DNA Aptamer-Drug Targeting Chemotherapy: Investigation of Cell Cycle Inhibition via S15 Aptamer – Norcantharidin Complex

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Abstract

Lung Cancer is the leading cause of cancer related death in both men and women in the United States (Bray et al., 2018). Cancer treatments are often non-specific and kill many dividing cells within a patient causing unwanted side effects. Norcantharidin (NCTD) is a synthetic FDA approved treatment for cancers including non-small cell lung cancers (NSCLC). NCTD suppresses cell proliferation by inhibiting cells from exiting the G2 phase of the cell cycle. Aptamers are short single-stranded DNA or RNA molecules with ligand directed self-annealing capabilities allowing selective binding to specific targets. This paper will discuss the hypothesized effects of using a single-stranded DNA aptamer bound to NCTD to reduce proliferation of A549 cells, an adenocarcinoma cell line often used to study NSCLC (Foster et al., 1998 & Giard et al., 1973).
DNA Aptamer-Drug Targeting Chemotherapy: Investigation of Cell Cycle Inhibition via S15 Aptamer – Norcantharidin Complex

**Introduction**

Cancer is the second leading cause of death in the United States and mortality rates are growing worldwide (Bray et al., 2018, Siegal et al., 2019). Specifically, lung cancer has the highest mortality rate of deaths caused by cancer universally. Lung cancer is most prevalent in the form of NSCLC but also presents in the form of Small Cell Lung Cancer (SCLC) (Luan et al., 2010). Optimal treatment plans for NSCLC include surgical resections; however, 70% of patients present with advanced or metastatic disease when diagnosed (Molina et al., 2008). Novel anti-cancer therapeutics have been introduced to attack NSCLC cells, including targeting the cell cycle. NCTD (Figure 1) is a synthetic FDA approved cancer drug derived from the Cantharidin of blister beetles. The drug suppresses the cell cycle in the G2 phase of proliferation, thus reducing proliferation of tumors. NCTD alone functions as a protein phosphatase inhibitor, an antimitotic, and blocks vital cancer protein receptor pathways (Qiu et al., 2017).

This study will describe the effects of the drug when coupled to the S15 single-stranded DNA aptamer. Aptamers can be used to deliver a drug to enhance binding a specific target, while selecting against non-immunogenic and toxic substances. Aptamers can target specific biomarkers or whole cells. This project will treat a common adenocarcinoma cell line in NSCLC research, the A549 cell line, with NCTD bound directly to the S15 aptamer. Previous studies have shown that the S15 aptamer has a high affinity (>85%) for A549 NSCLC cells and low affinities for SCLC and other cancer
Figure 1

*Chemical structure of Norcantharidin*

![Chemical structure of Norcantharidin](image)

*Note.* Norcantharidin is chemically demethylated form of Cantharidin to enhance the anti-tumor capabilities and reduce toxicity.

lines (Zhao et al., 2009). The S15 aptamer will also be applied to a human cervical cancer line, HeLa cells, in which the S15 aptamer has low affinity. The HeLa cells should experience a lower rate of apoptosis in comparison to the A549 cells.

**Cancer Drug Targets and Effects**

Cancer is a major medical concern not only in the United States but worldwide. This research topic is of great importance which may aid in reforming treatments of NSCLC and potentially other types of cancer. One of the major barriers remaining in developing successful clinical targeting therapeutics is the variability between each patient and their cellular signaling pathways. Current cancer treatments target all rapidly dividing cells thus attacking not only cancer cells, but many cells in the body, including immune cells. This may cause suppressed immune responses and death to cells that are
vital to normal physiology. The goal of targeting anti-cancer drugs is to reduce negative side effects during cancer treatments by killing only malignant, rapidly dividing cells. Anti-cancer drugs can target and block highly specific regions of a signaling pathway involved in replication. Blocking a vital transcription factor or preventing a chemical reaction from occurring results in the cessation of rapid cell replication and tumor growth.

This paper proposes to deliver NCTD via direct aptamer conjugation to decrease the attack on healthy dividing cells while targeting cancerous cells. Novel therapies in cancer treatments are being investigated, but this targeting mechanism of chemically binding the S15 aptamer to NCTD has not been investigated to the knowledge of the research team. NCTD is a common cancer therapeutic drug which typically uses a conjugated liposomal delivery method (Zhu et al., 2018). Targeted liposomal delivery of NCTD has been shown to be effective. However, liposomal delivery also triggers the body’s autonomic immune response. The body often expels the liposome containing the drug before the drug can be released to the targeted cells. This project seeks to find a more effective delivery system for NCTD to avoid liposome eradication. The delivery mechanism will be replaced with chemically bound aptamer targeting. This paper will attempt to demonstrate the efficacy of the proposed mechanism by evaluating evidence of successful coupling of the aptamer to NCTD and inhibition of cell proliferation in targeted cells only.

Targeting anti-cancer therapeutics have focused on preventing proliferation, angiogenesis, and metastasis as well as increasing apoptosis. Inhibition of the Vascular
endothelial growth factor (VEGF) pathway is one of the most researched areas of targeted cancer treatments. VEGF is involved in neovascularization and survival of cells. Overexpression occurs in many cancers and is often associated with disease progression and decreased survival rates. Drugs targeting VEGF inhibition are FDA approved for colorectal cancer, first line lung cancer, and metastatic renal cell carcinomas (Duda et al., 2007). Cell targets for this treatment are pluripotent as VEGF is expressed in endothelial cells in tumors, subsets of hematopoietic cells, stromal cells, and other malignant cell types. Several mechanisms of action have been proposed to describe this pathway because there are receptors on a variety of different cells, but it is most commonly suggested that an anti-VEGF agent should normalize vasculature. Blocking the VEGF/VEGF-R interaction demonstrates 74% tumor growth inhibition in a rhabdomyosarcoma when compared to a control group (Eytech Study Group, 2002). This aptamer targeting treatment produces mild toxicity and symptoms that can be treated with medications; serious complications have appeared on rare occasions. Further research is to be completed to gain a more complete understanding of the mechanism in which the aptamer complex interacts with the cell surface biomarker to enhance treatments. This study demonstrates the success and potential that aptamers have in clinical applications and specifically as an anti-cancer targeting method.

Aptamer targeting research has also involved other cancerous pathways including PI3K/AKT and MAPK signaling, HER2 (ErbB-2), PD-1/PD-L1, CXCL12/SDF-1, nucleolin and other proteins. These avenues focus on inhibiting phosphorylation of tumor promoters, blocking tumor growth signals, blocking IL-2 secretion to prevent tumor
growth and CD8 cytotoxicity, preventing angiogenesis and metastasis, and preventing oncogene activation, respectively (Morita et al., 2018). This study will focus primarily on inhibiting proliferation at specific cell cycle points. Malignant cells often deregulate the cell cycle machinery. Thus, focusing a treatment on the inhibition of a checkpoint or major transition in the cell cycle will prevent tumorigenic cells from rapidly proceeding through the cell cycle. This should prevent the hyperactivation of cell cycle machinery. These treatments often focus on the G1 or G2 checkpoints. The G1 checkpoint occurs prior to DNA synthesis and involve the phosphorylation of the pRb protein. When phosphorylated and activated, pRb releases E2F as a transcription factor. E2F forms a heterodimer with DP1 and DNA synthesis begins for the round of replication (Carnero, 2002). E2F genes often function as oncogenes, and recent studies have shown E2F knockouts can function as tumor suppressors. The G2 checkpoint occurs before chromosomes segregate to daughter cells; this is controlled by various CDKs, cyclins, and their regulators. Activation of the CDK1 gene is required for the cell to progress past the G2/M checkpoint. Wee1 and cell division control protein 25C (CDC25C) are primary regulators of CDK1; these regulators are dictated by polo-like kinase 1 (PLK1) and checkpoint kinase 1 (CHK1). Wee1 activation prevents cell cycle progression when localized in the nucleus while CDC25C induces cell cycle progression when localized in the nucleus (Gooijer et al., 2017). Wee1 is downregulated by PLK1 via phosphorylation of Ser53 leading to degradation; CHK1 antagonistically upregulates Wee1 by phosphorylating Ser642 leading to nuclear localization. Figure 2 presents a map of the primary and secondary regulation of the G2 checkpoint of the cell cycle. This study will
use NCTD, which inhibits the progression through the G2 checkpoint.

The G2 checkpoint functions as a tightly controlled biomolecular switch to ensure proper DNA stability and ploidy before segregation. Arrest at this point allows cells to repair DNA if possible or completely prevent the cell from replication and directing it towards apoptosis. By inhibiting the phosphorylation and sequestering these regulators in the cytoplasm, the cell cycle of malignant cells is effectively inhibited, and cell death is ensued. This study will examine this effect using an S15-NCTD complex to target these regulators.

**History of Chemotherapy**

The first form of chemotherapy appeared in research trials in a collaboration between Yale University and the Office of Scientific Research and Development in 1942. Prior to these trials, cancer was difficult to approach as a physician. Treatments included surgical resection of moveable tissues and radiation therapy. Surgical removal of tumors became the treatment of choice but could only be effective in early stages (Evolution of Cancer Treatments: Surgery, 2014). At this time physicians were forced to decide which organs would suffer in the process of removal; when anesthesia became available in 1846 surgical patient outcomes increased. As radiation became available as a diagnostic and a therapy, it was discovered that the radiation could cause cancer if the doses were continually too high (Evolution of Cancer Treatments: Radiation, 2014). During World War II, the U.S. Army studied chemicals related to mustard gas because numerous military personnel developed toxic changes to their bone marrow after exposure to mustard gas. Nitrogen mustard, an alkylating agent, was developed as a more
Figure 2

Schematic map of G2/M Checkpoint of cell cycle progression

Note. The CDK1 gene is the central control and is regulated primarily by Wee1 and CDC25C and secondarily by CHK1 and PLK1.

effective warfare agent and was found to also kill cancer cells of a lymphoma. It was discovered that alkylating agents prevent effective cell replication by adding an alkyl group to guanine bases and preventing the proper formation of a double helix in the DNA strands. This provided an avenue for researchers to study other alkylating agents and their ability to kill rapidly dividing cells by damaging their DNA (Evolution of Cancer Treatments: Chemotherapy, 2014). Sidney Farber of Boston then made a ground-
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breaking discovery on the molecular and organismal level regarding the treatment of cancers. His studies demonstrated that aminopterin contained an active agent that blocked a chemical reaction that is required in DNA replication. This was used as a treatment for acute leukemia which resulted in these children entering remission. This began the era of chemotherapy as chemicals were used to block various reactions or functions of the cell cycle.

History of NCTD

NCTD (7-oxabicyclo[2.2.1] heptane-2,3-dicarboxylic anhydride) is a water-soluble synthetic molecule of naturally occurring Cantharidin (CTD). CTD is commonly isolated from body fluid of blister beetles and used medicinally (Hsieh et al., 2013). Located throughout the midwest, south and entire east coast of the United States as well as Central America and worldwide, Traditional Chinese medicine (TCM) is credited with the discovery of the medicinal properties of a cytotoxin contained in CTD. CTD was historically administered orally to treat ulcers, venomous worms, and abdominal masses. As the drug gained popularity in other continents, it was used to treat dropsy, taken as an aphrodisiac, and used in attempt to “purify blood” (Moed et al., 2001, p. 1358). CTD was removed from the market in 1962 as safety issues and poisonings arose from certain uses. In 1997, CTD was added to the “Bulk Substances List” which provides that a pharmacist or physician can prescribe specific amounts under necessary circumstances. Literature has uncovered mechanisms of action for CTD which have changed the route of administration. Absorption of CTD occurs through the lipid bilayers of epidermal cell membranes; CTD is now often used dermatologically because a topical administration
leads to the release of a neutral serine protease which dissipates the desmosomal plaque connecting epidermal cells. Warts and other dermal targets are eliminated via acantholysis and healed with minimal to no scarring. The blistering process begins within 24-48 hours of application and the severity or degree of blistering is determined by the frequency of cleaning the site of application.

The cytotoxin contained in CTD is typically used as a defense mechanism by male blister beetles (Selender, 2000). This, now FDA approved treatment, was initially used for dermal treatments of warts and other skin conditions. Recently, further research has discovered potential anticancer properties. However, if CTD is administered orally or intravenously, toxic side effects in the gastrointestinal and urinary tract may occur. In the past couple of decades CTD has been analyzed in vitro in hopes of reducing these unwanted side effects in humans. NCTD is a demethylated small molecule analogue of CTD; both of these are shown in Figure 3. CTD can be demethylated in the laboratory to create NCTD; Figure 4 shows synthetical NCTD formation via a Diels-Alder reaction using furan and maleic anhydride. Studies have shown that the methyl groups are not the active functional group of the anticancer activity. By synthetically modifying CTD, the toxicity of the drug to the gastrointestinal and urinary tract is reduced without affecting the anticancer properties of the molecule (G.-S. Wang, 1989). NCTD is a multifaceted anticancer therapeutic. It has been shown to induce apoptosis, inhibit angiogenesis and metastasis, and alter pathways controlling cell proliferation. NCTD also inhibits normal cells including inhibiting peripheral blood mononuclear cells and blood stem cell maturation and plays a role in Multi-Drug resistance (MDR). Thus,
Figure 3
Chemical structures of Cantharidin and Norcantharidin

Note. Chemical structures (a) Cantharidin is a naturally occurring methylated compound extracted from blister beetles. (b) Norcantharidin is a synthetically demethyalted analogue of CTD which reduces toxicity in the human body and increases anticancer activities.

Figure 4
Chemical reaction of furan and maleic anhydride to produce NCTD

Note. The Diels-Alder reaction occurs via a [4+2]-cycloaddition of a conjugated diene and a dienophile.
NCTD has become the more common drug of focus in these anticancer studies because of its inhibition of the cell cycle with substantially fewer negative side effects of methylated CTD.

**NCTD Mechanisms of Action**

NCTD acts on the G2 checkpoint of the cell cycle to inhibit tumor growth. Research has shown that mitotic arrest via NCTD action may occur through the CDK1/cyclin B pathway. Studies completed at the China Medical College Hospital (CMCH) used cell viability assay, microscopy, flow cytometry, protein analysis, TUNEL assay and DNA electrophoresis to produce clear results indicating time and dose dependent tumor growth inhibition via NCTD (Chen et al., 2002). In order for a cell to proceed through the checkpoint into mitosis, CDC25C must be phosphorylated to become active. CDC25C is a phosphatase that activates CDK1/Cyclin B1 kinase. This study showed that NCTD induces a hyperactivation of CDC25C leading to a prolonged activation CDK1/Cyclin B1 activity. Results also demonstrated that premature, unscheduled, or prolonged activation of this complex induces mitotic arrest and apoptotic morphologies of cells. Cell death began to occur after 24 hours and maximum cell death was observed at 72 hours. Therefore, CMCH demonstrated that NCTD induces inappropriate activation of CDK1, the G2 checkpoint control, which leads to apoptosis of cells.

Other studies have also investigated multiple pathways related to the G2 checkpoint to describe the mechanism of action. This involves the p53 tumor suppressor gene and the Bcl-2-Bax pathway. Each respective pathway is commonly researched.
regarding apoptosis. Studies completed in Tokyo have also demonstrated that NCTD
affects the Bcl-2-Bax signaling pathway (Luan et al., 2010). Under normal physiological
conditions, Bcl-2 is an antiapoptotic protein and Bax is a proapoptotic protein. Both of
these proteins vary the potential, structure, and permeability of the mitochondrial
membrane. These changes can result in the release of cytochrome c from the
mitochondria to the cytoplasm, leading to caspase-involved apoptosis. Bcl-2 is normally
located within the inner mitochondrial membrane and functions to inhibit cell death. Bax
is normally located in the cytoplasm and moves into the membrane to induce cell death
when cell viability is compromised or if chromosomal status is not correctable at the G2
checkpoint. Bcl-2 and other pro-survival proteins share a similar hydrophobic anchor
region including BH1, BH2, BH3, and BH4 domains. Bax proteins often contain only
BH3 domains. The complexity of the caspase cleavages that produce the various domains
on Bax proteins allow the dimerization with other proteins such as Bcl-2. This may also
contribute to the binding of other C-terminal hydrophobic domain insertions; both
interactions appear to contribute to the apoptotic response (Pawlowski & Kraft, 2000).
Both Bcl-2 and Bax proteins can form ion channels in the mitochondrial membrane
independently of one another but can also interact to inhibit one another to regulate
apoptosis. This interaction creates the Bcl-2-Bax signaling pathway.

The proposed mechanism of interaction between Bcl-2 and Bax proteins occurs
via binding of Bax to the BH1, BH2, and BH3 domains of Bcl-2. The ratio of each
protein varies, but most likely strongly contributes to the regulation of apoptosis. Higher
levels of Bcl-2 are found in malignant cells as apoptosis is suppressed and cells
proliferate freely. A study completed in 2010 at Tokyo Noko University treated A549 cells with NCTD to examine morphologic changes and Bcl-2-Bax protein levels via Western Blot Assay (Luan et al., 2010). Apoptotic changes appeared in cell bodies 48 hours after treatment with NCTD. The Western Blot confirmed that NCTD up-regulates the expression of Bax proteins and down-regulates the expression of Bcl-2 proteins (Luan et al., 2010). Increasing levels of Bax while decreasing cellular levels of Bcl-2 will cause a reduction in the Bcl-2/Bax ratio leading to a greater rate of cell death. Another study completed at Zhongnan Hospital of Wuhan University completed various experiments producing similar results (Chang et al., 2010). They demonstrated that a malignant cell line began to rapidly enter apoptosis 24+ hours after treatment with NCTD. Flow cytometry assays displayed apoptotic cell surface morphologies after treatment. Western Blot assays also indicated mitochondrial membrane potential changes, increased cytochrome c release, and a substantially increased level of Bax proteins resulted from treatment (Luan et al., 2010). The cells treated with NCTD demonstrated decreased Bcl-2-Bax ratios and proceeded to enter apoptosis rapidly. Therefore, the overexpression of Bax proteins and the under expression of Bcl-2 proteins is a viable mechanism of action for cell death induced by NCTD.

**Aptamer Targeting**

Aptamers are single-stranded oligonucleotides of either DNA or RNA that bind specifically to ligands with complimentary pairing. Aptamers are often referred to as chemical antibodies as the specific three-dimensional folding allows for highly specific interaction with the target (Reyes-Reyes et al., 2010). They can be selected to have a high
affinity for a desired target of various disorders including human malignancies. Chemical aptamers are of interest in novel clinical applications because of this ability to target and mark cell surface biomarkers. Aptamers can penetrate tissue barriers quicker and more efficiently than their protein counterparts because of the smaller size. Protein antibodies are typically 150 kDa whereas synthetic nucleic aptamers range from 8-25 kDa (Sun et al., 2014). Aptamers are also advantageous to protein antibodies as they are nearly nonimmunogenic whereas antibodies are highly immunogenic, specifically after repeat injections. Aiming to reduce immunogenic responses in cancer therapeutics, roughly 60% of targeting cancer therapies currently use cell surface biomarkers, making this pathway optimal for research in aptamer targeting.

To develop an aptamer for a specific biomarker, SELEX technology (“systematic evolution of ligands by exponential enrichment” has been developed [Sun et al., 2014, p. 2162]). This technology selects an aptamer by incubating a 20-100-nt sequence flanked by fixed primer regions at the 5′ and 3′ ends with a target molecule in a library pool of $10^{15}$ variants. Variants that bind with a high specificity are recovered and amplified. Amplification occurs via polymerase chain reaction (PCR) or reverse transcription-polymerase chain reaction (RT-PCR) depending on the composition of the aptamer. Next, the single stranded pool is regenerated for several more rounds via in vitro transcription to identify the selected aptamer. This is feasible because of the thermal stability of aptamers. Aptamer-ligand interaction can be through specific base pairing or the geometry of the aptamer which includes loops, stems, hairpins, pseudoknots, bulges, or G-quadruplexes (Sun et al., 2014). These secondary structures interact to produce unique
tertiary interactions with the ligand. These are essential for aptamer specificity and include hydrophobic interactions, electrostatic interactions, hydrogen bonding, van der Waals forces, shape complementarity, and base stacking interactions. Direct aptamer-ligand conjugation specificity can be described by the lock and key model. The aptamer will interact only with the target due to the high level of specificity of binding. It has been demonstrated that aptamers can distinguish between one amino acid mutation, one functional group, and conformational isomers (Morita et al., 2018). The aptamer-drug complex mechanism is demonstrated in Figure 5. Pharmacologically, the drug of choice is often bound to the aptamer covalently via a linker or directly inserted into the nucleotide. Once complexed with the drug of choice, the aptamer binds to the cell biomarker as demonstrated in Figure 6.

**Significance of S15 Aptamer**

Previous research has demonstrated that of the SELEX selected binding options, the 85 base long ssDNA S15 aptamer provides the highest affinity with A549 cells. A study completed through a partnership between the Beijing National Laboratory in China and the University of Florida in the United States in 2009 used SELEX to identify four aptamers that could potentially detect NSCLC (Zhao et al., 2009 & Q. Wang et al., 2014). These four aptamers, S1, S6, S11a-f, and S15, were analyzed for their affinity with healthy Alveolar Type II (ATII) and cancerous adenocarcinomas. Equilibrium dissociation constant values ($K_d/nM$) were used to determine the affinities of each aptamer in conjugation with cell types. A549 cell lines were used to represent adenocarcinomas. $K_d$ values of each aptamer are shown in Table 1. The S15 aptamer has
**Figure 5**

Schematic diagram of aptamer-drug complexation via covalent and non-covalent binding

Figure 6

Schematic diagram of aptamer-target interaction


The highest affinity (>85%) of each examined aptamer; this study also demonstrated low affinities for other NSCLC cell lines and SCLC cell lines as found in Table 2. The S15 aptamer effectively binds to a surface biomarker of the A459 cells as binding properties were depleted when A549 cells were treated with a Protein Kinase K which destroys surface proteins (Zhao et al., 2009). A study was also completed by the Israel Cancer Association which demonstrated the high affinity of the S15 aptamer with A549 cells (Engelberg et al., 2019). This study also examined a clathrin-mediated endocytic delivery mechanism to overcome immunogenic reactions when liposomal delivery is used. The liposomal delivery method often results in expulsion of the liposome before the contents.
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Table 1
Sequences and equilibrium constants (Kd) of S1, S6, S11, and S15 aptamers.

<table>
<thead>
<tr>
<th>Aptamer type</th>
<th>Sequences of random region</th>
<th>Kd nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>GGTTCGTAGGCGGTGGGAGGAGGAGGAGTTGGTATAGGCATACACAGG</td>
<td>40.9 ± 6.2</td>
</tr>
<tr>
<td>S6</td>
<td>GTGCGCAGTATCATATGAGAGGAGGAGTTGGTATAGGCATACACAGG</td>
<td>28.2 ± 5.3</td>
</tr>
<tr>
<td>S11</td>
<td>AGGTGCGGCTGGGTGGGAGGAGGAGTTGGTATAGGCATACACAGG</td>
<td>66.9 ± 5.0</td>
</tr>
<tr>
<td>S15</td>
<td>TGGCGGTTAAATTTTTGGGGAGGAGGAGTTGGTATAGGCATACACAGG</td>
<td>47.0 ± 3.3</td>
</tr>
</tbody>
</table>


Table 2
Recognition of various cancer cell line by S1, S6, S11, and S15 aptamers.

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Cell lines</th>
<th>S1</th>
<th>S6</th>
<th>S11</th>
<th>S15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoarcinoma (NSCLC)</td>
<td>A549</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Large cell carcinoma (NSCLC)</td>
<td>HLAMP</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Large cell carcinoma (NSCLC)</td>
<td>NCI-H460</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Large cell carcinoma (NSCLC)</td>
<td>NCI-H292</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Squamous carcinoma (NSCLC)</td>
<td>NCI-H520</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Squamous carcinoma (NSCLC)</td>
<td>NCI-H517</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>SCCL</td>
<td>NCI-H3246</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Human cervical carcinoma</td>
<td>HeLa</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Human breast carcinoma</td>
<td>MCF7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>HepG2</td>
<td>+</td>
<td>++</td>
<td>++++</td>
<td>++</td>
</tr>
</tbody>
</table>


can be released and become effective; aptamer targeting seeks to provide a superior delivery mechanism. Clathrin-mediated endocytosis is adaptable in nature and can be modulated for the needs of the specific cargo (McMahon & Boucrot, 2011). The successful delivery of the drug bound directly to the S15 aptamer leading to increased
cell death specifically in A549 cells has promising implications for both drug delivery and diagnostics. This study seeks to extend this application in progressing a novel chemotherapeutic method that reduces negative side effects, lowers the required drug dosages, and reduces the cost of chemotherapy.

**Aptamer Based Chemotherapy**

Previous research has implemented aptamers into chemotherapeutics to target malignant proliferating cells. Aptamers are administered intravenously or orally, with the goal of binding to a specific target. Applications of aptamers vary widely from uses in basic research, food safety, diagnostics, and therapy. Clinical applications may include blocking a protein-protein interaction or binding antagonistically to a receptor ligand. In blocking protein-protein interactions, a vital reaction in the cell cycle pathway cannot occur and the cells cannot replicate. For example, interfering with interactions localizing CDK1 prevents a cell from proceeding past the G2 checkpoint and continuing through the cell cycle which effectively inhibits cell proliferation. To be effective as an anti-cancer therapeutic, the aptamer usage must evade renal filtration, nuclease degradation, and the safety profile. Effective anti-cancer drugs must remain in circulation for extended periods of time to increase the probability that the drug interacts with the cancerous cells. Aptamer stability has demonstrated the ability to overcome these standards as an effective anti-cancer agent. Renal filtration is the primary process to excrete drugs, but the low molecular weight of aptamers (8-25kDa) alongside the small diameter averaging less than 5 nm allow aptamers to evade excretion. The filtration threshold of the glomeruli is about 50 kDa. When conjugated to a larger drug, the aptamer may be filtered
by the kidneys, but the effective time of circulation is greatly increased. Both endonucleases and exonucleases are abundant in the body and cleave the phosphodiester bonds of oligonucleotides. Thus, aptamers are often chemically modified to avoid cleavage and increase their half-life from mere minutes to days or weeks. This could be attained by replacing the 2’ hydroxyl group with a 2’ amino group of the ribose sugars (Morita et al., 2018). The safety profile of aptamers includes the selection against immunogenic responses often conferred with protein antibodies. Traditional chemotherapeutics confer not only inflammation, irregularities of bone marrow hematopoiesis, and lymphatic pathways, but this immune suppression creates a susceptibility to more illnesses in all cells (Chemotherapy Side Effects, 2019). Due to these negative side effects, chemotherapy drugs must be given at low doses over longer periods of time; this is not an ideal method of treatment as the patient’s immune system is lowered and at risk for extensive periods of time. Because aptamers bind directly to the anti-cancer drug and interact only with their specific target, there is no attack on self-immune cells (Sun et al., 2014). This avoids humoral and cell-mediated responses of the body’s autonomic immune response.

**Research Design and Methods**

This project will use information from peer-reviewed scientific articles and journals to coordinate starting values of NCTD and S15 aptamer. Experiments will be performed under the supervision of Dr. L. Stevenson alongside undergraduate student Brandon Reynolds.
This study will use the human adenocarcinoma cell line, A549, to represent an aggressive NSCLC to examine the efficacy of the S15-NCTD targeting complex. The A549 cell line was isolated from a 58-year old Caucasian male by Giard et al. (1973) and is commonly used now for research both \textit{in vitro} and \textit{in vivo}. These squamous alveolar cells are involved in water and electrolyte transport in normal physiology, and the multitude of proteins and mechanisms they can perform have allowed scientists to understand more oncologic pathologies. The tendency of malignant alveolar cells to metastasize alongside their short half-life which averages 23 hours even while outside the body, will enhance this research. This study will also use the human cervical cancer cell line, HeLa, as a non-target for the S15-NCTD targeting complex. The HeLa cell line was isolated from a 31-year old African American woman in 1951 and has become vital to oncology research (Lyapun et al., 2019). The isolation of the immortal HeLa cells was a breakthrough scientifically and led to the culture of multiple other cell lines. It is expected that the A549 cells will be targeted and killed by the S15-NCTD complex while the HeLa cells will have no substantial interaction with the complex.

The first step in evaluating the proposed hypothesis will be to evaluate aptamer binding and target cell specificity. Aptamers possess the ability to anneal to themselves to create specific three-dimensional conformations specific to a protein or cell. The S15 aptamer should target and bind directly to the A549 cell after the aptamer drug complex is applied. First, the aptamer can bind to the drug covalently through the tail with the use of a linker protein or directly intercalate the DNA (Sun et al., 2014). The direct aptamer-drug binding will be visualized using a gel shift assay of 20\% polyacrylamide gel stained
with Ethidium Bromide. The aptamer will be applied to cell cultures of A549s and HeLa cells. After visualization on the polyacrylamide gel, a PCR process will be used to confirm that the complex is bound directly to the cells. Cells will be washed and lysed; a PCR reaction will be used to amplify and detect the presence of aptamers, thus indicating that the drug aptamer complex is bound. Current literature has shown that S15 aptamers bind to A549’s with a high affinity as conjugation experiments proceed at a rate greater than 85% (Zhao et al., 2009); cells will be treated with the S15 aptamer as well as an aptamer of the same length and a different sequence to evaluate the success rate of binding. It is expected that visualization will show successful binding of the S15 aptamer to the A549 cells and unsuccessful binding to the HeLa cells. It is also anticipated that the scrambled aptamer will not bind to the A549 cell line. Once the presence of the aptamer is confirmed specificity can be evaluated. This proposed method will allow NCTD to selectively attack only cancerous cells using the 3D oligonucleotide conformation of the aptamer that is complimentary to surface markers displayed on A549 cell membranes.

Targeted anti-cancer drugs must show the ability to kill rapidly diving cancerous cells without a significant decrease of healthy proliferating cells. NCTD has previously shown to be effective against a variety of cell lines, including A549 cells. We will complete a kill curve to show the effectiveness of NCTD on cancer cell lines. Cells will also be treated with DMSO as a control for cell death and PBS as a control for normal growth. These cells will be used as a comparison to determine the effectiveness of the S15-NCTD complex. This hypothesis proposes that when NCTD is chemically coupled to the S15 aptamer, it will maintain the same antimitotic ability, but will only attack cells
with surface markers complimentary to the aptamer. That is, the drug complex will preferentially decrease proliferation in only A549 cells. This will ensure any decrease in proliferation is due to the aptamer drug complex rather than aptamer alone or drug alone. Cell viability after treatment will be determined based on cell metabolic activity. A MTT assay will be used to assess the ability of NCTD to successfully inhibit proliferation of A549 cells while allowing HeLa cell proliferation. It is expected that NCTD alone decreases proliferation in the A549 cells and that the aptamer drug complex successfully decreases proliferation of A549 cells to a similar effectivity. It is also expected that the aptamer drug complex has little to no effect on the HeLa cells; this will show the specificity of the surface markers that the S15 aptamer will bind to. This will provide evidence of a successful aptamer delivery method for NCTD.

**Conclusion**

This research is vital and should be conducted because there is a lack of understanding in the functionality of aptamer targeting cancer therapeutics. Current cancer treatments cannot combat unwanted side effects and often suppress immune responses. The direct conjugation of the single-stranded S15 DNA aptamer to NCTD should provide an anti-cancer therapeutic that effectively inhibits the proliferation of cancer cells without inhibiting healthy proliferating cells. Thus, there will be a reduction in negative side effects during the treatment of NSCLC. With the analysis of this research project, this mechanism can continue to progress to in vitro and human testing to become a novelty in the field of chemotherapy.
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