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In Vitro Efficacy of Combined Macrophage-Mediated Plasmonic Photothermal Therapy and Chemotherapeutic Agents

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IN VITRO EFFICACY OF COMBINED MACROPHAGE-MEDIATED PLASMONIC
PHOTOTHERMAL THERAPY AND CHEMOTHERAPEUTIC AGENTS

by

En Chung Shih

Bachelor of Science in Biological Science
University of California, Irvine
Irvine, CA
2011

A thesis submitted in partial fulfillment
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**En Chung Shih**

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ABSTRACT

Although both chemotherapy and hyperthermia therapy have their inherent advantages, the non-specific targeting of chemotherapy and ineffectiveness of hyperthermia therapy alone suggests a better method can be developed. To overcome this problem, the combined treatment of chemotherapeutic agents with plasmonic photothermal therapy (PPTT) is proposed. This is the first study to investigate the efficacy of combined chemotherapy and PPTT using monocytes as delivery vehicles for gold nanoshells. The results of this investigation shows that PPTT combined with cisplatin resulted in some degree of synergism while additive effects were observed for concurrent treatments of PPTT and doxorubicin and PPTT and bleomycin.
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CHAPTER 1

INTRODUCTION

1.1 Hypopharyngeal Squamous Cell Carcinoma

Hypopharyngeal cancers are relatively uncommon: each year approximately 2,500 new cases are diagnosed in the U.S (American Cancer Society, 2014). These cancers can be divided into upper hypopharyngeal and lower hypopharyngeal cancers. Upper hypopharyngeal cancers are more closely linked with heavy drinking and smoking, in comparison to the lower hypopharyngeal, or the postcricoid, cancers which are more frequently associated with nutritional deficiencies. Currently, the majority of hypopharyngeal cancers are mucosal squamous cell carcinomas (SCC). Current treatment for many cancers such as brain, hypopharyngeal squamous cell carcinomas and breast include surgical resection of the tumor followed by various concurrent or subsequent treatments such as chemo and/or radiotherapy. The goal of subsequent treatments is to eliminate the malignant cells residing in the margin surrounding the resection cavity thereby reducing or eliminating recurrence. Unfortunately, in many cases, none of the standard treatment regimens have proven to be successful suggesting that alternative therapeutic approaches are required.

Almost all cancers in the hypopharynx originate from thin, flat cells of squamous cells located in the innermost layer of the epidermis (Fig. 1) Cancers originating in this layer are called squamous cell carcinoma or squamous cell cancer. Most squamous cell cancers begin as a pre-cancerous condition of dysplasia, and when seen under a microscope, the cells look abnormal but not yet cancerous. Often this condition resolves
on its own without any treatments, especially if the underlying cause is addressed. Most patients with dysplasia do not have any symptoms unless it is on the vocal cord. In some cases, the dysplasia will progress to carcinoma *in situ* (CIS). In this condition, the cancer cells are seen in the epithelial lining of the hypopharynx, but have not grown into deep layers or spread to other parts of the body. Carcinoma *in situ* is the earliest form of cancer and can be cured at this stage. If carcinoma *in situ* is not treated, it can develop into an invasive squamous cell cancer which can destroy nearby tissues and spread to other parts of the body.

![Figure 1: Right hypopharyngeal wall squamous cell carcinoma extending from the tonsil down to the pyriform sinus (Ghorayeb, 2014)](image)

Clinically, hypopharyngeal cancers have the tendency to be aggressive: they are characterized by diffuse local and distant spread and, as such, they tend to metastasize in their early stages. In a large study of patients with SCC of the pharynx and hypopharynx,
87% of patients with pyriform sinus SCC were found to have stage III or stage IV disease; 82% of patients with SCC of the posterior pharyngeal wall were found to have stage III or stage IV disease (American Cancer Society, 2014).

In general, hypopharyngeal cancer treatments are controversial. To some extent, this is attributed to its low incidence and the inherent difficulty in conducting adequately powered, prospective, randomized clinical studies (Trinidad et al., 2014). For this reason, it is difficult to identify the ideal therapy for a specific site or stage for this type of cancer. Primary treatment consists of surgery and radiation therapy however, in recent years, chemotherapy has been added for some select advanced hypopharyngeal cancers.

The overall poor prognosis of hypopharyngeal squamous cell carcinomas is due to a number of factors including presentation at a late stage, multisite involvement within the hypopharynx, unrestricted soft-tissue tumor growth, an extensive regional lymphatic network allowing metastases development, and restricted surgical options for complete resection. Local and regional recurrences are the most common causes of treatment failure. Following a definitive treatment, most failures occur within the first 2 years. In addition to the risk of delayed regional metastases, the risk of developing a second primary tumor in patients with tumors of the upper aerodigestive tract has been estimated to be 4 to 7% per year (American Cancer Society, 2014). Surveillance of patients with hypopharyngeal cancer should be lifelong because of these risks. Five year survival rates for hypopharyngeal cancer are summarized in Table 1
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<tr>
<td>I</td>
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<tr>
<td>II</td>
<td>39%</td>
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<td>III</td>
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*Table 1 Survival rates of hypopharynx cancer by stage (AJCC Cancer Staging Manual, Seventh Edition)*

1.2 Human Hypopharyngeal FaDu (HTB-43) Head and Neck Cell Monolayer

An epithelial cell line was established from a hypopharyngeal squamous cell carcinoma during the course of a study on the adenovirus-induced T-antigens in human oral and pharyngeal tumor tissues. This cell line was named FaDu, in honor of the patient from which the tissue was removed. This is a squamous cell carcinoma of the laryngopharynx (Grade II) which has been cultured since 1968. After the initial thirty serial subpassages, this cell line has retained the epithelial morphology and orderly growth as a monolayer sheet.

From the first tissue culture (Fig. 2), the polygonal-shaped and compactly packed epithelial cells migrated out of the explant three days after the beginning of the culture. Furthermore, many fibroblastic-type cells were on the outward growth from the explant, and many mitotic cells were observed in the peripheral regions of the epithelial outgrowth. The monolayer sheet of cells was obtained from the cellular outgrowths similar to the epithelial elements observed in the primary cultures (Fig. 3). Due to the early stage difficulties of cellular repopulating in the subpassage cultures, complete
isolation of individual cells from the cell sheet was not achieved until the thirtieth passage level.

Morphologically, the cells were and continue to be epithelial in nature. In phase contrast microscopy images, dense granular material in the cytoplasm and around the nucleus is seen in the cells at all passage levels (Rangan. 1972). In the monolayer, multilayered focal regions of the cells are frequently observed. The average population doubling time of the cell line at the 16th passage level was found to be approximately 50 hours (Fig. 3). With regards to the malignant properties of the cell line, the original study by Rangan (1972) showed two of the four ten-day-old chick-chorioallantoic membranes seeded with cell suspension developing single discrete nodular growth. From histological
studies, the nodules were found to contain identical cellular elements to those in the tissue culture.

![Graph of growth curve of cell line at the 16th passage level](image)

*Figure 3 Growth curve of the cell line at the 16th passage level (Rangan, 1972)*

In the preliminary virus infection studies, the cell line was found to be susceptible to some viruses containing either RNA or DNA. In particular, the parainfluenza viruses, types 2 and 3 were found to infect the cells. Additionally, cultures infected with adenoviruses, type 3 and 12, revealed typical cytopathic changes and numerous intranuclear aggregates of virus particles in thick sections of the cell (Rangan, 1972), however, the cells were not susceptible to SV40 virus.

1.3 P388D-1 Murine Lymphocytic Monocytes

Monocytes are precursor cells that differentiate into macrophages once they have migrated from the circulation into the tissue where they reside (Owen et al., 2004; Murdoch and Lewis, 2005). In general, macrophages are categorized as white blood cells
originating from myeloid progenitor cells of the bone marrow and they are the first to mediate host immune responses against foreign objects. In addition to their role in phagocytosis of invading pathogens, macrophages also release several growth factors such as cytokines and interleukins (e.g. transforming growth factor-β (TGF-β) and platelet-derived growth factor), and they are involved in a number of other inflammatory responses as well as in the repair of damaged tissues (Metz et al., 2004; Hsiao et al., 2008).

Macrophages play a central regulatory and effector role in the immune response of the body against neoplasia (Fidler et al., 1988). They are transformed into an activated state from the resting state in response to a variety of foreign stimuli like bacteria, viruses, and tumors. These activated macrophages release TNF, IL-1, nitric oxide, lysozymes, and other cytokines which can kill tumor cells and activate the host immune response (Adams, 1992).

It has been demonstrated that certain antineoplastic drugs confer a dual functionality to macrophages. i.e., these drugs act as both tumoricidal agents and immunopotentiators; a well-documented example of this phenomenon is cisplatin. It has been reported that murine macrophages treated with cisplatin showed enhanced capacity to lyse tumor cells in vitro and also resulted in increased production of IL-1, TNF, reactive oxygen intermediates, reactive nitrogen intermediates, lysozyme and arginase (Singh et al., 1991; Alaparthi et al., 1991). Furthermore, P388D-1 cells treated with different concentrations of cytosine arabinoside, vincristine sulphate, cyclophosphamide, mitomycin C, and hydroxyl urea showed increased IL-1 activity, and enhanced cytotoxicity (Pai et al., 1997).
In addition to neoplastic cells and blood vessels, tumors are also composed of leukocytes, specifically macrophages. These macrophages are known as tumor-associated macrophages (TAMs) which are monocytes that are recruited into the tumor and comprise up to 50% of the tumor’s cellular mass (Murdoch and Lewis, 2005; Knowles and Harris, 2007). In the case of squamous cell carcinomas, the tumor-infiltrating cells consist mainly of T lymphocytes, monocytes and macrophages. For a tumor to be able to utilize macrophages for their benefit, the macrophages must first be recruited, and to achieve this, tumors induce a chemo-attractive gradient by secreting a number of factors, the most important of which are chemokines (Choi et al., 2007).

Macrophages have become a very attractive carrier for the delivery of therapeutic agents due to their inherent ability to migrate and aggregate within and around the tumor, especially in hypoxic regions. A key benefit of using a cell-based delivery system is the increase in the nanoparticle’s systemic half-life because macrophages act as protectors against reticulo-endothelial elimination. From a clinical perspective, the basic procedure for such a strategy involves isolation of macrophages from a given patient followed by macrophage loading of the agent of interest (e.g. nanoparticles) which are then injected into the patient (Owen et al., 2004; Murdoch and Lewis, 2005; Knowles and Harris, 2007).

Cell-based vectorization is one method that can target and maintain an elevated concentration of nanoparticles at the tumor site and prevent their spread into normal tissue (Madsen et al., 2012a). Employing macrophages, in conjunction with nanoparticle delivery for plasmonic photothermal therapy (PPTT) has interesting potential for cancer treatment since they are attracted to hypoxic and necrotic regions within tumors.
Macrophages have been shown to internalize a sufficient number of gold-silica nanoshells to be useful for PPTT in \textit{in vitro} systems consisting of cell monolayers or 3-D multicellular spheroids (Baek et al., 2011; Trinidad et al., 2014).

1.4 Plasmonic Photothermal Therapy

The use of hyperthermia in the destruction of solid tumors has been under investigation for some time. The efficacy of thermal therapy through a variety of heat sources including laser light, focused ultrasound, and microwaves has been studied by numerous investigators. Compared to normal tissue, tumors have a poor blood supply and, therefore a reduced heat tolerance which makes them susceptible to hyperthermia treatments. In addition, hyperthermia causes irreversible cell damage by loosening the cell membrane and denaturing critical proteins. There are numerous benefits of thermal therapy over conventional resection: most approaches are minimally or non-invasive, relatively simple to perform, and have the potential of treating embedded tumors in vital regions where surgical resection is not feasible. Ultimately, the difficulty of this type of therapy is that the activating energy source must have significant penetration into tumor tissues while, at the same time, sparing normal tissues. Simple heating techniques have difficulties discriminating between tumors and surrounding healthy tissue, and often result in the heating of intervening tissue between the source and the target site. Several groups have investigated treatment of tumors through hyperthermia using deep penetrating near infrared (NIR) lasers with or without contrast enhancing agents, however, success with current systems has been modest (Chen et al., 1997; Prudhomme et al., 1996; Chen et al., 1996).
The emerging use of laser light to achieve controlled and confined thermal damage to the tumor tissue has revolutionized cancer therapy. Lasers have been used in medical applications since the 1960s, and the first reports of tumor eradication in 1965 sparked a wide interest in their use in oncologic applications. The laser light can either be transmitted from an optical fiber tip to exposed tumors or be delivered into a confined space via insertion of the fiber into the center of the target tumor, which is often called interstitial laser hyperthermia (Bown 1983; Steger et al., 1989; Masters and Bown 1990; Masters and Bown 1992a; Masters and Bown 1992b). Although reasonable tumor selectivity can be achieved with interstitial laser hyperthermia, this is an invasive technique. Light absorbing dyes have been used to increase tumor selectivity. Following light excitation of the dye molecule, electrons transition from the ground state to the excited state. Typically, the dye molecules relax via nonradiative decay channels resulting in heating of the local environment around the light absorbing agents. If the temperature increase is sufficient, the process leads to localized tissue destruction. The photoabsorbing agents can be naturally occurring chromophores in the tissue or externally added dye molecules such as indocyanine green, naphthalocyanines, and porphyrins coordinated with transition metals (Huang et al., 2008). Unfortunately, natural chromophores are characterized by very low absorption and therefore they are inefficient hyperthermia agents. A potential solution to this problem is the laser activation of NIR-absorbing metallic nanoparticles, termed plasmonic photothermal therapy (PPTT) (Anderson and Parrish 1983) (Fig 4).
Figure 4 NIR light ablated PC3 prostate tumor cells with nanoshell treatment. In vitro cellular ablation with bare gold/gold sulfide nanoparticles following incubation and laser application. Yellow circle indicates the laser spot; live/dead stain for viability shows dead cell as red while viable cells appear green. White bars = 100 microns. (Gobin et al., 2011).
In recent years, the rapid development of nanotechnology has provided a variety of nanostructures with unique optical properties that may prove useful in biology and biomedical applications (El-Sayed, 2001; Katz and Willner 2004). From the cancer therapy point of view, noble metal nanoparticles are very useful PTTT agents due to their enhanced absorption cross sections, which are four to five orders of magnitude larger than those offered by conventional photoabsorbing dyes which make them more effective photothermal coupling agents (Huang et al., 2008). Nanoshells are a new class of optically tunable nanoparticles composed of a dielectric core (silica) coated with an ultrathin metallic layer of gold (Oldenburg et al., 1999). Through adjusting the thickness of the core and shell, nanoshells can be manipulated to absorb or scatter light at a desired wavelength across visible and NIR wavelengths (Fig. 5). This optical tunability permits the fabrication of nanoshells with a peak optical absorption in the NIR, a region of light absorption.
where optical penetration through tissue is optimal (Weissleder, 2001). In general, the efficacy and stability of the metal shell on the nanoshell converts absorbed light to heat with an efficiency that far exceeds that of conventional dyes. Furthermore, the absorption properties of the nanoshells are dependent upon the material’s rigid metallic structure rather than the more labile molecular orbital electronic transition of conventional dyes, which in turn makes the nanoshells less susceptible to photobleaching. The efficacy of nanoshells as a NIR absorber has already been demonstrated in a series of in vivo magnetic resonance thermal imaging (MRTI) studies examining temperature profiles of nanoshell-loaded tumors irradiated with NIR light. These studies found that nanoshells absorbed NIR light and generated increased temperatures sufficient to produce irreversible photothermal damage to subcutaneous tumors (Hirsch et al., 2003a).

To improve the biocompatibility of the nanoshells, ‘stealthing’ polymers like poly(ethylene glycol) (PEG) can be crafted to nanoshell surfaces using simple molecular self-assembly techniques (Hirsch et al., 2003b). It has been demonstrated that stealthing liposomes as well as other biomolecules and materials with PEG suppresses immunogenic responses, improving blood circulation times and overall material/implant performance (Chen and Scott 2001; Harris et al., 2001).

In PPTT electromagnetic radiation (usually in the visible to near-infrared wavelengths) incident on gold-based nanoparticles is converted to heat and, as such, this technique can be used as a therapeutic approach to destroy cancer cells. The maximum absorption wavelength depends on a number of factors including nanoparticle composition, size and geometry. Ideally, nanoparticles should demonstrate significant absorption in the near-infrared since this wavelength region has high penetration in
biological tissues (Trinidad et al., 2014). This provides the rationale for using gold-silica nanoshells with silica core diameters of 120 nm and gold shells of 15 nm thickness as they have maximum light absorption around 800 nm. In addition to their favorable absorption properties, 150 nm gold-silica nanoshells are also ideally suited for passive delivery to tumors via the enhanced permeability and retention (EPR) effect (Puvanakrishnan, 2012). Preferential accumulation of 20-300 nm particles in tumors occurs due to the leaky tumor vasculature (enhanced permeability) and poor lymphatic drainage (enhanced retention).

The efficacy of chemotherapeutic agents is sensitively dependent on the ability to deliver these compounds in therapeutic concentrations to the tumor site. Most chemotherapeutic agents demonstrate a high efficacy with a variety of tumors. Nevertheless, nonspecific delivery leads to significant normal tissue toxicities and limits dosages to levels far below those required to destroy most malignancies. To overcome this problem, the combined treatment of chemotherapeutic agents with PPTT is proposed. Heat treatment, called hyperthermia, has been demonstrated to augment the cytotoxicity of some chemotherapeutic agents, resulting in similar cure rates and reduced morbidity with lower drug doses (Hahn et al., 1975; Hildebrandt et al., 2002; Jones et al., 2005; Tang and McGoron 2009).

1.5 Bleomycin

One of the anti-cancer agents under investigation for this research is a chemotherapeutic compound called bleomycin (BLM) (Fig 6). Bleomycin is a 1.5 kDa glycopeptide antibiotic produced from the bacterium Streptomyces verticillus. It was
discovered by Umezawa et al. in 1966 and was found to be cytotoxic to eukaryotic cells due to its ability to induce DNA breaks (Suzuki, 1970). Bleomycin is typically used for the treatment of squamous cell carcinomas of the head and neck, esophagus, bronchus, and skin, as well as testicular cancer and Hodgkin’s and non-Hodgkin's lymphoma (Mir et al., 1996; Berg et al., 2005). Side effects include pneumonitis (in 46% of patients) and lung fibrosis (in 3% of patients) which can occur after a cumulative dose of 300 mg m\(^{-2}\) (Mir et al., 1996). Patients are typically treated two to three times per week or given a continuous low dose for several days. The drug has a biological half-life in blood of 2-4 hours after intravenous injection, with 70% excreted after the first 24 hours (Mir et al., 1996).

![Figure 6 Structure of bleomycin. (Barber et al. 1981)](image)

**Structures of bleomycins**

- \( R = \text{OH} \) bleomycinic acid, \( R = \text{NH}_2 \) bleomycin B\(_1\),
- \( R = \text{NH}(\text{CH}_2)_3.\text{Me}_2 \) bleomycin A\(_2\), \( R = \text{NH}(\text{CH}_2)_4 \text{NH}.\text{C-NH}_2 \) bleomycin B\(_2\)

Bleomycin has a large hydrophilic structure comprised of four major parts: terminal amine, bithiazole, pseudopeptidic, and glycannic (Mir et al., 1996). The
terminal amine group interacts with nucleic acids, anchoring the bleomycin to the double helix strand. The bithiazole domain serves to bind with DNA and interacts with the DNA minor groove. The pseudopeptidic group is responsible for binding to transition metals and functions as DNA sequence selectivity and strand cleavage. Last, the function of the glycannic domain is still under investigation. Bleomycin forms metal oxide complexes by both binding to oxygen as well as redox-active transition metal ions Fe$^{2+}$, Co$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, and Cu$^{2+}$ (Suzuki, 1970). Bleomycin has been shown to amass in endocytic vesicles and has limited ability to penetrate the cell membrane. Hence, bleomycin typically enters the cell through receptor-mediated endocytosis by binding to a 250 kDa membrane receptor (Berg et al., 2005). Though bleomycin generally demonstrates some potency once inside the cell, it still needs to diffuse through the nuclear membrane in order to interact with the DNA.

The primary biochemical action of bleomycin is through induction of DNA strand breaks, and some suggest bleomycin inhibits incorporation of thymidine into the DNA strand (Van Meir et al., 2010). In vitro, bleomycin DNA cleavage depends on oxygen and metal ions, but the exact mechanism of DNA strand scission is still unresolved. It has been suggested that bleomycin chelates metal ions, primarily iron, producing a pseudoenzyme that reacts with oxygen to produce superoxide and hydroxide free radicals which can cleave the DNA. An alternative hypothesis proposes that bleomycin may bind at specific sites in the DNA strand and induce scission through abstracting the hydrogen atom from the base, which would result in strand cleavage as the base undergoes a Criegee-type rearrangement or forms an alkali-labile lesion (Quada et al., 1998). The intracellular response to bleomycin damage is illustrated in Figure 7.
1.6 Doxorubicin

Doxorubicin is the trade name of the liposomal formulation Doxil, also known as hydroxydaunorubicin (Fig. 8). It is an anthracycline antibiotic, closely related to the natural product daunomycin. Like all anthracyclines, it works by intercalating DNA, with the most serious adverse effect being life-threatening heart damage (Tacar et al., 2013). It is commonly used in the treatment of a wide range of cancers, including hematological
malignancies, soft tissue sarcomas and many types of carcinomas, including those of the breast (Tacar et al., 2013).

Figure 8 Structure of doxorubicin

Doxorubicin interacts with DNA by intercalation and inhibition of macromolecular biosynthesis (Hahn et al., 1975). This inhibits the progression of the enzyme topoisomerase II, which relaxes supercoils in DNA for transcription (Pigram et al., 1972). Doxorubicin stabilizes the topoisomerase II after it has broken the DNA chain for replication which prevents the DNA double helix from being resealed and therefore stops the process of replication. The planar aromatic chromophore portion of the molecule intercalates between two base pairs of the DNA, while the six-membered daunosamine sugar sits in the minor groove and interacts with flanking base pairs immediately adjacent to the intercalation site, as evidenced by several crystal structures (Pang et al., 2013). By intercalation, doxorubicin can also induce histone eviction from
chromatin. As a result, DNA damage response, epigenome and transcriptome are deregulated in doxorubicin-exposed cells (Tang and McGoron, 2009). A summary of the action of doxorubicin is illustrated in Figure 9.

Figure 9 Action of doxorubicin in a stylized cancer cell. (Thorn et al. 2010)
1.7 Cisplatin

Cisplatin is one of the most potent antitumor agents known, displaying clinical activity against a wide variety of solid tumors. Cisplatin, cisplatinum, or cis-diamminedichloridoplatinum(II) (CDDP) is a member of a class of platinum-containing anti-cancer drugs which now also includes carboplatin and oxaliplatin (Fig. 10). These platinum complexes react in vivo, binding to and causing crosslinking of DNA, which ultimately triggers apoptosis (programmed cell death) (Pruefer et al., 2008)

![Cisplatin Structure](image)

**Figure 10 Structure of cisplatin**

Cisplatin’s cytotoxic mode of action is mediated by its interaction with DNA to form DNA adducts, primarily intrastrand crosslink adducts, which activate several signal transduction pathways, including those involving ATR, p53, p73, and MAPK, and culminate in the activation of apoptosis (Fig. 11). DNA damage-mediated apoptotic
signals, however, can be attenuated, and the resistance that ensues is a major limitation of cisplatin-based chemotherapy (Siddik. 2003). Cisplatin induces DNA crosslinks in several different ways thereby interfering with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible (Liu et al., 2008). Most notable among the changes in DNA are the 1,2-intrastrand cross-links with purine bases. These include 1,2-intrastrand d(GpG) adducts which form nearly 90% of the adducts and the less common 1,2-intrastrand d(ApG) adducts (Matysiak et al., 2009). 1,3-intrastrand d(GpXpG) adducts occur but are readily excised by nucleotide excision repair (NER). Other adducts include inter-strand crosslinks and nonfunctional adducts that have been postulated to contribute to cisplatin's activity (Matysiak et al., 2009). Interaction with cellular proteins, particularly HMG domain proteins, has also been advanced as a mechanism of interfering with mitosis, although this is probably not its primary method of action (Matysiak et al., 2009).

Cisplatin also activates human natural killer cells and monocytes and upregulates interleukin-2-induced lymphokine activated killer cells (Sodhi et al., 1992). It is thought that cisplatin-mediated cancer remission is a combined outcome of its immunostimulating and cytotoxic effects. (Pai et al., 1997).
1.8 Scope of Work

The purpose of this research is to investigate the combined effects of a number of commonly used chemotherapeutic agents (bleomycin, doxorubicin, cisplatin) with gold-silica nanoshell-induced hyperthermia on a human head and neck cancer cell line. This will be accomplished by comparing the toxicity of each treatment (chemotherapy or
photothermal therapy) with the combined treatment. In the proposed *in vitro* studies, macrophages will be used as nanoshell delivery vehicles.

For comparative purposes, a separate set of experiments will be performed employing a conventional hyperthermia technique in which the monolayers will be exposed to elevated temperatures in an incubator. In all cases, a cell proliferation colorimetric assay will be used to determine therapeutic efficacy.

![Figure 12 Model of macrophage endocytosis of nanoparticles and incorporation into tumor spheroids followed by laser-mediated thermal ablation (Makkouk and Madsen 2013)](image)

Hypothesis: The combined macrophage-mediated chemo-thermal treatment will yield greater cell toxicity than individual treatments consisting of either chemotherapy or photothermal therapy. The increased cell toxicity will be similar to that observed in monolayers subjected to combined chemotherapy and conventional hyperthermia. There are two particularly novel aspects associated with the proposed work: (1) the use of nanoshell PPTT-induced hyperthermia combined with chemotherapeutic agents, and (2) macrophage-mediated delivery of nanoshells.
CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines

All experiments were performed in vitro utilizing a human hypopharyngeal squamous cell carcinoma line (FaDu) and murine lymphocytic monocytes (P388D-1). All work was done inside a Labconco Class II Biohazard Cabinet (Labconco Corp., Kansas City, MO) at the University of Nevada, Las Vegas (UNLV) unless otherwise specified. Cells were incubated at 37°C, 5.0% CO₂, and 95% humidity inside a VWR incubator (VWR International, LLC. West Chester, PA). The cell line was kept in Gibco Dulbecco’s Modified Eagle medium (DMEM) (Invitrogen Corp., Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES buffer (pH 7.4), and penicillin (100 U/ml). All monolayer cells were sub-cultured using the same technique.

After checking the confluence, the T-75 flask was emptied of all media and the cells washed with 5 ml of Gibco phosphate buffered saline (PBS) with pH of 7.2 (Invitrogen Corp., Carlsbad, CA). Next, 5 mL of Gibco 0.25% Trypsin-EDTA (Invitrogen Corp., Carlsbad, CA) was added and the flask slowly rocked approximately twenty times. The trypsin was removed after 5 min. Trypsin is a proteolytic enzyme, which functions to detach adherent cells from the flask surface. Following the 5 min. trypsin incubation, 5 mL of DMEM was added to the flask and the suspended cell media was pipetted up and down several times to ensure the separation of cells. Thereafter, 2 mL of the media was added to 13 mL of fresh DMEM inside a new T-75 flask. Two
flasks were seeded in this fashion to ensure a consistent supply of cells for the experiments.

2.2 Monolayer Plating

The monolayer plating consisted of a mixture of FaDu cells and P388D1 murine monocytes loaded with gold nano-shells (AuroShell™), purchased from Nanospectra Biosciences Inc. (Houston, TX). Gold nanoshells (4.3 x 10⁹) were incubated with monocytes (2.0 x 10⁶ cells ml⁻¹) under standard conditions for 24 h prior to the cell plating. After 24 h of incubation, monocytes were treated with mitomycin C for one hour to prevent cell division. Monocytes were washed twice with PBS in order to remove non-ingested nanoparticles and mitomycin C residues.

On the day of plating, FaDu cells (70% confluence) were harvested from the growth flask and transferred to a 15 ml centrifuge tube with 11 ml of DMEM with a cell concentration of 20,000 FaDu cells ml⁻¹ (Total of 2.2 x 10⁶ cells). 1.1 x10⁵ loaded monocytes were added to the 15 ml centrifuge tube making the ratio of FaDu-to-murine monocytes 2:1. The 15 ml centrifuge tube was slightly vortexed for 10 seconds to achieve better mixing of the cells. The cell solution was then transferred to a 50 ml reservoir and 100 µl of the cell solution was placed in each well of a 96-well plate resulting 2,000 FaDu cells and 1,000 monocytes per well.

For the chemo agent toxicity plates and heat simulation experiment, each well in the 96-well plate was filled with the cell solution except the 12th column which was left empty.
2.3 Cell Proliferation Assay (MTS)

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTiter 96® AQueous One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], inner salt, MTS, and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium (Figure 13). This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The quantity of formazan product, as measured by absorbance at 490 nm, is directly proportional to the number of living cells in culture. The absorbance at 490 nm in each well is measured using a microplate reader.

Figure 13 Chemical Structure of MTS to Formazan
For every measured 96 well plate, 30µl of MTS reagent was added into each well containing cells. 150 µl DMEM and 30µl of MTS reagent were added into each well of the 12th column of each plate. This last column contained no cells and thus provided a measure of the background noise of the MTS assay. The plates were incubated in the VWR incubator for one hour. After incubation, the plates were transferred to an Infinite® M1000 PRO microplate reader (TECAN Group Ltd., Männedorf, Germany) for absorbance reading at 490 nm. The results were recorded on Microsoft Excel worksheets.

2.4 Drug Toxicity

The FaDu cell/nanoshell-loaded P388D1 murine monocyte suspension was injected into each well of a 96-well plate (except the 12th column). A total of three well plates were prepared (one for each chemotherapeutic drug). Drugs were added to DMEM medium to obtain concentrations of: 0.1-10 µg ml⁻¹ (bleomycin), 0.001-0.05 µg ml⁻¹ (doxorubicin) and 1-75 µg ml⁻¹ (cisplatin). The drugs were added to each well in 7 of the 11 columns containing the cell/loaded monocyte suspension 24 h after the initial cell seeding. Two non-drug columns served as positive and negative controls, respectively while two columns containing cell mixtures were unused. Typically, each column (eight wells) on a plate contained one drug concentration. In all cases, suspensions and chemotherapeutic agents were incubated for five days in an incubator (VWR International, LLC. West Chester, PA). Following incubation, 0.1 ml of 90% ethanol was added to each well of one of the non-drug columns (eight wells total) of each of the three plates. These columns served as positive controls since ethanol is toxic to cell suspensions. Any signal emanating from these wells was considered background noise,
i.e., a reference point for total cell death. Following the 15 min. ethanol incubation, MTS assays were performed to determine viability. The MTS reagent (30 µl) was added to each well and after 60 min incubation, the plate was placed in a microplate reader (Infinite® M1000 PRO) and the fluorescence emission from each well was recorded. The background signal (MTS column #12) and the ethanol treated column was subtracted from each well and the resultant signal was normalized to the signal from the negative control group, i.e., the column containing no drugs (cell suspensions only). The pharmacological potency resulting in approximately 60-90% survival was used in subsequent combined therapy studies.

2.5 Photothermal Toxicity

A fiber-coupled diode laser (Intense, North Brunswick, NJ) was used to irradiate cells at a wavelength of 805 nm. Each well was irradiated separately using an irradiance of 7 W cm\(^{-2}\). Three irradiation times were investigated: 5, 7.5 and 10 min corresponding to radiant exposures of 2.10, 3.15 and 4.20 kJ cm\(^{-2}\). The radiant exposure resulting in approximately 90% survival was used in subsequent combined therapy studies. All irradiations were performed in a specially designed incubator in order to maintain physiological conditions, in which the incubator was kept at a constant temperature of 37°C and acetic acid was added to sodium bicarbonate for CO\(_2\) production. The 600 µm dia. optical fiber (Model FD1; Medlight, Ecublens, Switzerland) connected to the diode laser was inserted through an opening at the top of the incubator and cells in individual wells of the 96-well plate were irradiated by placing the fiber in contact with the bottom of the plate (irradiation beam diameter = 8 mm). Only every third column and every third row
of the well plate contained cells resulting in a total of 12 wells per plate and a total of two plates for this experiment. The empty wells provided a barrier preventing scattered light from irradiating cells in adjacent wells. As previously described, the 12th column contained MTS reagent and no cells and one column with cells served as positive control. After the irradiation, the plates were incubated in the VWR incubator for 5 d. Following incubation, MTS assays were performed as previously described. The background signal from the MTS-only column and the ethanol treated column was subtracted from the treatment columns and results are normalize to the no PPTT treatment control group.

2.6 Combined Chemo- and Photothermal Treatment

Based on the toxicity results for each of the three drugs, concentrations resulting in 70-90% survival were chosen, i.e., 5 µg ml⁻¹ bleomycin, 10 µg ml⁻¹ cisplatin, and 0.005 µg ml⁻¹ doxorubicin were added to the combined FaDu cells and P388D1 murine monocytes monolayer in the 96 well plates prior to the irradiation; Only every third column of the well plate contained cells, resulting in a total of 6 wells per treatment group and 12 wells per plate. The empty wells provided a barrier preventing scattered light from irradiating cells in adjacent wells. The control groups were established with the same concentrations of chemo agents as in the corresponding combined PPTT and chemo treatment groups in one 96 well plate (eight well total for each group) and were maintained in the VWR incubator for the duration of the trial. Each photothermal treated well was irradiated separately using an irradiance of 7 W cm⁻² (8 mm beam diameter) for 7.5 minutes resulting in a radiant exposure of 3.15 kJ cm⁻². Otherwise, the irradiation protocol using the 805 nm fiber-coupled diode laser was identical to that previously
described in section 2.5. Following treatment, the plates were incubated for 5 d and MTS assays were performed at the end. The background signal from the MTS-only column and the ethanol treated column was subtracted from the treatment columns, and results were normalized to the no treatment control group.

2.7 Conventional Hyperthermia

Bleomycin (5 µg ml⁻¹), cisplatin (10 µg ml⁻¹), and doxorubicin (0.005 µg ml⁻¹) were added to the combined FaDu cells and P388D1 murine monocytes monolayer in three 96 well plates (total of eight wells per drug in each plate) prior to the hyperthermia treatments. As previously described, the interval between cell seeding and addition of drugs was 24 h. Immediately thereafter, each 96-well plate was placed in the specially designed irradiation incubator for 45 min. at one of three temperatures: 37, 41 or 44 °C. During the 45 min. treatment, aceticylic acid was added to sodium bicarbonate for CO₂ production. Following treatment, each plate was transferred to the main incubator. After 5 d of incubation, the MTS assay was performed. The background signal from the MTS-only column and the ethanol treated column was subtracted from the treatment columns and results were normalized to the 37 °C no-drug control group.

2.10 Statistical Analysis

Data analysis was performed in Microsoft Excel. All data were normalized to the control group of each individual experiment and all error bars represent standard deviations. In order to determine the degree of interaction between PPTT and the chemo-
agents the following equation was used (Drewinko et al. 1976 – not found in reference section – please add):

\[ \alpha = \frac{SF^{PPTT} \times SF^{Chemo}}{SF^{PPTT+Chemo}} \] (Eq. 1)

The numerator includes the product of the surviving fraction (SF) of the individual treatments separately and the denominator includes the surviving fraction of the combined treatments. A value of \( \alpha = 1 \) indicates an additive effect. A value of \( \alpha < 1 \) or \( \alpha > 1 \) indicates an antagonistic or synergistic effect, respectively.
CHAPTER 3

RESULTS

3.1 Drug Toxicity

In order to determine the optimal drug levels for evaluating the effects of combined chemotherapy and PPTT, titrations were performed for each drug. The results are shown in Figures 14 – 16 for each respective chemotherapeutic agent. In all cases, the surviving fraction was normalized to the negative (no drug) controls. As expected, the results show a decrease in survival with increasing drug concentrations. As illustrated in Figure 14, the 50% survival dose (LD$_{50}$) for doxorubicin in this cell mixture is approximately 0.0075 µg ml$^{-1}$. All cells were killed at a concentration of 0.05 µg ml$^{-1}$.

The bleomycin titration results (Figure 15) show that the LD$_{50}$ is approximately 5 µg ml$^{-1}$ which is lower than the corresponding value for cisplatin (Figure 16). Collectively, the data show that, for this particular cell line, doxorubicin has the highest toxicity while cisplatin is the least toxic. Based on the previously established criterion of 60-90% survival, the results in Figures 14-16 suggest that doxorubicin, bleomycin and cisplatin, concentrations of 0.005, 5, and 10 µg ml$^{-1}$, respectively should be used in the combined treatments.
Figure 14 Doxorubicin toxicity titration from 0.001 to 0.05 ug ml⁻¹. Each data point represents mean ± standard deviation.

Figure 15 Bleomycin toxicity from 0.1 to 10 ug ml⁻¹. Each data point represents mean ± standard deviation.
3.2 Photothermal Toxicity

As illustrated in Figure 17, a 7 W cm\(^{-2}\) irradiance had little effect on overall survival for the treatment times investigated: irradiation times of 7.5 and 10 min. resulted in only 15% cell kill. Based on these results, an irradiation time of 7.5 min. was chosen for the combined PPTT and chemotherapy studies.

*Figure 16 Cisplatin Toxicity titration from 1 to 75 μg ml\(^{-1}\). Each data point represents mean ± standard deviation.*
Figure 17 Photothermal therapy potency using fiber-coupled diode laser at 810 nm. Each data point represents mean ± standard deviation.

3.3 Combined Chemo- and Photothermal Treatment

As illustrated in Figure 18, surviving fractions for bleomycin, cisplatin, and doxorubicin were 68.2 ± 5.7 %, 68.1 ± 4.43% and 81.8 ± 3.9 % respectively. The toxicity of photothermal therapy was 79.4 ± 11.2 %. Surviving fractions for combined photothermal and chemo treatments were 65.08 ± 10.2 %, 49.2 ± 5.1 %, and 30.9 ± 5.8 % for doxorubicin, bleomycin and cisplatin, respectively. The corresponding alpha values, calculated from equation 1 are shown in Table 2. The alpha values suggest slight additive effects for the PTT + doxorubicin and PTT + bleomycin combinations, while a synergistic effect was noted for the PTT + cisplatin treatment.
Figure 18 Combined chemo and photothermal treatment. Surviving fraction was normalized to the positive (no treatment) controls. Each data point corresponds to the mean and standard error of three trials.

<table>
<thead>
<tr>
<th>Trial Group</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPTT + doxorubicin 0.005 µg ml⁻¹</td>
<td>0.83±0.19</td>
</tr>
<tr>
<td>PPTT + bleomycin 5 µg ml⁻¹</td>
<td>1.09±0.20</td>
</tr>
<tr>
<td>PPTT + cisplatin 10 µg mL⁻¹</td>
<td>2.10±0.50</td>
</tr>
</tbody>
</table>

Table 2 Alpha values of combined chemo and photothermal treatments

3.4 Conventional Hyperthermia

As shown in Figure 19, surviving fractions for doxorubicin, bleomycin, and cisplatin at 37 °C were 87.7 ± 4.1 %, 78.2 ± 3.5 %, and 66.9 ± 5.4 % respectively. Surviving fractions for cells exposed to 41 and 44 °C were 88.0 ± 5.2 % and 95.4 ± 2.4 %, respectively. Surviving fractions for combined 41 °C hyperthermia and chemo treatments were 86.2 ± 2.6 %, 81.4 ± 3.4 %, and 67.6 ± 7.6 % for doxorubicin, bleomycin and cisplatin, respectively. The corresponding values for combined treatments at the higher
temperature (44 °C) were 65.4 ± 3.0 %, 51.0 ± 4.2 %, and 37.3 ± 8.8 %. The corresponding alpha values calculated from equation 1 are shown in Table 3. Alpha values for the combined treatments at the lower temperature (41 °C) suggest a slight additive effect while, at the higher temperature, a small degree of synergism is observed for each drug.

![Figure 19 Combined hyperthermia and chemotherapy treatments for three different drugs. Survival fraction was normalized to the positive (no treatment) controls. Each data point represents mean ± standard deviation.](image_url)

<table>
<thead>
<tr>
<th>Trial Group (41 °C)</th>
<th>Trial Group (44 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat + doxorubicin 0.005 µg ml⁻¹</td>
<td>Heat + doxorubicin 0.005 µg ml⁻¹</td>
</tr>
<tr>
<td>0.89±0.07</td>
<td>1.28±0.11</td>
</tr>
<tr>
<td>Heat + bleomycin 5 µg ml⁻¹</td>
<td>Heat + bleomycin 5 µg ml⁻¹</td>
</tr>
<tr>
<td>0.85±0.07</td>
<td>1.46±0.16</td>
</tr>
<tr>
<td>Heat + cisplatin 10 µg mL⁻¹</td>
<td>Heat + Cisplatin 10 µg mL⁻¹</td>
</tr>
<tr>
<td>0.87±0.13</td>
<td>1.71±0.43</td>
</tr>
</tbody>
</table>

*Table 3 Alpha values of combined hyperthermia and chemo treatments*
CHAPTER 4

DISCUSSION

Current treatment for many cancers such as brain, hypopharyngeal squamous cell carcinomas and breast cancers include surgical resection of the tumor followed by various concurrent or subsequent treatments such as chemo and/or radiotherapy. The goal of subsequent treatments is to eliminate the malignant cells residing in the margin surrounding the resection cavity thereby reducing or eliminating recurrence. Unfortunately, in many cases, none of the standard treatment regimens have proven to be successful suggesting that alternative therapeutic approaches are required. This research investigated the utility of combining traditional chemotherapy agents with nanoshell mediated laser-induced hyperthermia in an in vitro system consisting of murine monocytes and human hypopharyngeal cancer cells.

Chemotherapy is one of the standard treatment options for cancer patients. The efficacy of chemotherapeutic agents is sensitively dependent on the ability to deliver these compounds in therapeutic concentrations to the tumor site. Most chemotherapeutic agents demonstrate a high efficacy with a variety of tumors. Nevertheless, nonspecific delivery leads to significant normal tissue toxicities and limits dosages to levels far below those required to destroy most malignancies. Heat treatment, called hyperthermia, has been demonstrated to augment the cytotoxicity of some chemotherapeutic agents, resulting in similar cure rates and reduced morbidity with lower drug doses (Hahn et al., 1975; Hildebrandt et al., 2002; Jones et al., 2005; Tang and McGoron, 2009).

Hyperthermia treatments have been under clinical investigation for some time. Compared to normal tissues, tumors are more susceptible to hyperthermia because of
their poor blood supply and subsequent reduced heat tolerance. Furthermore, heat modifies the cytotoxicity of many chemotherapeutic agents. The extent of ‘thermal chemosensitisation’ both in vitro and in vivo can be quantified by comparing tumors treated with drug alone and those treated with a combination of drug and hyperthermia. Several studies have shown S-phase specific cell lethality of cells exposed to a variety of chemotherapeutic agents (Grisham et al., 1980). Observations suggest that moderate heat treatment itself does not induce chromosomal DNA strand breaks directly, but can alter chromatin structure influencing DNA repair (Kampinga and Dikomey, 2001). In addition, heat treatment induces both apoptosis and necrosis, and the form of death changes from apoptosis to necrosis above a certain threshold temperature (Harmon et al., 1990).

To overcome the non-specific targeting of chemotherapy, the combined treatment of chemotherapeutic agents with PPTT is proposed. This is the first study to investigate the efficacy of combined chemotherapy and PPTT using monocytes as delivery vehicles for gold nanoshells.

The effects of bleomycin, cisplatin and doxorubicin have been investigated in a wide variety of cancer cell lines. For example the LD\(_{50}\) for bleomycin in a F98 rat glioma cell line ranged from 0.5 to 0.75 µg ml\(^{-1}\) (Mathews et al., 2012; Madsen et al., 2009) while the corresponding values in human breast carcinomas was between 0.75 and 1.75 µg ml\(^{-1}\) (Mathews et al., 2012). These values are significantly lower than the LD\(_{50}\) observed for bleomycin in the present study employing the FaDu cell line (5 µg ml\(^{-1}\)). The LD\(_{50}\) for cisplatin in three human breast cancer cell lines (MCF-7, MDA-MB-435 and MDA-MB-231) was approximately 93 µg ml\(^{-1}\) while for doxorubicin it was 10 – 20 µg ml\(^{-1}\) in the MCF-7 cell line and 0.5 – 0.75 µg ml\(^{-1}\) in the MDA-MB-435 and MDA-
MB-231 breast cancer lines (Mathews et al., 2012). In comparison, the LD$_{50}$ in FaDu cells was approximately 15 and 0.0075 µg ml$^{-1}$ for cisplatin and doxorubicin, respectively. Differences in LD$_{50}$ values between the present work and the results of Mathews et al. (2012) are likely due to the different cell lines investigated. The observation that different cell lines have wide varying sensitivities to chemotherapeutic agents is illustrated in the doxorubicin data of Mathews et al. (2012) which show that, even for the same type of cancer (breast), there can be a significant variation in sensitivities to chemotherapeutic drugs. Other reasons for the observed differences between the present study and those of others include differences in: (1) survival assays (MTS vs. clonogenic survival) and (2) growth conditions.

In all cases, low drug concentrations resulted in survival fractions exceeding 100%. This was likely due to a phenomenon known as hypergenesis in which cancer cells are stimulated to divide in response to external stimuli.

PPTT has been shown to be very effective for inducing rapid temperature elevations in tumors containing NIR absorbing gold nanoshells while minimizing damage to surrounding normal tissues. Since squamous cell carcinomas of the head and neck contain significant concentrations of macrophages, these tumors are ideally suited for macrophage-mediated therapies (Trinidad et al., 2014). Both stromal and tumor cells produce a wide variety of chemokines and growth factors which have the ability to recruit circulating monocytes and differentiate them into macrophages (Lewis and Pollard, 2006; Galdiero et al., 2013). The tendency of macrophages to migrate and accumulate in tumors, especially hypoxic regions, makes them attractive delivery vectors for both therapeutic and diagnostic agents including nanoparticles.
PPTT-induced cytotoxic effects depend on the total light energy (radiant exposure) delivered. Therefore, higher incident laser powers (irradiances) allow for shorter treatment times. For example, doubling the irradiance reduces the treatment time by one-half. Effective PPTT requires temperatures in the range of 46 to 60 °C, however, higher temperatures may be required in hypoxic, low pH environments found in many tumors (Huang et al., 2008). PPTT efficacy is dependent on a number of factors, including light distributions in tissues. Due to the limited penetration depth of 810 nm light in brain tissues (0.5 – 1.0 cm; Madsen and Wilson 2012), PPTT may require direct light delivery via optical fibers. In addition to the light distribution, the concentration of nanoparticles in the tumor tissue is also important and should be optimized to produce sufficient temperature elevations for efficient PPTT (Trinidad et al., 2012). Since PPTT is oxygen-independent, it’s ideally suited for the treatment of tumors with high fractions of hypoxic cells.

A number of studies have demonstrated the efficacy of PPTT using gold nanoshell loaded macrophages or monocytes in in vitro systems consisting of human glioma spheroids (Baek et al., 2011; Chhetri et al. 2014.) and human head and neck squamous carcinoma cell lines (Trinidad et al. 2014). The results of Trinidad et al. (2014) are particularly relevant to the present work since the same cell line (FaDu) was used. The irradiation parameters (7 W cm\(^{-2}\); 5 – 10 min. exposures) and FaDu to monocyte ratio (2:1) used in the present study were chosen based on the results of Trinidad et al (2014) who showed that an irradiance of 7 W cm\(^{-2}\) for 5 min. (radiant exposure of 2.1 kJ cm\(^{-2}\)) resulted in approximately 95% survival in a 2:1 mixture of FaDu cells and nanoshell-loaded macrophages. This is in excellent agreement with the results presented
in Figure 17. Trinidad et al. (2014) found that significant cytotoxicity (20% survival) required irradiances of 28 W cm\(^{-2}\) delivered over 5 min. (radiant exposure of 8.4 kJ cm\(^{-2}\)).

These investigators also observed no cytotoxicity in empty macrophages exposed to 8.4 kJ cm\(^{-2}\). A similar finding was noted by Chhetri et al. (2014) who found no growth inhibition in human glioma spheroids subjected to identical radiant exposures. Collectively, these results suggest that a radiant exposure of 8.4 kJ cm\(^{-2}\) is insufficient to produce hyperthermic effects in cells devoid of nanoshells. Based on these results, a laser-irradiated only control group of FaDu cells was deemed unnecessary.

The results in Figure 17 show that PPTT is a very inefficient treatment modality. For example, a radiant exposure of 4.2 kJ cm\(^{-2}\) produced only 15% cell kill. In comparison, a radiant exposure of 0.75 J cm\(^{-2}\) used in photodynamic therapy, resulted in 50% survival of FaDu cells (Trinidad et al., 2014). As previously discussed, the advantage of PPTT is that, unlike photodynamic therapy, it is an oxygen-independent treatment modality and, as such, it is useful for the treatment of hypoxic cells.

In order to investigate the degree of interaction between the chemotherapeutic agents and PPTT, suboptimal levels of both modalities were determined. The results (Figure 18 and Table 2) show an additive effect (\(\alpha \approx 1\)) with concurrent treatments of both PPTT and bleomycin and PPTT and doxorubicin. A synergistic effect (\(\alpha > 1\)) was noted for concurrent PPTT and cisplatin. The mechanism of synergism is not known, but it likely has several components. Most importantly, all three drugs are incorporated into DNA causing damage which often results in cell death unless the damage is repaired. Since hyperthermia inhibits DNA repair, it’s postulated that the addition of heat enhances the cytotoxicity of the chemotherapeutic agents via inhibition of DNA repair.
PPTT-induced hyperthermia was compared with conventional incubator-induced hyperthermia. The conventional hyperthermia data (Figure 19, Table 3) show that the degree of interaction between the two modalities was sensitively dependent on temperature: at 41 °C, all interactions were additive (or mildly antagonistic), while at 44 °C all interactions were weakly synergistic. These results are in qualitative agreement with studies showing additive effects for doxorubicin and synergistic effects for bleomycin and cisplatin at 43.5 °C (Issels, 2008). The reason for the differences in alpha values between the PPTT and conventional hyperthermia data is not known, but it’s likely due to a temperature threshold effect. As illustrated in Table 3, synergistic effects are more likely at higher temperatures. Since temperatures were not recorded during the PPTT treatments, it’s possible that the laser irradiation parameters chosen were insufficient to induce the threshold temperatures required for synergism with bleomycin and doxorubicin.
CHAPTER 5

CONCLUSIONS

The overall objective of the work was to investigate the effects of combined PPTT and chemo agents on monolayers of FaDu hypopharyngeal squamous cell carcinoma. Suboptimal levels of both drugs and PPTT were determined. The results showed that PPTT combined with cisplatin resulted in some degree of synergism while additive effects were observed for concurrent treatments of PPTT and doxorubicin and PPTT and bleomycin. Concurrent treatments of conventional hyperthermia and chemotherapeutic drugs demonstrated that the degree of interaction between the two modalities was sensitively dependent on temperature: only additive (or mildly antagonistic) interactions were observed at 41 °C while weakly synergistic interactions were observed in all three cases at the higher temperature (44 °C). Compared to the standard hyperthermia data, the lack of synergism between PPTT and bleomycin and PPTT and doxorubicin might be due to an inability to attain the required temperature threshold with the chosen laser irradiation parameters. In future experiments, this discrepancy might be resolved by careful monitoring of temperatures during PPTT.

Although mechanistic studies were not performed, the likely explanation for the observed synergism is the ability of hyperthermia to inactivate DNA repair mechanisms thus resulting in increased cell death. This could be investigated in future studies using chemotherapeutic agents that do not interact directly with DNA. Performing these experiments in other cell lines may also shed some light on the mechanisms of action. Finally, in vivo studies should be performed to evaluate the effectiveness of the combined
treatments. This is important since vascular effects will most certainly play a major role in the tumor’s response to these treatments.
Reference


Curriculum Vitae

Education

Master of Science-Health Physics Emphasis Medical Physics

Bachelor of Science-Biological Science

Clinical Skills

Shadowed Dr. Ali Meigooni at Comprehensive Cancer Centers of Nevada Apr. 2014-Present

Performed with proper records and correspondence pertaining to quality control and quality assurance, calibration, radiation safety and compliance in accordance to TG-40, TG-51, TG-142 reports
Calibrated radiation sources and treatment machines under the supervision of the Chief Physicist
Performed quality assurance of patient charts weekly for adequate and accurate documentation and dosage
Assisted with maintenance schedules, component replacement and necessary modifications and calibration of continued safe operation with service engineers
Observed HDR treatments
Performed VMAT and IMRT Quality Assurance
Performed and assisted with MLC motor change
Observed the physicists troubleshoot clinical problems
Performed and assisted with Annual QA
Performed and assisted with HDR source change
Observed and practiced the treatment planning with externally delivered radiation
Assisted with DailyQA machine calibration and computer upgrade
Observed Varian Engineer perform on the LINAC

Therapeutic Radiological Physics Clinical Rotation and Lab Jan. 2014-May 2014

Monthly QA of linear accelerators, including TG-51, TG-40 and TG-142.
Familiar with Acceptance tests and Annual QA of the Linear accelerators.
Performed IMRT/VMAT quality assurance with MapCHECK
Dosimetry of high-energy photon and electron beam
2nd Physics Chart Check with External Beam Treatment Planning with Pinnacle

Assisted with HDR Source Change
Performed Daily Quality Assurance with Daily QA 3
Weekly Chart Check with MOSAIQ
Observed HDR Brachytherapy with Nucletron
CT Simulation

Varian Course EC102 Inverse Planning Operations and RapidArc Operations
Varian Course EC101 Eclipse Operation
Varian Course EC201 Eclipse Commission
Varian Course CL 101 On Board Imaging for Therapist
Varian Course Optimizing Work Flow

**Employment**

**University of Nevada, Las Vegas**

**Graduate Assistant - Biological Lab Manager**

Las Vegas, NV


- Developed programs to improve the effectiveness of the research and overall operation of the laboratory
- Ensured that all laboratory staffs followed health and safety standards
- Conducted orientations and training to new staffs and ensured capability to perform assigned analytical methods
- Evaluated effectiveness of research programs and recommended improvements
- Supervised laboratory staffs to finish testing within deadlines
- As needed, developed and modified analytical methods
- Efficiently interact with professor and students in achieving research goals
- Supervises purchase of supplies and maintains equipment inventories

**Graduate Assistant - Instructor of Freshmen Seminar**


- Instructed and communicated with culturally, racially and linguistically diverse students
- Established rapport with students by creating positive and respectful environment and encouraging dialogue through regular communication
- Kept accurate student-progress records
- Provided presentations to students regarding different areas of research and ensured students full comprehend all aspects of the curriculum

**Quest Diagnostic**

San Juan Capistrano, CA

**Lab Associate**


- Performed daily and monthly machine maintenances on mass spectroscopy
- Supported CLS for clinical biological assays
- Prioritized and organized assay samples
- Troubleshoot and resolved administrative errors on patient documentations

**University of California, Irvine - Beckman Laser Institute**

Irvine, CA

**Junior Scientist**


- Research and designed the procedure of *in vitro* study of gene transfection with photochemical internalization effect
- Recorded and analyzed data with MS Excel in order to make concise graphic representation
- Discussed current data with Principal Investigator in accomplishing research goals
- Performed experiments based on protocols
- Operated and maintain laboratory instruments and equipment
- Documented detail protocols for future research procedural reference
- Routine maintenance of cell cultures

**Publications**

54. doi: 10.1002/lsm.22082


**Leadership and Affiliations**

Executive committee member of Taiwanese Student Association at University of California, Irvine. 2010.

Representative of School of Allied Health to Graduate and Professional Student Council. Summer 2013.

Member of American Association of Physicist in Medicine and Alpha Phi Omega Service Fraternity