have introduced many substituents at the para position of azobenzene molecule and studied the shift of their absorption maximum and change in thermal relaxation rates\textsuperscript{43}. The presence of an alkyl carbon atom at the para position makes the absorption maximum to be 342 nm with a half-life of 43 hours for thermal relaxation. The replacement of this carbon atom with an amide group red shifts the absorption maximum to 366 nm and the half-life of thermal relaxation changes to 12 minutes. The presence of a urea substituent at the para position changes the absorption maximum to 382 nm and the thermal relaxation becomes extremely fast with a half-life of 80 seconds. The structures of these compounds have been shown in Figure 1.3.3. Thus by introducing different substituents at the para position the absorption maximum and thermal relaxation of azobenzene photoswitches can be controlled.
Figure 1.3.3 Azobenzene Derivative with substituents: Azobenzene derivatives with carbon substituent (A), amide substituent (B) and urea substituent (C) at para position
1.3.4 Use of Azobenzene Photoswitches in Controlling Peptide Conformations

Various derivatives of azobenzene molecules have been used to control the conformation of peptides\textsuperscript{44,45}. Peptides form an integral part of many biological systems and having a spatiotemporal control over its conformation would help to modulate and study many biological processes.

Schutt and group have cyclized a heptapeptide containing the RGD sequence (Arg-Gly-Asp) with 4-aminomethylphenylazobenzouic acid (AMPB)\textsuperscript{46,47}. It is known that the RGD sequence interacts with αVβ3 integrin- a cell surface receptor which mediates the adhesion between cells and the extracellular matrix\textsuperscript{48}. The AMPB bound RGD peptides binds to the αVβ3 with higher affinity only when the isomer is in the trans form. This was a control over the attachment of RGD sequence with αVβ3 using AMPB.

Woolley and coworkers have also used various derivatives of azobenzene to control the secondary structure of peptides by attaching the linker to the peptides through cysteines placed at (i, i+4), (i, i+7) or (i, i+11) positions\textsuperscript{49,50}. Molecular modelling helped them to decide which isomeric form of the linker was a better fit at these positions. They developed the azobenzene linker 3,3′-bis(sulfonato)-4,4′-bis(chloroacetamido)azobenzene (BSBCA)\textsuperscript{51}. The end to end distance of the trans form of this linker would fit well at the (i, i+11) positions of a peptide making it helical while the length of the cis form was not compatible with this spacing. So on exposure to UV radiation when the trans isomer photoswitched to the cis form the helicity of the peptide decreased.

Thus much work has been done by researchers to use azobenzene derivatives in controlling the conformation of peptides.
1.4 Cell Penetrating Peptides in Drug Delivery

Cell penetrating peptides (CPP)\(^{52,53,54,55,56,57}\) are emerging as an important means of delivery of different types of cargo like ribonucleic acids, quantum dots, nano particles or insoluble drugs into the cells. The transport of the insoluble drugs has made the CPP more important in therapeutic studies. Tat\(^{58}\), penetratin\(^{59}\) and nona-arginine\(^{60}\) are examples of some cell penetrating peptides. CPP undergo internalization by endocytosis or by interacting with the cell surface glycans. TAT undergoes internalization by endocytosis whereas the positively charged arginine of nona-arginine interacts with the heparin sulfate proteoglycans of the cell membrane and enters the cell.
CHAPTER 2
PHOTOCAGED PERMEABILITY-
A NEW STRATEGY FOR DRUG RELEASE

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2.1 Introduction

Off-target toxicity plagues conventional cancer chemotherapy. One strategy to enhance selectivity of anti-cancer drugs involves un-masking the cytotoxicity of a molecule in the vicinity of the tumor\textsuperscript{61}. This type of activation can be mediated by enzymes\textsuperscript{61,62}, changes in pH\textsuperscript{63}, or exogenous factors such as temperature\textsuperscript{64} or light\textsuperscript{65,66,67}. Light is an ideal external stimulus since it provides a broad range of adjustable parameters\textsuperscript{68} that can be optimized for biological compliance. Several approaches that use light for biomolecular activation have been reported\textsuperscript{69,70,71,72,73}. One established method to enable selectivity of drug action using light is photodynamic therapy (PDT). In PDT,\textsuperscript{74,75} light activation of a photosensitizer generates cytotoxic singlet oxygen killing only illuminated cells. PDT is currently used in several types of malignancies including skin, lung, esophageal, bladder, head and neck, and prostate cancer. A related strategy, photochemical internalization,\textsuperscript{75,15} also uses photosensitizers. In this case, the photosensitizers are used to release macromolecular cytotoxins from endosomes, enabling their entry into the cytosol. However, these approaches suffer from disadvantages,\textsuperscript{76} including unpredictable drug uptake rates, the limited diffusion and lifetime of $^{1}$O$_{2}$, and the requirement for moderate levels of O$_{2}$ which may not always be available in the tumor environment.

In this communication, we report a new light-targeted drug delivery system, which operates independently of the creation of $^{1}$O$_{2}$. The basis of the system is the attachment of a cell impermeable small molecule to a drug via a linker that can be removed in presence of light, allowing cellular entry (Fig. 2.1). We call this new strategy photocaged permeability (PCP).

More specifically, we report the controlled release of the anti-cancer drug, doxorubicin (Dox) (Fig. 2), upon illumination. To prevent entry of Dox in the dark we attached Dox to EDANS (Fig. 2), a small fluorophore, chosen because it contains a sulfonic acid moiety known to hinder cellular
To connect Dox to EDANS we utilized a light-cleavable nitroveratryl (1) linker. The nitroveratryl moiety has been used previously as a photocaging group for a wide variety of biomolecules.

Figure 2.1. 1 Photocaged Permeability Strategy for Drug Delivery

Figure 2.1. 2 a). The anticancer drug doxorubicin. b). Cell-impermeable EDANS
2.2. Materials and Methods:

2.2.1: Synthesis of Dox-EDANS Drug Conjugate:

The synthesis (Scheme 1) began with commercially available nitroveratryl carboxylic acid (1). The N-hydroxy succinimide ester was prepared followed by coupling with propargylamine to give amide (1). This compound was converted to the p-nitrophenyl carbonate which was then treated with doxorubicin to generate photocaged doxorubicin carbamate (3). EDANS was coupled with azido benzoic acid (5) and was finally attached to the photocage using click chemistry to generate the final Dox-EDANS conjugate (6).
Scheme 2.1: Synthesis of photocaged-cell impermeable drug conjugate. Reagents and conditions:
(a) NHS, EDC-HCl; (b) propargylamine, Et3N;
(c) bis p-nitrophenylcarbonate, Et3N; (d) doxorubicin-HCl, Et3N; (e) NHS, EDC-HCl; (f) EDANS, EtN(iPr)2; (g) CuSO4·5H2O, sodium ascorbate, tris-(benzyltriazolymethyl)amine, DMSO : water (1 : 1).
2.2.2 Cell Studies:

The Esophageal cancer cell line (JH-EsoAd1) was cultured at 37°C and in a humidified atmosphere of 5% CO2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). For 96 well plate experiments, the JH-EsoAd1 cells were seeded at 4450 cells/well in 100uL of media in 96 well plates; experiments were carried out one day after seeding. MTT assays were analyzed using a Bio-Tex µQuant plate reader at 562 nm. FACS was performed BD FACS Aria II using BD FACS Diva software at the VCU Flow Cytometry Core Facility. A minimum of 20,000 cells within the gated region were analyzed. Confocal microscopy images were acquired at room temperature with a Leica confocal laser scanning microscope.

2.2.2.1 Illumination Time-Dependent Toxicity:

JH-EsoAd1 cells were treated with media alone, media supplemented with EDANS (20 µM), or media supplemented with Dox-EDANS (20 µM) in a 96 well clear-bottom, opaque plate. Cells were exposed to light (9 mW/cm2) for various times (0-40 min) regulated by an aluminum foil mask. During the experiment the plate was kept in ice to reduce the heating by the UV lamp. After the lamp was turned off, the plate was covered with foil and placed at room temperature for an additional 80 min. The media was removed, and the cells were thoroughly washed with PBS (3x, 200µL); then 200 µL of fresh media was added to each well. The plates were incubated for 72 hours at 37°C, following which the media was removed and 100 µL of MTT (2mg/ml) in phosphate buffered saline (PBS) was added to each well. After 3 hours, the MTT solution was removed and replaced with 100 µL of DMSO. The absorbance at 562 nm was analyzed via a plate reader.
2.2.2.2: Concentration Dependent Cell Viability Studies:

JH-EsoAd1 cells were treated with media supplemented with various concentrations of doxorubicin (0-6.4 µM) or Dox-EDANS (6) (0-16 uM) in a 96 well clear-bottom, opaque plate. Control wells were covered with aluminum foil while the remaining wells were exposed UV light (9 mW/cm²) for 20 min. The plate was kept in ice during light exposure in order to minimize the heating. After irradiation, the plate was kept at rt in the dark for another 100 minutes. The cells were washed and treated as above (time dependent toxicity experiments) to determine viability via the MTT assay.

2.2.2.3: Flow cytometry analysis of permeability:

JH-EsoAd1 cells were seeded at 150,000 cells/well in 1.5 mL of media in 6 well plates. The cells were treated with media alone or media supplemented with 10 µM of EDANS-Dox (6). The plate was placed on ice, and the light treated cells were exposed to UV light (9 mW/cm²) for 20 minutes. Following the light exposure the plates were kept at room temperature for 100 minutes. Then, the cells were washed with sterile PBS and trypsinized. For quantification of Dox fluorescence, treated cells were trypsinized, harvested, and washed with PBS at approximately 1x106 cells/mL. Pellets were fixed for 15 min at 37°C with 3% paraformaldehyde in PBS. Samples were washed thoroughly with PBS, resuspended in PBS and analyzed by flow cytometry.
2.2.2.4: Confocal microscopic analysis of permeability:

JH-EsoAd1 cells were seeded at 30,000 cells/well on 4 well glass chamber slides, and were treated as above (Flow cytometry analysis). The cells were treated with media alone or media supplemented with 10 μM of EDANS-Dox (6). The plate was placed on ice, and the light treated cells were exposed to UV light (9 mW/cm²) for 20 minutes. Following the light exposure the plates were kept at room temperature for 100 minutes. The cells were washed extensively with PBS and fixed for 15 min at 37°C with 3% paraformaldehyde in PBS. Slides were rinsed, dried and mounted under coverslips using Vectashield mounting media (Vector Laboratories, Inc.).

2.2.3: UV Lamp:

The UV lamp used in all studies consisted of a simple aquarium light fixture containing two Philips PL-S 9w/2P BLB bulbs. The intensity of the lamp was measured in the lab of Prof. M. Bertino (VCU) with the help of Michael Foussekis (VCU). The intensity was measured at different distances from the lamp and was found to decrease with increasing distance from the lamp.
Table 2.2. Measurement of Intensity of UV Lamp

<table>
<thead>
<tr>
<th>Distance of Photodetector from UV Lamp</th>
<th>Intensity of Lamp $^2$ (mWatts/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exactly Below lamp</td>
<td>9</td>
</tr>
<tr>
<td>1 inch below lamp</td>
<td>6.6</td>
</tr>
<tr>
<td>3 inches below lamp</td>
<td>3.9</td>
</tr>
<tr>
<td>4 inches below lamp</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Where diameter of the detector window was 9mm

2.3 Results

2.3.1: Effect of Light on the IC$_{50}$ of Doxorubicin

The dox-EDANS drug conjugate that we would use to validate our hypothesis has doxorubicin in it. It was important to see if the presence of the UV light affects the IC$_{50}$ of doxorubicin itself in Jh-EsoAd1 cells. The conclusion of this assay would help us compare the cytotoxicity induced in JhesoAd1 cells by the Dox-EDANS drug conjugate under dark and light conditions with that induced by doxorubicin itself under such conditions. In order to determine the effect of light on cytotoxicity of doxorubicin, we treated the Jh-EsoAd1 cells with (0.025uM, .05 µM, 0.1 µM, 0.25 µM, 0.4 µM, 0.8 µM, 1.6 µM, 3.2 µM and 6.4 µM) of doxorubicin in the presence and absence of UV lamp. The fraction of surviving cells was evaluated using the absorbance of the formazan product of MTT reduction. The IC$_{50}$ plot of the JH-EsoAd1 cells with doxorubicin in light and dark conditions is shown in Figure 2.3.1. The cytotoxicity in the presence of UV light as shown
by the red curve is similar to that under dark conditions as shown by the blue curve respectively in the Figure 2.3.1. Therefore, doxorubicin is cytotoxic to the cells to the same extent under illumination or in darkness. The value of IC$_{50}$ remains $1.0 \pm 0.4 \mu$M under both conditions.

![Figure 2.3.1 IC$_{50}$ of Doxorubicin in Jh-EaoAd1 Cells: IC$_{50}$ of Doxorubicin in Jh-EsoAd1 cells in the presence of UV light (Red Curve) and in Dark (Blue Curve) conditions.](image)
2.3.2: Effect of EDANS in Jh-EsoAd1 Cells under Light and Dark Conditions:

Since our conjugate also contain EDANS, we needed to show that it did not contribute to the cytotoxicity in the presence of light. The cells were treated with 20µM of EDANS and the assay was completed following the protocol mentioned in section 2.2.2.1. The effect of EDANS on the Jh-EsoAd1 cells over this time period has been shown in Figure 2.3.2 which is an average of three individual experiments. EDANS does not affect the cells prior to 20 minutes in the light and then the cell viability decreases thereafter. Under the dark conditions EDANS is not cytotoxic to the cells over the entire time period.
Figure 2.3.2 Effect of EDANS in Jh-EsoAd1 Cells: Effect of EDANS on treatment with Jh-EsoAd1 cells in the presence of UV light (blue curve) and in dark (red curve).
We proceeded to investigate the extent to which the release of Dox with light would lead to enhanced cellular toxicity. Indeed, increased illumination lead to decreased survival as measured by an MTT assay as shown in Figure 2.3.5. The cells with the Dox-EDANS drug were exposed to UV light for 1 min, 5 mins, 10 mins, 20 mins and 40 mins respectively. As the illumination time increased the cellular toxicity increased and the cellular viability almost reduced to 20 at the 40th minute as shown in the curve with solid diamonds in the figure 2.3.3. Whereas the Dox-EDANS conjugate is almost non toxic to the cells under the dark conditions as evident from the dotted curve with open squares in the same figure. This effect was not seen with doxorubicin and EDANS and clearly the cellular cytotoxicity is arising from the light controlled release of doxorubicin from the Dox-EDANS drug conjugate.
Figure 2.3.3 Light-dependent cytotoxicity of EDANS-Dox: Cells were treated with 10 mM of Dox-EDANS with (solid diamonds) or without (open squares) light for the specified times.
2.3.3 Concentration Dependent Cytotoxicity Study of Dox-EDANS on Jh-ESoAd1 Cells

The Jh-ESoAd1 cells were treated with Dox-EDANS drug at concentrations of 0.02 µM to 16 µM under illumination and dark conditions. The cells were exposed to UV light for 20 minutes and then kept incubated with the drug as mentioned in the section 2.2.2.2 (Concentration dependent Cell Viability Studies). From Figure 2.3.4 it is clearly evident that cytotoxicity is induced in the presence of the UV light (black diamonds) whereas in the dark (open squares) cell viability is almost intact. The IC\textsubscript{50} has been found to be 1.6 ± 1.0 µM
Figure 2.3.4 Concentration-dependent IC$_{50}$ of EDANS-Dox: Cells were treated with EDANS-Dox at various concentrations in the dark (open squares) or with UV light for 20 min (black diamonds).
2.3.4 FACS and Confocal Studies to Study Cell Permeability of Dox-EDANS Drug

We then investigated whether the attachment of EDANS to Dox via the veratryl linker would enable drug delivery. JH-EsoAd1 cells were incubated with Dox-EDANS or untreated with the drug in the dark or with illumination. Cell permeability was measured with flow cytometry; upon illumination a significant enhancement of cellular Dox fluorescence was observed (Figures: 2.3.5; 2.3.6 and 2.3.7). From Figure 2.3.5 it is clearly evident that the cells without the drug under light (red curve) or dark (blue) conditions do not show enhanced fluorescence. In the presence of the drug under light (green curve) the fluorescence intensity is enhanced compared to the dark condition (orange curve). A Representative dot plot of the relative fluorescence intensity of EDANS-DOX in Light or Dark has been shown in Figure 2.3.6 where the gate shown in the left panel (A) was used to derive the % Fluorescence data shown in the right panel (B). The Dox fluorescence quantified in the right panel (B) clearly indicates the enhanced % Fluorescence of Dox in the presence of UV light.
Figure 2.3.5 Flow cytometry of JH-EsoAd1 cells with and without EDANS-Dox: The flow cytometry histogram displays the relative fluorescence intensity of untreated controls in the dark (blue) or light (red) or JH-EsoAd1 cells treated with EDANS-Dox in the dark (orange) or light (green).
Figure 2.3.6 Relative fluorescence intensity of EDANS-DOX in Light or Dark: Flow cytometry analysis of EDANS-Dox with JH-EsoAd1 cells were performed in the dark and upon illumination (9.0 mW/cm²) for 20 min. Left Panel (A): Representative dot plot of the relative fluorescence intensity of EDANS-DOX in Light or Dark. The gate shown was used to derive the % Fluorescence data shown in the right panel. Right Panel (B): Quantification of DOX fluorescence for the indicated treatment conditions. Data are representative of two independent experiments, N=9.
This enhancement of fluorescence was mirrored in confocal studies with the same cell line (Figure 2.3.11); only light-treated cells show significant Dox fluorescence in the nucleus, where it is known to accumulate.

**Figure 2.3.7 Confocal images of JHEsoAd1 cells with and without EDANS-Dox:** These are representative confocal images of JHEsoAd1 cells treated with EDANS-Dox in the dark or light. The red channel shows Dox fluorescence, the gray channel is a DIC image, and the overlay represents the cellular localization of Dox fluorescence. Data are representative of two independent experiments of N=9.
2.4: Discussion

We have developed a new and efficient strategy for drug release based on photocaged permeability (PCP). In this first report, we have focused on applying PCP to the light-stimulated delivery of Dox into esophageal adenocarcinoma cells.

From the FACS studies (Figures 2.3.5, 2.3.6,) it is clearly evident that the Dox-EDANS conjugate does not enter the cells in dark. In the presence of light the nitroveratryl group is removed and the doxorubicin is released and is cell permeable. Doxorubicin upon entering the cells interacts with the DNA in the nucleus. The localization of doxorubicin in the nucleus in the presence of light is evident from Figure 2.3.7.

In principle, the PCP approach could be applied to any small, cell permeable molecule that has a free amine, hydroxyl, or carboxylic acid group for attachment of the veratryl-EDANS molecule. Further experiments will focus on use of other light-scissile linkers that can operate at longer wavelengths that are able to penetrate farther into tissues, as well as the use of other molecules that block permeability.
CHAPTER 3

PHOTOCAGED PERMEABILITY (PCP)

IN RECEPTOR MEDIATED DRUG DELIVERY
3.1: Introduction:

Targeted therapies are being used to treat different types of diseases in order to prevent various side effects that are part of the conventional treatments. The photocaged permeability (PCP) concept that has been introduced in chapter 2 is specific in terms of its light controlled drug release. To make this method dually specific we have tried to modify the conjugates such that a receptor binding molecule is attached to the photocaged entity. The receptor binding molecule would be specific for a particular receptor and once the drug conjugate gets internalized then upon shining light the photocaged molecule will be removed releasing the cytotoxic doxorubicin leading to death.

Targeted therapy is being widely used in cancer treatment where the overexpressed receptors on cancer cells are being used for drug delivery. Folate receptor\textsuperscript{79,80,81,82,83} is one of the receptors that are overexpressed in cancer cells. Much work has been done to develop folate-drug conjugates that are taken up by cancer cells through the folate receptors\textsuperscript{84}. Radioactive imaging agents have also been internalized using the folate receptors\textsuperscript{85}. The drug conjugate that we have developed has the photocaged molecule attached to folic acid (Dox-Folate conjugate) as shown in Figure 3.1.1. The folic acid of the drug conjugate would interact with the folate receptor and be internalized via endocytosis. The drug is not released until the drug conjugate is exposed to light.

Beyond folic acid, this strategy would open the doors to the use of photocaged technologies with other cell targeting molecules such as DUPA (2-[3-(1, 3-dicarboxy propyl)-ureido]) that binds to PSMA (prostate specific membrane antigen)\textsuperscript{86} which is overexpressed in prostate cancer cells.
Figure 3.1.1 Structure of the Photocaged Doxorubicin-Folate (Dox-Folate) Conjugate
3.2 Materials and Methods

3.2.1 Dox-Folate Drug Conjugate

The Dox-Folate conjugate shown in Figure 3.1.1 has been used for the study.

3.2.2 Solubility and Concentration of the Doxorubicin-Folic Acid Drug Conjugate

The Dox-Folate conjugate was initially dissolved in DMSO and it was precipitating. To overcome this problem 4 µL of 1 (N) acetic acid was added to a 20 µL solution of the drug conjugate in DMSO. This changed the pH of the solution from 10.5 to 6.5 as indicated by the pH paper and the precipitation of the drug conjugate stopped. So for the cell studies the drug conjugate was dissolved in DMSO with 1(N) acetic acid.

The concentration of the doxorubicin-folic acid drug conjugate was measured on nano drop using molar extinction coefficient of 11500 for doxorubicin in DMSO at 480nm.

3.2.2 Cell Studies

The initial experiments were done with FRα-null (R2) and FRα-(RT16) Chinese hamster cells (CHO) which were obtained from Prof. Larry H. Matherly, Department of Pharmacology, School of Medicine, Wayne State University. The R2 cells were cultured in α-minimal essential medium (MEM) supplemented with 10% fetal bovine serum (USA Origin, heat inactivated), glutamine and penicillin-streptomycin solution. The RT16 cells were cultured in α-MEM medium supplemented with 10% fetal bovine serum (USA Origin, heat inactivated), glutamine, penicillin-streptomycin solution and G418 (1.5 mg/mL). For the cytotoxic assays the R2 cells were cultured in RPMI 1640 with 10% dialyzed fetal bovine serum (USA Origin, heat inactivated), glutamine
and penicillin-streptomycin solution for 2 passages before the cytotoxic experiments. Similarly the RT16 cells were cultured in folate-free RPMI media supplemented with 10% dialyzed fetal bovine serum, glutamine, penicillin–streptomycin solution and G418 (1.5 mg/mL) for two passages prior to the cytotoxic experiments. The other cell line used for the experiments were KB-3-1 cells obtained from Prof. Jamal Zweit, Department of Radiology, Virginia Commonwealth University. These cells were cultured in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin solution. All the cell cultures were carried out in a humidified atmosphere of 5 % CO₂.

For 96 well plate experiments, the cells were seeded at 5000 cells/well in 100uL of media in 96 well plates; experiments were carried out one day after seeding. MTT assays were analyzed using a Bio-Tex µQuant plate reader at 562 nm. FACS was performed BD FACS Aria II using BD FACS Diva software at the VCU Flow Cytometry Core Facility. A minimum of 20,000 cells within the gated region were analyzed. Confocal microscopy images were acquired at room temperature with a Leica confocal laser scanning microscope.

The UV lamp used in all studies consisted of a simple aquarium light fixture containing two Philips PL-S 9w/2P BLB bulbs placed on top of the surface of the plate.

3.2.2.1 Concentration Time point assay:

The cells were treated with media alone in a 96 well clear-bottom, opaque plate. Cells were exposed to light (9 mW/cm²) for various times (0-120 mins) regulated by an aluminum foil mask. During the experiment the plate was kept in ice to reduce the heating by the UV lamp. The media was removed, and the cells were thoroughly washed with PBS (3x); then 200 µL of fresh media was added to each well. The plates were incubated for 72 hours at 37°C, following which the media
was removed and 100 µL of MTT (2mg/ml) in phosphate buffered saline (PBS) was added to each well. After 3 hours, the MTT solution was removed and replaced with 100 µL of DMSO. The absorbance at 562 nm was analyzed via a plate reader.

### 3.2.2.2 Flow Cytometry analysis

The cells were seeded at 200,000 cells/well in 1.5 mL of media in 6 well plates. The cells were treated with media alone, media with 1 µM of Dox-Folate conjugate (4), media with 1 µM of doxorubicin, media with folic acid (20 µM) + 1 µM of drug conjugate or media with 1 µM of doxorubicin + folic Acid (20 µM) at various time points. For the inhibitory studies in presence of folic acid at first, the cells were pre-incubated with 20 µM of folic acid for an hour and then the drug conjugate or doxorubicin was added to the wells and incubated for time period of 30 minutes, 3 hours and 24 hours. Then, at the end of each time point the cells were washed with sterile PBS and trypsinized. The trypsinized cells were centrifuged and resuspended in PBS supplemented with 10% FBS at 37°C and analyzed by flow cytometry. The fluorescence of doxorubicin was used to monitor the cell permeability of the drug. The cells were treated with media alone or media supplemented with 1 µM Doxorubicin-Folic acid conjugate (4). The plate was placed on ice, and the light treated cells were exposed to UV light (9 mW/cm²) for 20 minutes. Following the light exposure the plates were kept at room temperature for 100 minutes. Then, the cells were washed with sterile PBS and trypsinized. The trypsinized cells were centrifuged and resuspended in PBS supplemented with 10% FBS at 37°C and analyzed by flow cytometry.
3.3 Results:

The transport of folates across cell membrane takes place by different receptors. Folate receptor α (FRα)\textsuperscript{90,91,92,80} is one such receptor that helps the transport of folates across the cell membrane by receptor mediated endocytosis. These FRα receptors are overexpressed in cancer cells. So many folate conjugated cytotoxins or radionuclitides have targeted these FRα\textsuperscript{93,94} receptors to destroy the cancer cells. The drug that we have designed is a Dox-Folate conjugate which is believed to be taken up by these FRα folate receptors. In order to test for folate-receptor dependent uptake we needed cell lines with variable folate expression. R2 cells are CHO cells where the α-folate receptor has been knocked out whereas in the RT16 cells the folate receptors are over expressed.

3.3.1 Effect of UV light on R2 and RT16 Cells

The studies of the R2 and RT16 cells with the doxorubicin-folic acid drug conjugate would be carried out in the presence of UV light. It is important to see if the UV light has a role in causing cell death when these cell lines are exposed to the UV light over a certain interval of time. MTT assays were done with both R2 and RT16 cells where the cells were treated with media and exposed to UV light over an interval of (0-120) minutes regulated by an aluminum foil mask. The MTT assay was completed as mentioned in section 3.2.2.2. The survival plots for R2 cells and RT16 cells have been shown in Figures 3.3.1 and 3.3.2 respectively. From the Figure 3.3.1 it is clear that the R2 cells have a high viability close to 80 % till 40\textsuperscript{th} minute and after that there is a decrease in cell viability. For the RT16 cells the viability is close to 90% till the 80\textsuperscript{th} minute and then there is a drop. Both these cell lines have a 100 % viability for the first 20 minutes. Thus 20 minutes was chosen to be the time frame for the experiments to be conducted with the doxorubicin-folic acid drug conjugate in order to avoid toxicity from the light.
**Figure 3.3.1 Effect of UV Light on R2 cells**: R2 cells were exposed to UV light over a time period of (0-120) minutes.
Figure 3.3.2 Effect of UV light on RT16 cells: RT16 cells were exposed to UV light over a time period of (0-120) minutes.
3.3.2 FACS Studies

3.3.3.1 Cell Permeability Studies in R2 and RT16 cells with and without Folic Acid in the dark

We expected that folate receptor-null R2 cells would not show any uptake as they lack the folate receptors to promote the uptake of the drug whereas the RT16 cells will show the drug uptake due to the presence of the folate receptors. In addition, we performed studies with and without folic acid to confirm receptor-dependent uptake at various time points (Figure 3.3.3). These studies were performed in the dark in order to evaluate uptake prior to doing the release studies.

The results of flow cytometry experiments for R2 cells at different time points and conditions have been shown in Figure 3.3.3. It clearly shows that neither doxorubicin alone nor Dox-Folate drug enters the R2 cells that lack the folate receptors. For the competition studies the cells were preincubated with folic acid for an hour prior to the addition of the Dox-Folate drug. In this study it was found that in presence of folic acid there seemed to be a slight increase in the uptake. The plot of % fluorescence at 24 hr time point with doxorubicin, Dox-folate drug conjugate and Dox-folate drug conjugate with folic acid has been shown in Figure 3.3.4.
Figure 3.3.3 Histograms of R2 cells with doxorubicin, Dox-Folate + and – Folic Acid: Histograms of R2 cells only at t=30 mins (Panel A), with doxorubicin (1 µM) at t=30 mins, 3 hrs and 24 hrs from top to bottom (Panel B), with Dox-Folate conjugate (1 µM) at t =30 mins, 3 hrs and 24 hrs from top to bottom (Panel C) and with the Dox-Folate conjugate (1µM) + Folic acid (20µM, incubated for 1 hr with cells before adding the drug) at t=30mins, 3 hrs and 24 hrs from top to bottom (Panel D)
Figure 3.3. 4 Percentage Fluorescence Plot of R2 Cell: Plot of percentage fluorescence of doxorubicin, Dox-folate conjugate and Dox-folate conjugate with folic acid in R2 cells.
The flow cytometry experiments for RT16 cells at different time points and conditions have been shown in Figures 3.3.5 and 3.3.6. It clearly shows that both doxorubicin and Dox-Folate conjugate in the absence or presence of folic acid do not enter the RT16 cells even though they have overexpressed folate receptors. In the competition studies it was found that there was an increase in the uptake of Dox-Folate conjugate (Panel C of Figure 3.3.5) and of doxorubicin in the presence of folic acid (Panel B of Figure 3.3.6). It was necessary to do a control study where the cells were treated with only folic acid to see if that labels the cell. The study with just folic acid (Panel C of Figure 3.3.6) clearly shows that it does not label the RT16 cells that had the overexpressed folate receptors. The folic acid clearly blocks the folate receptors. The plot of % fluorescence at 24 hr time point with doxorubicin, Dox-folate drug conjugate and Dox-folate drug conjugate with folic acid has been shown in Figure 3.3.7.
Figure 3.3.5 Histograms of RT16 cells with doxorubicin, Dox-Folate + and –Folic Acid: Histograms of RT16 cells only at t=30 mins (Panel A), with doxorubicin (1 µM) at t=30 mins, 3 hrs and 24 hrs from top to bottom (Panel B), with doxorubicin-folic acid drug conjugate (1 µM) at t =30 mins, 3 hrs and 24 hrs from top to bottom (Panel C) and with the Dox-Folate conjugate (1µM) + Folic acid (20µM, incubated for 1 hr with cells before adding the drug) at t=30mins, 3 hrs and 24 hrs from top to bottom (Panel D)
Figure 3.3.6 Histograms of RT16 cells doxorubicin + Folic Acid, Folic Acid: Histograms of RT16 cells only at t=30 mins (Panel A), with doxorubicin (1 µM) + Folic acid (20µM, incubated for 1 hr with cells before adding doxorubicin) at t=30mins, 3 hrs and 24 hrs from top to bottom (Panel B) and with only folic acid (1µM) at t=30 mins, 3 hrs and 24 hrs from top to bottom (Panel C).
Figure 3.3. 7 Percentage Fluorescence Plot of RT16 Cells: Plot of percentage fluorescence of doxorubicin, Dox-folate conjugate and Dox-folate conjugate with folic acid in RT16 cells.
We do have an explanation as to why cell permeable doxorubicin did not accumulate in the cells. CHO cells are known to express P-glycoprotein 1 which is known to pump out doxorubicin as soon as it enters the cell. It is likely that the Dox-Folate drug conjugate also gets pumped out of both cell types.
3.3.3.2 Cell Permeability Studies in KB-3-1 cells with and without Folic Acid:

Since our ultimate goal was to measure doxorubicin toxicity, we decided to change the cell line to KB-3-1 cells. These cells have overexpressed folate receptors and are expected to uptake the drug conjugate in the absence of folic acid.

The fluorescence of doxorubicin was used to monitor the fluorescence and study the cell permeability via flow cytometry. From Figure 3.3.8 as expected the cells show a clear time dependent uptake of doxorubicin. The uptake of the Dox-Folate conjugate also increases with time as evident from panel C of Figure 3.3.8. But the inhibitory study did not give a satisfactory result. The panel D of this figure shows that even in presence of folic acid the drug conjugate is entering the cells which might indicate some different path of entry apart from the folate receptor for the drug. This is all the more clear from Figure 3.3.9 which clearly shows that the drug uptake in presence of folic acid (Panel C of Figure 3.3.9) is enhanced compared to experiments where cells are incubated alone with the Dox-Folate conjugate (Panel B of Figure 3.3.9). The plot of % fluorescence at 24 hr time point with doxorubicin, doxorubicin folate conjugate with and without folic acid has been shown in Figure 3.3.8.
Figure 3.3.8 Histograms of KB-3-1 cells with doxorubicin, Dox-Folate + and – Folic Acid:
Histograms of KB-3-1 cells only at t=30 mins (Panel A), with doxorubicin (1 µM) at t=30 mins, 4 hrs and 9 hrs from top to bottom (Panel B), with doxorubicin-folic acid drug conjugate (1 µM) at t=30 mins, 4 hrs and 9 hrs from top to bottom (Panel C) and with the Dox-Folate conjugate (1 µM) + Folic acid (20 µM, incubated for 1 hr with cells before adding the drug) at t=30 mins, 4 hrs and 9 hrs from top to bottom (Panel D)
Figure 3.3.9 Histograms of KB-3-1 cells with Dox-Folate, Dox-Folate + Folic acid: Histograms of KB-3-1 cells only at t=30 mins (Panel A), with Dox-Folate conjugate (1 µM) at t=30 mins, 4 hrs, 9 hrs, 12hrs and 24 hrs left to right (Panel B) and with the Dox-Folate conjugate (1µM) + Folic acid (20µM, incubated for 1 hr with cells before adding the drug) at t=30mins, 4 hrs, 9 hrs, 12 hrs and 24 hrs from left to right (Panel D)
Figure 3.3. 10 Percentage Fluorescence Plot of KB-3-1 Cells: Plot of percentage fluorescence plot of doxorubicin, Dox-folate conjugate and Dox-folate conjugate with folic acid in KB-3-1 cells.
3.4: Discussion

The FACS studies with the CHO cells showed no uptake of the drug due to the pumping out of doxorubicin by the P-gp pumps in these cells. The P-gp<sup>95,96,97</sup> pumps expressed on these cell membranes identify foreign substances like drugs or toxins and pump them out of the cells using an ATP-dependent efflux pump. To avoid this issue, the cell line was changed to KB-3-1 cells. In these cell lines the drug was taken up by the cells but the inhibitory study in the presence of folic acid unexpectedly showed enhanced uptake of the drug. There could be various reasons for this. We surmise that pre-incubation of the cells with folic acid have activated endocytosis, pulling membrane bound-drug into the cells. The relatively slow uptake of the folate-dox conjugates suggests that this activation may be operative. Alternatively, the pre-incubation with folic acid could have increased the folate receptor concentration on the cell surface to ultimately enhance uptake. In either case, our inability to show selective blocking of uptake with folate led us to abandon these experiments.

3.5 Future work

Much of the work with folate inhibitors have been done with KB<sup>85b</sup> cells. So it may be fruitful to change the cell line to KB cells for further studies with the doxorubicin-folic acid drug conjugate. The other point that also needs to be addressed is the drug concentration used in the FACS studies. Most of the work done with these cell lines have folate conjugates added in nM range whereas we had used 1 µM. We used this high concentration in order to detect the penetration of the weakly fluorescent doxorubicin. Using such a high concentration of the drug might have enhanced non-specific binding that increased the drug uptake and obscured the folate specific update.
CHAPTER 4

EFFECTS OF IODOACETAMIDE AZOBENZENE ON THE SECONDARY STRUCTURE OF PEPTIDES
4.1 Introduction

The α-helix is an integral part of the secondary structure of proteins and it plays an important role in its biological function. Yet peptides excised from the α-helical domains of proteins typically do not retain their helical structure. Many studies have been done to investigate the factors that stabilize and enhance α-helicity in peptides. The helicity could be stabilized by adding metal chelates, by introducing helix initiator templates or by using intramolecular peptide cross linkers. Verdine and coworkers have developed hydrocarbon stapled peptides with improved helicity. A ruthenium catalyzed olefin metathesis reaction takes place between the olefin side chains of the amino acids to form a hydrocarbon staple that improves the helicity of the peptides. Paramjit Arora and group have developed hydrogen bond surrogate helices where the intramolecular hydrogen bond is replaced by a C-C bond to form a better helix.

To have a spatial and temporal control over the secondary structure of peptides photoisomerizable linkers have been introduced to reversibly control the secondary structure of peptides. One of the most commonly used photoswitchable linker is azobenzene. Different derivatives of azobenzene have been used to control the secondary structure of peptides. Woolley and coworkers have used iodoacetamide, shown in figure 4.1.1 to improve the helicity of peptides. They have shown that this linker in its trans form when introduced at (i, i+7) residues of a peptide destabilizes the helicity as its length is too long to allow the adjacent residues to adopt an α-helical form. Whereas the length of the cis form of the linker is compatible with the (i, i+7) spacing and increases the helicity of the peptides and thus acts as an entropic helix stabilizer. The peptide with the trans form of the linker in the randomly coiled state when exposed to UV radiation changes to the more helical cis form as shown in Figure 4.1.2. The sequences on which most people have worked to study change in peptide helicity upon addition of linkers have been derived from the sequence.
(EAAAR)_n^{103}. The basic sequence (EAAAR)_n is already helical due to the presence of ion pair interactions between glutamic acid (E) and arginine (R). Therefore it is unclear how much contribution to the helicity is made by the azobenzene and how much is due to the intrinsic helicity of the peptide sequence itself. We reasoned that we could investigate the role of iodoacetamide azobenzene itself in affecting the secondary structure of peptides by systematically removing such ion pair interactions and by comparing the uncyclized to the azobenzene crosslinked peptides in each state.

The initial sequence that we took for our study is Ac-EACARV1AACAAARQ-NH$_2$ where 1= $\alpha$-amino isobutyric acid. The peptide was cyclized and circular dichroism was used to measure the change in helicity upon cyclization under dark and light conditions. We then systematically mutated the glutamic acids to arginine, to remove the ion pairs and replace them with the somewhat repulsive Arg-Arg pairs. A series of such peptides have been generated with different arginine mutations and cyclized with the iodoacetamide azobenzene linker. This study would give us an insight into the ability of the iodoacetamide linker itself to affect the secondary structure of peptides in the absence of helix promoting factors like ion-pair interactions.
Figure 4.1.1 4, 4' Iodoacetamide Azobenzene
Figure 4.1.2 Conformational Control of Peptides by Azobenzene

Trans linker and Randomly coiled Peptide

Cis linker and helical Peptide

370 nm

450 nm or
Thermal Relaxation
4.2 Materials and Methods

4.2.1 Synthesis of Linker

The synthesis of the iodoacetamide linker was done based on the literature report\textsuperscript{43} . 4, 4'-Diaminoazobenzene (225mg, 1.2 mmol) was dissolved in 20mL of anhydrous tetrahydrofuran. It was then stirred under argon for 15 mins and was protected from light during this time. Then triethylamine (3 equivalents, 500uL, 3.61 mmol) was added to the above solution followed by the dropwise addition of chloroacetyl chloride (3 equivalents, 287 µL, 3.61 mmol). The reaction was then stirred at room temperature for another 30 minutes and then vacuum filtration was done to remove the precipitate. The tetrahydrofuran was removed by rotary evaporation. The product obtained was dissolved in a 1:1 solution of tetrahydrofuran and dichloromethane and was extracted with water acidified with a few drops of 1% HCl. Anhydrous MgSO\textsubscript{4} was use to dry the filtrate and it was then vacuum filtered. The solvents were then removed by rotary filtration to form 4, 4'-Dichloroacetamide azobenzene. Then to form the iodo derivative from the chloro derivative at first a 20 mL solution of anhydrous acetone and 15 mL of anhydrous tetrahydrofuran were combined and degassed. 1 g (7.1 mmol) of sodium iodide was dissolved in the above degassed solvent and added to 150mg of dichloroacetamide acetamide. This solution was protected from light and was stirred for 12 hrs under argon. Sodium chloride was removed by filtration and the solvent were removed by rotary evaporation. The solid obtained was redissolved in a 1:1 solution of tetrahydrofuran and dichloromethane and vacuum filtration was used to remove excess of sodium iodide. The filtrate was extracted with water and anhydrous MgSO\textsubscript{4} was used to dry it. It was vacuum filtered, the solvent was removed by rotary evaporation and a solid product of 4, 4'-iodoacetamide azobenzene was obtained.
4.2.2 Peptide Synthesis:

Standard Fmoc-based solid phase peptide synthesis was used to make the peptides on a Liberty Automated Microwave Peptide Synthesizer (CEM). The peptides were made on a Fmoc-Rink amide MBHA Resin (0.65 mmol/gm). The formation of the peptides were confirmed by MALDI-TOF and they were purified by reverse phase HPLC on a Shimadzu system with Vydac (218TP C18 5µ) column. The mobile phases used for the purifications were 0.1% TFA in water (A) and CH₃CN (B) and they were monitored at 215nm. The elution time for these purifications were 30 minutes. The gradients used for the purification of the peptides and the mass obtained from the MALDI-TOF have been summarized in Table 4.2.1.
Table 4.2.1 Linear Peptides with HPLC gradient and MALDI-TOF values

<table>
<thead>
<tr>
<th>Linear Peptides</th>
<th>HPLC Gradient Used for Purification</th>
<th>MALDI-TOF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OBSERVED (Da)</td>
</tr>
<tr>
<td>Ac-EACARV1ACEAAARQ-NH₂ (Pep R2)</td>
<td>10-65% of B</td>
<td>1645.94</td>
</tr>
<tr>
<td>AC-RACARV1AACRAARQ-NH₂ (Pep R4)</td>
<td>10-60% of B</td>
<td>1700.43</td>
</tr>
<tr>
<td>Ac-RACRRV1RACRAARQ-NH₂ (Pep R6)</td>
<td>10-55% of B</td>
<td>1850.54</td>
</tr>
<tr>
<td>Ac-RACRRV1RACRRARRQ-NH₂ (Pep R8)</td>
<td>10-60% of B</td>
<td>2041.33</td>
</tr>
<tr>
<td>Ac-EACARV1ACEAAARAAARQ-NH₂ (Pep R3)</td>
<td>10-60% of B</td>
<td>2015.52</td>
</tr>
<tr>
<td>AC-RACARV1AACRAARAAARQ-NH₂ (Pep R5)</td>
<td>10-60% of B</td>
<td>2070.43</td>
</tr>
</tbody>
</table>

Where 1 = α-Amino isobutyric Acid, 
B = 0.1% TFA in CH₃CN
4.2.3 Peptide Cross-Linking

Peptide crosslinking with iodoacetamide azobenzene linker was performed according to a literature report. The uncrosslinked peptide (1.13 mM), Tris.Cl buffer (pH=8) and Triscarboxyethyl phosphine (1.13 mM) were combined in a volume of 355µL and stirred under Argon for 2 hrs to reduce the cysteine residues. To this a linker solution of 0.56 µmol in 500µL of DMSO was added and stirred in the dark for 10 min. A second aliquot of 10 mM solution of 55.8µL the linker was added to the solution and stirred in the dark for 10 min. Then a third aliquot of 10 mM of the same volume of linker was added and stirred in dark for 10 minutes. This was followed by an exposure of the solution to light for 20 minutes. The unreacted linker was removed by acetone precipitation. The cyclized peptides were purified using a Shimadzu system with Vydac (218TP C18 5µ) column using mobile phases 0.1% TFA in water (a) and CH₃CN (B) and were monitored at 370 nm. The elution time for these purification was 30 minutes. MALDI-TOF was used to confirm the formation of the cyclized peptides. The gradients used for the purification of the cyclized peptides and the mass obtained from MALDI-TOF has been summarized in table 4.2.2.
### Table 4.2.2 Cyclized Peptides with HPLC gradient and MALDI-TOF values

<table>
<thead>
<tr>
<th>Cyclized Peptides</th>
<th>HPLC Gradient Used for Purification</th>
<th>MALDI-TOF</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OBSERVED</td>
<td>CALCULATED</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Da)</td>
<td>(Da)</td>
<td></td>
</tr>
<tr>
<td>Ac-EACARV1AACEAAARQ-NH₂ (Pep R2)</td>
<td>10-65% of B</td>
<td>1937.82</td>
<td>1937.43</td>
<td></td>
</tr>
<tr>
<td>AC-RACARV1AACRAARQ-NH₂ (Pep R4)</td>
<td>10-60% of B</td>
<td>1993.56</td>
<td>1993.54</td>
<td></td>
</tr>
<tr>
<td>Ac-EACARV1AACEAAARAAARQ-NH₂ (Pep R3)</td>
<td>10-60% of B</td>
<td>2306.10</td>
<td>2306.34</td>
<td></td>
</tr>
<tr>
<td>AC-RACARV1AACRAARAAARQ-NH2 (Pep R5)</td>
<td>10-65% of B</td>
<td>2361.92</td>
<td>2361.76</td>
<td></td>
</tr>
</tbody>
</table>

Where

- \( l \)= \( \alpha \)-Amino isobutyric Acid,
- \( B \)= 0.1% TFA in CH₂CN
4.2.4 Circular Dichroism

Circular Dichroism was measured on an OLIS DCM 1000 spectrophotometer. All measurements were done with a quartz cuvette of 0.1 cm pathlength. The samples were dissolved in 5 mM of phosphate buffer of pH=7. The reported spectra are an average of 3 CD experiments. The response time was 4 sec. The mean residue weight of the peptide and cross-linked peptides were assumed to be the same for the respective peptides and were calculated by dividing the molecular weights of the peptides by the number of amino acid residues in the sequence. The peptide concentration was measured on a nanodrop ND 1000 spectrophotometer using a molar extinction coefficient of 28,000 for the iodoacetamide azobenzene linker in the dark state.

4.2.5 Agadir Calculation

The software Agadir (http://agadir.crg.es/) was used to calculate the percentage helicities of the uncyclized peptides. The conditions used for the calculations include a buffer concentration of 5 mM, temperature of 20°C and ionic strength of 0.03.
4.3 RESULTS

4.3.1 Design of Azobenzene Linker

The design of the azobenzene linker has been based on the work done by Andrew G. Woolley. The trans form of the linker is more stable than the cis form in the dark. In the paper (PNAS 2000, 8, 3803) it has been shown that the iodoacetamide azobenzene linker when attached to the cysteine residues of the peptides at (i, i+7) positions disrupts and reduces its helicity. On shining light on the cyclized peptide in dark for 5 minutes the linker photoswitches to the cis form and in turn increases its helicity. We incorporated the azobenzene linker at the (i, i+7) positions of the peptides that we designed and studied the effect of the linker on its secondary structure upon photoisomerization.

4.3.2 Design of Peptides

We designed a series of peptides with varying number of arginines and studied the effect of the azobenzene linker on its secondary structure. The basic sequence used was Ac-EACARV1AACEAAARQ-NH2 (Peptide R2) where 1=α-amino isobutyric acid (AiB) described by Woolley101. Molecular modeling had suggested that introduction of bulky amino acids at (i, i+3) and (i, i+4) positions between the linker attachment points would destabilize the trans conformation further. Thus the amino acids valine and AiB were introduced at the (i, i+3) and (i, i+4) positions with the purpose of creating steric interactions between these residues and the linker that would further destabilize the helicity of the peptides in the dark state. So upon photoisomerization of the linker a greater change in helicity is expected. A set of peptides were generated from this basic sequence by systematically replacing the glutamic acid with arginine to
replace the ion pairs with Arg-Arg repulsive pairs. The second sequence was obtained by replacing 
the glutamic acids in peptide R2 to generate the peptide R4 with 4 arginines as shown in the table
4.3.1. Agadir software was used to generate the next set of peptides. It’s an algorithm based 
prediction which uses the helix coil transition theory. It predicts the helicity of monomeric peptides 
and takes into account factors such as pH, temperature, ionic strength, short range interactions and 
also terminal modifications of the peptides. The basic sequence of peptide R2 was used and 
arginine mutations were done to generate peptides with 6 and 8 arginines with the least helicity 
with the purpose of studying their change in helicity upon cyclization with the azobenzene linker.
This led to the sequences of peptide R6 and R8 as shown in the table 4.3.1. The peptide R2 and 
R4 when extended by amino acids AAAR along the C terminus was found to have an increased 
helicity. This led to the generation of peptides R3 and R5 as shown in the table below. The 
sequences used for the studies have been summarized in Table 4.3.1.
Table 4.3.1 Peptide Sequence

<table>
<thead>
<tr>
<th>Peptide Sequences</th>
<th>(Pep R#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-EACARV1AACEAAARQ-NH₂</td>
<td>(Pep R2)</td>
</tr>
<tr>
<td>Ac-RACARV1AACRAARQ-NH₂</td>
<td>(Pep R4)</td>
</tr>
<tr>
<td>Ac-RACRRV1RACRAARQ-NH₂</td>
<td>(Pep R6)</td>
</tr>
<tr>
<td>Ac-RACRRV1RACRRRAARQ-NH₂</td>
<td>(Pep R8)</td>
</tr>
<tr>
<td>Ac-EACARV1AACEAAARAAARQ-NH₂</td>
<td>(Pep R3)</td>
</tr>
<tr>
<td>Ac-RACARV1AACRAARAAARQ-NH₂</td>
<td>(Pep R5)</td>
</tr>
</tbody>
</table>

Where 1 = \( \alpha \)-Aminoisobutyric acid
4.3.3 Effect of the Photolinker on Secondary Structure of the Peptides

The secondary structure of the peptides were measured using circular dichroism. At first the helicity of the uncylcized peptides were measured at 20°C as shown in Figure 4.3.1. Peptide R3 is the most helical among the six peptides with 21.2 % helicity and peptide R6 has the least helicity of 3.5 % as shown in Table: 4.3.2. The ion pair interaction between glutamic acid and arginine and the presence of an extra turn of the helix along the C terminus is responsible for the high helicity of peptide R3. The replacement of the ion pair interactions between glutamic acid and arginine with the arginine-arginine repulsive pairs reduce the helicity and thus accounts for the less helicity in peptides R4, R5, R6 and R8.

The peptides were cyclized with the iodoacetamide linker. The circular dichroism of the cyclized peptides were measured in dark and then they were exposed to light at 370nm for 5 minutes and then the circular dichroism were again measured. The change in secondary structure of the linear peptides R2, R3, R4 and R5 upon cyclization has been shown in Figure 4.3.2. It is evident that there is not much change in helicity of peptide R2 upon cyclization as denoted by A in this figure. The helicity of R4 and R3 decreases upon cyclization as shown by B and C in this figure. On the other hand the helicity of R5 increases on cyclization. In peptide R4 in addition to the absence of the Glu-Arg ion-pair interaction the trans form of the linker further reduces its helicity. Whereas in peptide R3 the only destabilizing factor for the helicity is the trans form of the linker that reduces its helicity to 15.1 % from 21 %. In peptide R5 the repulsive interaction between the Arg-Arg residues is probably overcome to some extend by the trans linker that increases its helicity from 8.2 % to 14.6 %.
Figure 4.3.1 CD of Linear Peptides: CD spectrum of Linear Peptides in 5mM Phosphate Buffer at pH=7 and 20°C.
Table 4.3. 2 Percentage Helicity of Linear and Cyclized Peptides

<table>
<thead>
<tr>
<th>Peptide Sequences</th>
<th>% Helicity of Uncyclized Peptide</th>
<th>% Helicity of Cyclized Peptide in DARK</th>
<th>% Helicity of Cyclized Peptide after Shining Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-EACARV1AACEAAARQ-NH$_2$ (Pep R2)</td>
<td>11.8</td>
<td>13.2</td>
<td>34.1</td>
</tr>
<tr>
<td>Ac-RACARV1AACRAARQ-NH$_2$ (Pep R4)</td>
<td>5.9</td>
<td>NIL</td>
<td>15.2</td>
</tr>
<tr>
<td>Ac-EACARV1AACRAARAAARQ-NH$_2$ (Pep R3)</td>
<td>21.1</td>
<td>15.1</td>
<td>32</td>
</tr>
<tr>
<td>Ac-RACARV1AACRAARAAARQ-NH$_2$ (Pep R5)</td>
<td>8.2</td>
<td>14.6</td>
<td>38.0</td>
</tr>
</tbody>
</table>
Figure 4.3.2 CD Spectrum of linear and Cyclized peptide R2 (A), R4 (B), R3 (C) and R5 (D)
The changes in helicity of R2, R4, R3 and R5 peptides upon photoswitching of the linker from trans to cis form has been shown in Figures 4.3.3. In each of these peptides the trans form of the linker changes to the cis form that is compatible with the (i, i+7) spacing and produces an increase in helicity. The greatest change in helicity is observed for peptide R5 which changes from 14.6 % to 38 %. This is followed by peptide R2 that changes from 13.2 % to 34.1 %.
Figure 4.3.3 CD Spectra of Peptides R2 (A), R4 (B), R3 (C) and R5 (D) under light and Dark Conditions
4.4 DISCUSSION

The effect of cyclization of the iodoacetamide azobenzene on the secondary structure of the peptides can be analyzed in the following categories.

4.4.1 Effect of Arg-Glu Ion-pair Interaction and Length of Peptides

In peptides R2 and R3 favorable Arg-Glu ion pair interactions exist. Upon cyclization the helicity of R2 does not change much whereas that of peptide R3 decreases. In peptide R3 there is an extra turn of helix along the C terminus where an additional Arg-Glu interaction exists. The azobenzene crosslinking position is at the cysteine adjacent to this Glu, and it is possible that a steric interaction disrupts this helix-stabilizing ion pair. The opposite result is seen in peptide R5. In this case, the cyclization linker appears to enhance helicity. The trans form of the azobenzene is structurally incompatible with an alpha-helical conformation, so this enhancement is challenging to explain. We postulate that the cyclization helps to segregate the N-terminal repulsive arginines from the rest of the peptide by orienting them in a direction away from those in the C-terminus, enabling the C-terminal portion to adopt a more helical structure. Thus we see that the degree of secondary structure present in any of these sequences is a very delicate balance of the existing ion-pair interactions, the length of the helix and the photoisomerization of the linker. In some cases the ion pair interaction overrides the cyclization while in other cases it’s the cyclization that overrides the ion pair interaction.
4.4.2 Dipole-Side Chain Interaction

The helicity of a peptide also depends on the interaction between the peptide dipole and the side chain of the amino acids. It has been found that the positive side chain of arginine when present at the N terminus interacts with the dipole and destabilizes the α-helix and if it’s along the C terminus then it stabilizes it. In all these peptides there is an arginine at the C terminus. So the presence or absence of arginine along N terminus makes a contribution to its stability. In peptide R2 one arginine is at the C terminus and the other is at the center and upon cyclization not much change in its interaction with the dipole occurs and so the secondary structure does not change much.

R4 has an arginine along the N terminus, 2 along the center and one at the C terminus. The arginine is almost equally distributed along the length of this peptide. Similar is the situation for peptide R5. So not much contribution to helicity is expected from this interaction. Upon cyclization probably the change in helicity observed in R4 and R5 is governed by other factors than this dipole–side interaction. Thus in these two peptides it’s the balance other factors like cyclization and Arg-Glu interactions that finally determine the helicity. In R3 peptide the presence of two arginines along the C terminus makes it helical. Upon cyclization the helicity of this peptide decreases probably a change in alignment of the arginines reduces it interaction with the dipole and so a lesser % of helicity is obtained upon cyclization.
CHAPTER 5

PHOTOSWITCHABLE PEPTIDES -

IN TARGETED THERAPY
5.1 Introduction

The chemotherapeutic treatment of cancer involves a variety of methods. The lack of specificity in the methods causes many side effects to the patients. In order to reduce these side effects and increase the specificity of the treatment the concept of targeted therapy has developed. The molecular targets like human epidermal growth factor receptor 2 (HER-2) are identified and are treated with monoclonal antibodies Trastuzumab (Herceptin). Small molecular drugs like Dasatinib (Sprycel) are used which inhibit tyrosine kinase enzymes. Cancer vaccines are another means to treat cancer where; the vaccine activates a person’s immune system to recognize and attack the cancer cells. Another method of targeted therapy that is highly selective as well as specific is the light mediated treatment of cancer. Photodynamic therapy and photochemical internalization fall under this category. In photodynamic therapy\textsuperscript{74} phosensitizers trigger the formation of singlet oxygen upon irradiation with light of a suitable wavelength. It operates only in presence of light so it is highly selective. It has some drawbacks as the amount of molecular oxygen in cancer cells is often not very high. In photochemical internalization a toxin is at first internalized in cells in the endosome and this reduces the efficiency of the process. Both in photodynamic therapy and photochemical internalization there is much disparity in the light to drug administration time probably due to the slow uptake of the phosensitizers and formation of singlet oxygen. The method we have developed is devoid of the problems described above. A photoswitchable peptide is used which, in the dark state is randomly coiled and cell impermeable, but upon illumination becomes helical and cell permeable. Then upon entering the cell the drug attached to the peptide would be released causing cell death.

The design of the peptides has been based on the work done by Woolley and co-workers.\textsuperscript{101} They have designed peptides and have used 4, 4’-bis (iodoacetamide)-azobenzene to control the helicity
of these peptides. The linker when attached to the peptides through cysteines at (i, i +7) and (i, i+4) position in the trans form makes the peptide less helical as their end to end distance does not match the distance between the two cysteines. Upon exposure to UV radiation at 370 nm the trans isomer photoswitches to the cis form. In the cis form the end to end distance of the linker matches with the spacing between the two cysteines and this makes the peptides helical. To make the peptides more randomly coiled in the dark state they have introduced branched amino acids like α amino isobutyric acid (AiB) and valine at (i, i+3) and (i, i+4) positions between the cysteines which are seven amino acids apart. The linker in the trans form interacts sterically with these branched amino acids and further destabilizes the cyclized peptide in the dark state. Thus on photoisomerization a greater change in % helicity is observed. It has been established by Alanna Schepartz and group that an arginine patch developed on the helical surface of an impermeable protein makes it cell permeable. Based on these facts and the photoswitchable peptides designed by Woolley we have designed our peptides. We want the peptide to be randomly coiled in the dark state and change to a helical form in the presence of light. This will be possible when it is linked by a light sensitive linker at the (i, i+4) and (i, i+7) positions as established by molecular modeling studies done by Woolley and his co-workers. The linker that we will use to control the conformation of the peptide is 4, 4' - bis (iodoacetamide)-azobenzene. In the trans form the peptide is randomly coiled and upon isomerization to cis form it would make the peptide helical as shown in Figure 4.1.2.
The initial sequence on which the work was started was Ac-2EACARV1AACEAAARQ-NH$_2$ where 2=Amidated cysteine and 1=α-amino isobutyric acid. To develop an arginine patch in this sequence the amino acids would be mutated to arginine. The formation of the arginine patch is clearly evident from the helical wheels in Figure 5.1.1 where replacement of the glutamic acids at position 2 and 12 with arginine generates an arginine patch as shown in helical wheel B.

Figure 5.1.1 Helical Wheel of peptides 8.1 and 8.2: Helical Wheel of peptides Ac-2EACARV1AACEAAARQ-NH$_2$ (A) and Ac-2RACARV1AACRAARQ-NH$_2$ (B).
The amidated cysteine denoted as 2 in the peptide sequences would serve as an attachment point for the drug through a Cathepsin B cleavable linker. In the trans form of the linker the peptide with the drug would be cell impermeable. Upon illumination at 370 nm the linker would change to the cis form making the peptide helical with the arginines aligned together to form the arginine patch that would make it cell permeable. This has been shown in Figure 5.1.2. This system could potentially be used to deliver drugs into cells in the presence of light.

Figure 5.1. 1 Light Induced Drug Delivery
5.2 Materials and Methods:

5.2.1 Synthesis of Linker:

The linker used is 4, 4′-bis-iodoacetamide azobenzene and the synthesis has been done according to the literature report. The synthesis has been reported in details in the section 4.2.1.

5.2.2. Peptide Synthesis:

Standard Fmoc-based solid phase peptide synthesis was used to make the peptides on a Liberty Automated Microwave Peptide Synthesizer (CEM). The peptides were made on a Fmoc-Rink amide MBHA Resin (0.65 mmol/gm). The formation of the peptides were confirmed by MALDI-TOF and they were purified by reverse phase HPLC on a Shimadzu system with Vydac (218TP C18 5µ) column. The mobile phases used for the purifications were 0.1% TFA in water (A) and CH$_3$CN (B) and they were monitored at 215nm. The elution time for these purifications were 30 minutes. The HPLC chromatograms of the peptides and their mass spectra have been reported in the appendix section.
5.2.3 Peptide Cross Linking

Peptide crosslinking with the iodoacetamide azobenzene linker has been done as mentioned in section 4.2.3. The cyclized peptides were purified using a Shimadzu system with Vydac (218TP C18 5μ) column using mobile phases 0.1% TFA in water (a) and CH$_3$CN (B) and were monitored at 370 nm. The elution time for these purification was 30 minutes. MALDI-TOF was used to confirm the formation of the cyclized peptides. The chromatograms for the purification of the cyclized peptides and their mass spectra have been reported in the appendix.

5.2.4 Circular Dichroism

Circular Dichroism was measured on an OLIS DSM 1000 spectrophotometer. All measurements were done with a quartz cuvette of 0.1cm pathlength. The samples were dissolved in 5 mM of phosphate buffer of pH=7. The reported spectra are an average of 3 CD experiments. The response time of the instrument was 4 second. The mean residue weight of the peptide and cross-linked peptides were assumed to be the same for the respective peptides and were calculated by dividing the molecular weights of the peptides by the number of amino acid residues in the sequence. The peptide concentration was measured on a nanodrop ND 1000 spectrophotometer using a molar extinction coefficient of 28,000 for the iodoacetamide azobenzene linker in the dark state at 367 nm.
5.2.5 Removal of Acm group of Amidated Cysteine

The amidated peptide (22mg, 3µmol) was treated with TFA containing 1 % of anisole. To this solution Ag\textsubscript{2}SO\textsubscript{4} (80mg, 480µmol) was added and was stirred at 4°C for 2 hours. Then the solution was concentrated under argon. Around 5mL of cold ether was used to precipitate the peptide from the above concentrated solution. The concentrated solution was vortexed and centrifuged. The precipitated peptide was treated with 1 mL of dithiothreitol (0.2M in 1M acetic acid) for 3 hours at room temperature. After 3 hrs this solution was filtered and injected on HPLC. The purified peptide obtained from HPLC was then subjected to mass spectra analysis to confirm the removal of the acm group. The mass spectra and HPLC traces of peptide 8.5 are there in the appendix.

5.2.6 FAM5 Labelling of Peptides

The peptides were labelled with 5(6) Carboxyfluorescein (Fam-5) [shown in Figure 5.2.1]. The labelling of the peptide was done on the resin, on which the peptide was synthesized. Fam-5 (0.3 mmoles, 112.8 mg) was dissolved in 4 mL of N, N-dimethyl formamide (DMF). It was covered in aluminum foil and put on a tumbler for 20 minutes that would help in the dissolution of Fam-5 in DMF. Then this Fam-5 solution was added to a tube containing the peptide-resin (0.01 mmoles, 27 mg). To this was added a solution of N, N-dicyclohexylcarbodiimide [DCC] (0.3 mmoles, 61.9 mg) dissolved in 200 µL of DMF and hydroxybenzotriazole [HOBT] (0.3 mmoles, 40.5 mg) dissolved in 200 µL of DMF. The tube with all these contents was covered with aluminium foil and kept on a tumbler overnight to complete the labelling of the peptide. The formation of the labelled peptide was confirmed with Ninhydrin test. In this test some of the beads are taken in a small test tube and washed with DMF, MeOH and DCM thoroughly. Then they were washed 3-4
times with ethanol. Then a few drops of Kaiser solution A [20 µL KCN (13 mg dissolved in 20 mL of water) with 980 µL of pyridine and to this was added 100 µL of Pyridine/ethanol solution (8g of phenol in 2 mL of absolute ethanol)] and Kaiser solution B [1.0 g of Ninhydrin in 20 mL of absolute alcohol] was added. The tube was kept in a heating block at 100° C for 2 minutes. The color of the beads were observed. The color of the beads remain yellow indicating they were labelled with Fam-5. If the color of the bead changed to blue then that would indicate the presence of free amine that had reacted with Ninhydrin to form the blue compound Ruhemanns blue. The labelling method has been summarized in Figure 5.2.2.

![Figure 5.2.1 Structure of 5(6)-Carboxyfluorescein](image)
5.2.7 Cell Studies

The Esophageal cancer cell line (JH-EsoAd1) was cultured at 37°C and in a humidified atmosphere of 5% CO2 in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). For 96 well plate experiments, the JH-EsoAd1 cells were seeded at 4450 cells/well in 100uL of media in 96 well plates; experiments were carried out one day after seeding. MTT assays were analyzed using a Bio-Tex µQuant plate reader at 562 nm. FACS was performed BD FACS Aria II using BD FACS Diva software at the VCU Flow Cytometry Core Facility. A minimum of 20,000 cells within the gated region were analyzed. Confocal microscopy images were acquired at room temperature with a Leica confocal laser scanning microscope.
5.2.7.1 Fluorescence Microscopy

The cell permeability of the cyclized labelled peptide was studied initially using fluorescence microscopy. Fam-5 Labelled Arg-9 peptide was used as a positive control as it is known to be highly cell permeable. Olympus inverted Fluorescence microscope was used for these experiments.

To determine the concentration of Arg9 needed to label the cells around 30,000 cells were plated in a 3mm sterile cell culture plate. The following day the cells were treated with Arg9Fam5 peptide at concentrations of 0.09 µM, 0.18 µM, 0.37 µM, 0.75 µM, 1.5 µM and 3 µM. The peptide was incubated with the cells for an hour. Then the media was removed and the cells were washed with PBS (3x) and fresh media was added. The plates were then observed under the fluorescence microscope. The minimum concentration of Arg9Fam5 needed to label JH-EsoAd1 cells were seeded at 5000 cells/well and treated with media alone, Fam-5 labelled Arg9 (Arg9Fam5) peptide and Fam-5 labeled cyclized peptides in a 96 well clear-bottom, opaque plate under light and dark conditions. Cells were exposed to light (9 mW/cm2) for various times (0-40 min) regulated by an aluminum foil mask. During the experiment, the plate was kept in ice to reduce the heating by the UV lamp. After the lamp was turned off, the plate was covered with foil and placed at room temperature for an additional 80 min. The media was removed, and the cells were thoroughly washed with PBS (3x); then 200 µL of fresh media was added to each well. The plates were then observed under the fluorescence microscope to check the permeability of Arg9 and Fam-5 labeled cyclized peptide.
5.2.7.2: Flow Cytometry Analysis for Cell Permeability:

JH-EsoAd1 cells were seeded at 200,000 cells/well in 1.5 mL of media in 6 well plates. The cells were treated with media alone or media supplemented with Arg9Fam5 or Fam-5 labeled cyclized peptides. The plate was placed on ice, and the light treated cells were exposed to UV light (9 mW/cm²) for 20 minutes. Following the light exposure the plates were kept at room temperature for 100 minutes. Then, the cells were washed with sterile PBS and trypsinized, centrifuged and resuspended in PBS and analyzed by flow cytometry.

5.3: Results:

5.3.1: Design of peptides:

The peptide sequence on which Woolley has studied the effect of photoswitching with iodoacetamide linker is Pep 8 of Table 5.3.1. In this peptide the cysteines are at (i, i+7) spacing. Based on this sequence a peptides 8.1 and 8.2 (shown in Table 5.3.1) were designed where arginine mutations were introduced to develop peptides with an arginine patch that would eventually help in their cell permeability. We also wanted to explore the effect of the iodoacetamide azobenzene on the helicity of the peptide when attached at (i, i+4) positions and so Pep 8.3 was synthesized. The peptides 8.1, 8.2 and 8.3 were cyclized and then the CD data was obtained. The problem encountered during the purification of these cyclized peptide was their extremely low yield. The amount of peptides obtained after the removal of Acm protecting group of amidated cysteine was extremely low. To improve the yield of the peptides it was decided to label them with a fluorescent tag. The fluorescent tag Fluorescein-5-isothiocyanate (FITC) was added at the N terminus to form Pep 8.4. During the cyclization of this peptide the resulting thiourea competed with the other two
cysteines to react with the linker and thus the expected cyclized peptide was not obtained. The sequences were further changed to Peptides 8.5 and 8.6 where FAM-5 was used as a fluorescent label. They were cyclized and purified and a good yield was obtained. The cell permeability of these two peptides were studied using Jh-EsoAd1 cells.
### Table 5.3. 1 Peptide Sequences

<table>
<thead>
<tr>
<th>Peptide Sequences</th>
<th>(Pep 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-<strong>E</strong>ACARV1AACEAAARQ-NH$_2$</td>
<td></td>
</tr>
<tr>
<td>Ac-<strong>E</strong>ACARV1AACEAAARQ-NH$_2$</td>
<td>(Pep 8.1)</td>
</tr>
<tr>
<td>Ac-<strong>R</strong>ACARV1AACRAAARQ-NH$_2$</td>
<td>(Pep 8.2)</td>
</tr>
<tr>
<td>Ac-<strong>R</strong>AAAREACARRCAARQ-NH$_2$</td>
<td>(Pep 8.3)</td>
</tr>
<tr>
<td><strong>5</strong> , <strong>3</strong> , <strong>4</strong>-RAAAREACARRCAARQ-NH$_2$</td>
<td>(Pep 8.4)</td>
</tr>
<tr>
<td><strong>6</strong>-EACARV1AACEAAARQ-NH$_2$</td>
<td>(Pep 8.5)</td>
</tr>
<tr>
<td><strong>6</strong>-RACARV1AACRAAARQ-NH$_2$</td>
<td>(Pep 8.6)</td>
</tr>
</tbody>
</table>

Where  
1= α-amino isobutyric acid, 2= S-acetamidomethyl-L-cysteine  
3= Fmoc-propargyl glycine, 4= Fmoc-6-aminohexanoic acid  
5= Fluorescein-5-isothiocyanate (FITC)  
6= 5(6)-Carboxyfluorescein (Fam5)
5.3.2 Effect of Linker on Helicity of Peptides

The secondary structure of the cyclized peptides was measured using circular dichroism in before and after irradiating with 370 nm light for 5 min.

5.3.2.1 Change in Helicity of Peptides with Linker attached at (i, i+7) and (i, i+4) position

We were interested to see how the iodoacetamide azobenzene linker would change the helicity of peptides when attached at a (i, i+7) and (i, i+4) positions. The CD spectrum of peptides 8.1, 8.2 and 8.3 were recorded in the dark, after irradiation with UV light for 5 minutes and 30 minutes after removal of light. The spectrum showed that the change in % helicity of peptides 8.1 and 8.2 with cysteines at (i, i+7) positions were higher than that of pep 8.3. So it was decided to pursue further studies with peptides attached to the linker at (i, i+7) positions. Among peptides 8.1 and 8.2 a greater change in % helicity was observed for pep 8.3. The CD spectrum of peptides 8.1 and 8.2 have been shown in Figures 5.3.1 and 5.3.2. The change in helicity for both peptides have been shown in Table 5.3.2.
Figure 5.3.1 CD Spectrum of Pep 8.1: CD Spectrum of Ac-2EACARV1AACEAAARQ-NH$_2$ (Pep 8.1) in phosphate buffer at pH=7
Figure 5.3.2 CD Spectrum of Pep 8.2: CD Spectrum of Ac-2RACARV1AACRAARQ-NH$_2$ (Pep 8.2) in phosphate buffer at pH=7.
Table 5.3. 2 Percent Helicity of Peptides 8.1 and 8.2

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>% Helicity of Pep 8.1</th>
<th>% Helicity of Pep 8.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark State</td>
<td>2.2</td>
<td>11.5</td>
</tr>
<tr>
<td>Upon Illumination</td>
<td>3.8</td>
<td>18</td>
</tr>
<tr>
<td>30 Minutes After Removal of Light</td>
<td>2.5</td>
<td>12.4</td>
</tr>
</tbody>
</table>

5.3.2.2 CD Spectrum of Pep 8.2 at 25°C and 37°C

The permeability of the peptides would eventually be studied in cells at 37°C and hence it was necessary to see how the helicity of the peptides change at this temperature. The CD spectrum of peptide 8.2 was measured at 25°C (Figure 5.3.3) and 37°C (Figure 5.3.4). The measurements were done in dark, immediately after shining light for 5 minutes and readings were taken for about an hour since the light was removed. It was found that the helicity change was greater at 25°C than at 37°C. The % change in helicity of pep 8.2 at 25°C and 37°C has been shown in Table 5.3.3 and 5.3.4 respectively. With the rise in temperature the kinetic energy of the molecules increase which leads to their unfolding. As the temperature increases from 25°C to 37°C the peptide 8.2 unfolds and so the helicity also reduces. Since the helicity is low at 37°C not much change in it is observed on exposure to UV radiation when the linker photoswitches. These studies indicate that to attain a decent amount of change in % helicity at 37°C the peptide sequence needs to be
modified. The simultaneous low yield of Pep8.2 also led to change the sequence to pep 8.5 and
8.6. Further CD studies would be done with these peptides.

The CD of the Fam-5 labelled cyclized Pep 8.5 gave a percentage helicity of 250.2 in dark to
266.3% upon illumination. Literature search showed that researchers working with Fam-5 labelled
peptides took the CD of the unlabeled peptides while they used the Fam-5 labelled peptide for cell
studies. Fam5 probably might interfere with the CD measurement and hence such numbers of %
helicity were obtained.
Figure 5.3.3 CD spectrum of Pep 8.2 at 25°C: CD spectrum of Ac-2RACARV1AACRAAARQ-NH₂ (Pep 8.2) at 25°C in phosphate buffer at pH=7
Table 5.3.3 Percent Helicity of Peptide 8.2 at 25°C

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Dark State</th>
<th>Upon Illumination</th>
<th>20 Minutes After Light Removal</th>
<th>35 Minutes After Light Removal</th>
<th>50 Minutes After Light Removal</th>
<th>77 Minutes After Light Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Helicity</td>
<td>9.7</td>
<td>16.8</td>
<td>13.2</td>
<td>10.8</td>
<td>9.9</td>
<td>8.2</td>
</tr>
</tbody>
</table>
**Figure 5.3.4 CD Spectrum of Pep 8.2 at 37°C:** CD Spectrum of Ac-2RACARV1AACRAARQ-NH$_2$ (Pep 8.2) at 37°C in phosphate buffer at pH=7
Table 5.3.4 Percent Helicity of Peptide 8.2 at 37°C

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Dark state</th>
<th>Upon Illumination</th>
<th>14 Minutes After Light Removal</th>
<th>44 Minutes After Light Removal</th>
<th>64 Minutes After Light Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Helicity</td>
<td>4.7</td>
<td>5.0</td>
<td>3.5</td>
<td>2.4</td>
<td>2.7</td>
</tr>
</tbody>
</table>

5.3.3 Measurement of Half Life of Cyclized pep 8.2

The Half-life of the azobenzene linker in cyclized peptide 8.2 was determined using HPLC. At first the cyclized pep 8.2 was exposed to UV radiation at 370nm for 5 minutes. Small volumes were removed at various times for direct analysis by HPLC. At the beginning when t=0 there were two peaks denoting the presence of both the cis and trans form of the cyclized peptide 8.2. As time increased from 32 mins to 96 mins one of the peaks decreased indicating that the cis isomer was thermally relaxing back to the trans isomer. The half-life was determined to be 50.58 minutes using the plot shown in Figure 5.3.6.

Half-life was calculated using the equation $y=Ae^{-kt}$ where $y$= quantity at time $t$, $A$= quantity at $t=0$, $k$=decay constant.
Figure 5.3.5 Chromatograms for Cyclized pep 8.2: Chromatograms for Cyclized pep 8.2 at 1=0mins, 2=32mins, 3= 64 mins and 4=96mins.
Figure 5.3.6 Half-life of cyclized peptide 8.2: Plot of % Cis isomer of cyclized peptide 8.2 with time to calculate half-life of cyclized peptide 8.2
5.3.4: Fluorescence Microscopy to Study Peptide Permeability

5.3.4.1 Concentration Dependent Permeability Study of Arg9Fam5

A concentration based permeability study of the control peptide Arg9Fam5 in JH-EsoAd1 cells was done using fluorescence microscopy. The plates were then observed under the fluorescence microscope. The minimum concentration of Arg9Fam5 needed to label the Jh-EsoAd1 cells was 1.5 µM as evident from Figure 5.3.7.

![Figure 5.3.7 Concentration dependent study of Arg9Fam5 with Jhe-EsoAd1 cells](image)
5.3.4.2: Permeability Study of Fam-5 labeled Peptides

A permeability study of the Fam-5 labeled Pep 8.5, Pep 8.6, control peptide Arg9Fam5 and negative control Fam5 was performed on JH-EsoAd1 cells. The concentration of all the peptides used in the assay was 1.5µM. Only the cells treated with Arg9Fam5 both under light and dark conditions were labelled as evident from Figure 5.3.8. The Fam5 labelled peptides Pep 8.5, Pep 8.6 and Fam5 itself did not label the peptides. As a concentration of 1.5µM of the labeled peptides was used for these studies it could be possible that a little higher concentration these labeled peptides are permeable. To validate these results flow cytometry studies were performed on JH-EsoAd1 cells with the lab labeled peptides at a higher concentration.

Figure 5.3.8 Fluorescence Microscopy studies with FAM5 labelled peptides: Cell Permeability study of Fam5 labelled Pep7, Pep8, Arg9Fam5 and Fam5 at concentration of 1.5µM
5.3.4.3 Flow Cytometry Study of labelled Peptides

To study the permeability of the Fam5 labelled peptides JH-EsoAd1 cells were plated at 150000 cells/well in 6 well cell culture plates. The Cells were treated with only media, media supplemented with Arg9Fam5 (1.5 µM), Pep 8.6 (Fam5-RACARV1AACRAAARQ-NH₂, 40uM and 76 uM) under dark and light conditions. For the experiments with light the cells were exposed to UV light for 20 minutes and then were kept in dark for 100 minutes. The media was removed, cells were washed with PBS (3x), trypsinized and then suspended in PBS for flow cytometry.

The cells were labelled extensively with ARg9Fam5 at 1.5 uM of the peptide used. On the other hand a large amount of Pep 8.6 was used for the studies to get a sufficient amount of labelling. From Figure 5.3.9 it is evident that there is not much difference under light and dark conditions for Pep 8.6. As per our hypothesis it was expected that the Pep 8.6 under dark condition would not label the cells whereas upon illumination as the linker would change to the cis form the peptide would become helical and cell permeable. But the results obtained were contrary to our hypothesis—the peptide was not cell permeable under either conditions.

The reason that not much difference in the light and dark condition is observed is probably because of the peptide’s poor % change in helicity at 37 degrees.
Figure 5.3. 9 Histogram of JH-EsoAd1 cells with FAM5-Pep8 and FAM5Arg9: Histograms of JH-EsoAd1 cells only and with Arg9Fam5 at 1.5 µM from top to bottom of Panel A, with Pep8 (40µM) under Dark (top) and Light (bottom) of Panel B, with Pep8 (76µM) under Dark (top) and light (bottom) of Panel C.
5.3.5 CD Studies of Peptides developed from Computational Studies

5.3.5.1 Computational Studies to Generate Peptide Sequences

Computational studies were done in collaboration with Prof. Glenn Kellogg’s lab to develop a series of peptides that would show some change in helicity when cyclized with meta-dibromo xylene linker. They built peptide sequences within SYBYL-X in α-helical conformation using charged termini and using the sequence of peptide 5 as the basic sequence as shown in Table 5.3.5. The other sequences were obtained by replacing alanine with AiB (α-amino isobutyric acid) as AiB is a helix promoter. A total combination of 91 sequences were obtained using all possible combinations by incorporating a maximum of four AiB amino acids. They were then subjected to minimization studies under TRIPOS force field. 10 sequences were selected out of this pool to do molecular dynamics simulation studies to predict their secondary structure. Finally a total of four peptides were selected for CD studies from this set of 10 peptides as shown in Table 5.3.5.

Table 5.3.5 Peptide Sequences from Computational Studies

<table>
<thead>
<tr>
<th>Peptide Sequences</th>
<th>(Pep 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAAAREACARRCAARQ</td>
<td></td>
</tr>
<tr>
<td>RAAARE1CARRCAARQ</td>
<td>(Pep 5.1)</td>
</tr>
<tr>
<td>RA1AREACARRCA1RQ</td>
<td>(Pep 5.2)</td>
</tr>
<tr>
<td>RA1AREACARRC11RQ</td>
<td>(Pep 5.3)</td>
</tr>
</tbody>
</table>

Where 1 = α-amino isobutyric acid
5.3.5.2 CD Studies of Peptides with AiB Mutations

The CD spectrum of the linear peptides obtained from computational studies clearly showed that these peptides had a very low helicity. This has been shown in Figure 5.3.10. The percentage helicity of the peptides were very low as shown in Table 5.3.6.

![Graph showing CD spectrum of Peptides 5, 5.1, 5.2, and 5.3.](image)

**Figure 5.3.10 CD of Peptides 5.5.1, 5.2 and 5.3:** CD Spectrum of Peptides 5, 5.1, 5.2 and 5.3 in phosphate buffer at 20°C in phosphate buffer.
Table 5.3. 6 Percentage Helicity of Peptides 5, 5.1, 5.2 and 5.3

<table>
<thead>
<tr>
<th>% Helicity</th>
<th>Peptide 5</th>
<th>Peptide 5.1</th>
<th>Peptide 5.2</th>
<th>Peptide 5.3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.3</td>
<td>2.7</td>
<td>3.3</td>
<td>3.3</td>
</tr>
</tbody>
</table>
5.4 Discussion

The cell permeability studies clearly indicated that cyclized peptides were not cell permeable both under dark and light conditions. The CD studies clearly showed that peptides had low helicity at 37°C. There are certain factors that needs to be modified in order to improve the helicity of the peptides. The peptide lengths and nature of amino acids in the sequences can be modified. It is also possible that the peptides had less amount of arginines in it. So if the sequence of peptide 8.5 which is Fam5-EACARV1AACEAAARQ-NH₂ can be modified by introducing more arginines at different positions and tested for cell permeability then some promising result might be obtained. A series of such peptides have been developed as shown in chapter 4 and they need to be tested for cell studies. Simultaneously computational studies with the basic peptide 5 with different AiB substitutions generated a total of 4 peptides and the CD studies also showed that these peptides had very less helicity. Computational studies with peptide 8 where the N terminus would have an acetyl and the C terminus would have an amide and with arginines at different positions might generate a series of peptides. The CD study with this set of peptides might give some new results.

5.5 Future Work

The basic peptide 8.5 would be mutated with arginines at various positions and labelled with Fam5 and used for cell studies on Jh-EsoAd1 cells both under light and dark conditions. At the same time computational studies would also help to decide on the sequences of peptides to be used.
5.6 Summary

In the first part of this thesis we have discussed the development of a new strategy called photocaged permeability (PCP). Using this strategy we have shown the light controlled release of doxorubicin in the esophageal adenocarcinoma cell lines. The concept of photocaged permeability has been further developed to introduce dual specificity in the drug delivery systems. The dox-photocage would be attached to a molecule that is specific for a receptor overexpressed on the cell surface. Thus attachment to a specific receptor and the release of doxorubicin from the internalized drug upon exposure to radiation would make the process dually specific. Folic acid has been attached to the photocaged doxorubicin to specifically target the overexpressed folate receptors on the cancer cells. The photocaged doxorubicin would be further attached to different chemical substances that are specific for other receptors overexpressed in cancer cells.

In later part of the thesis we talk about a series of designed peptides whose conformation was controlled by the photoswitch iodoacetamide azobenzene. The secondary structure of these peptides changed upon cyclization but did not show much cell permeability. So a new series of peptides with different number of arginine substitutions have been developed and they would be tested for cell permeability. Computational studies would also be done to generate a series of cyclized peptides that might possibly show more permeability upon exposure to radiation.
REFERENCES


73. Riggsbee, C. W.; Deiters, A., Recent advances in the photochemical control of protein function. *Trends in biotechnology* 2010, 28 (9), 468-75.


APPENDIX I MALDI-TOF ANALYSIS OF PEPTIDES

Pep R2
Obs. Mass=1646.08
Calc. Mass=1646.32

Pep R4
Cal: 1700.44
Obs: 1700.33
Pep R3
Cal: 2015.52
Obs: 2015.51

Pep R5
Obs. Mass=2069.48
Calc. Mass=2069.65
Pep R8
Cal: 2040.82
Obs: 2040.70

Cyclized Pep R2
Cal: 1937.52
Obs: 1937.43
Cyclized Pep R4
Obs. Mass=1993.56
Calc. Mass=1993.54

Cyclized Pep R3
Obs. Mass=2306.10
Calc. Mass=2306.34
Cyclized Pep R5
Obs. Mass=2361.92
Calc. Mass=2361.76

Pep 8.1
Obs. Mass: 1819.5
Cal. Mass: 1820.017
Cyclized Pep 8.1
Obs. Mass: 2114.10
Cal. Mass: 2214.13

Pep 8.3
Obs. Mass=2269.25
Calc. Mass=2269.27
Cyclized Pep 8.5
Obs. Mass=2016.68
Calc. Mass=2016.9

Pep 8.5
Obs. Mass=1832.06
Calc. Mass=1832.76
Acm Removed Pep 8.1
Obs. Mass: 2041.5
Cal. Mass: 2041.08

Pep 5
Obs.: 1760.60
Cal.: 1760.90
Pep 5.1
Obs.: 1774.61
Cal: 1774.45
Appendix II HPLC CHROMATOGRAM
Vita

Education:

M.Sc in Chemistry from Banaras Hindu University, India in 2007

B.Sc in Chemistry from Lady Brabourne College, Calcutta University, 2005

Publications:


Patent:

Light Enabled Drug Delivery (WO2013049521) published on 4th April, 2013
Inventors: Matthew C.T. Hartman, Martin Michael Dcona, Deboleena Mitra

Poster Presentations:

Title: Photoswitchable Peptides and Photocaged Molecules in Targeted Drug Delivery
Annual Poster Session, Department of Chemistry, Virginia Commonwealth University 2009-2013

Title: Photocontrolled Drug release from a Caged Drug Conjugate
Poster presentation at American Association of Cancer Research in April, 2013

Title: Photoswitchable Peptides and Photocaged Molecules in Targeted Drug Delivery
Massey Retreat 2012, MCV, Virginia Commonwealth University 2012

Title: Photocontrolled Drug Release from a Caged Drug Conjugate
Watts Day Poster session, MCV, Virginia Commonwealth University 2011

Research Experience:

1. Graduate Research Assistant, Massey Cancer Center, Virginia Commonwealth University
   Advisor: Prof. Matthew C.T. Hartman

   A. Photocaged Molecules in Targeted Drug Delivery:
   Developed photocaged doxorubicin prodrug which is cell impermeable in the dark but on exposure to UV light releases the cytotoxic doxorubicin leading to cell death. Techniques used include tissue culture, cell survival assays, flow cytometry and confocal microscopy.
B. Conformational Control of Secondary Structure of Peptides by Azobenzene Linker
Developed and studied the conformational control of α-helical structure of peptides crosslinked with azobenzene. Techniques used include solid phase peptide synthesis, HPLC, mass spectroscopy, circular dichroism, flow cytometry and confocal studies.

2. Master of Science, Indian Association for the Cultivation of Science, Kolkata, WB, India
Advisor: Prof. Amitabha Sarkar from May-July, 2006

Title: ‘Derivatization of Ferrocene for the Formation of C-C and C-N Bonds’

Teaching Experience:

Organic Chemistry Laboratory-Teaching Assistant (10 semesters, 50 students each semester). Supervised and instructed students in Organic Chemistry labs which included multiple step synthesis of compounds and various chemical techniques.

Awards:

1. Susan and Gerald Bass Scholarship, Department of Chemistry, College of Humanities and Sciences, VCU in 2013

2. Lidia Vallerino Scholarship, Department of Chemistry, College of Humanities and Sciences, VCU in 2012

3. VCU Global Education Office community connections program recognition certificate in 2011

4. Susan and Gerald Bass Scholarship, Department of Chemistry, College of Humanities and Sciences, VCU in 2009