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Cysteine Deleted Tachyplesin Peptide Analogs as Anti-Cancer Agents

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Abstract

Adenocarcinoma is a common type of non-small cell cancer that represents 80% of all diagnosed lung cancers. Typical treatment methods include surgery, radiation therapy, and chemotherapy, or some combination of the three. Although radiation therapy and chemotherapy treatments are effective at killing cancer cells, the side effects and symptoms associated with these treatments can cause severe damage to the patient’s body and even encourage some fatal illnesses, such as sepsis. Research has been focused on the development of other methods of treatment that would be less harmful or even non-harmful to patients. Antimicrobial peptides are known for their potential in modern antibiotics to treat bacteria-caused illnesses. The antimicrobial peptide, cysteine deleted tachyplesin (CDT), demonstrates both antibacterial as well as anticancer properties. To improve the development of CDT into a possible treatment for adenocarcinoma of the lung, analogs of the peptide CDT containing the hyaluronan binding sequence are being synthesized and tested on the cell line A549, with the hopes of improving their anticancer characteristics and to understand better the mechanism that allows CDT and its analogs to cause adenocarcinoma cell death.

Introduction

Adenocarcinoma

In 2016, the American Cancer Society issued a report indicating that 526,510 men and women were living with lung cancer, and a projected 224,390 additional people would be diagnosed before the end of that year. A year later, the 2017 report...
showed that lung cancer had become the second most diagnosed type of cancer in the United States in both men and women. The report estimated that 222,500 cases would be diagnosed before the conclusion of 2017, and, of those patients, an estimated 155,870 would die due to complications from the illness (American Cancer Society, 2017).

Adenocarcinoma of the lung is the most commonly diagnosed type of non-small cell lung cancer (American Thoracic Society 2014). Lung cancer can be divided into two sub-categories: small cell cancer and non-small cell cancer. Adenocarcinoma of the lung is a type of non-small cell cancer. The cancer forms in the outer tissues of the lung from cells that are responsible for making mucus (The Roche Group 2017). Most lung cancers are known to be asymptomatic until the late stages (III and IV; Miller et al. 2016). Adenocarcinoma of the lung is characterized as being a slow growing cancer, however, with the usual diagnosis of the cancer being in stage IV at a median age of 70 years old, recovery can be very difficult, even with aggressive treatment (American Cancer Society 2016).

Lung cancer, specifically adenocarcinoma, is directly linked to levels of environmental carcinogenic exposure (American Cancer Society 2017). Men and women who smoke are 25 times more likely to develop lung cancer (American Cancer Society 2017). Risk factors include smoking and exposure to radon gas, asbestos, diesel exhaust, radiation, and air pollution (American Cancer Society 2017). In a Croatian study conducted in 2014 by Kukulj et al., the lung cancer treatments of 212 newly diagnosed patients were monitored. Of the 212 patients, 186 were diagnosed with small cell lung cancer, and 89 of these patients had adenocarcinoma of the lung. As per the trend reported by the American Cancer Society, the study found that median age of diagnosis was 65 years old, and 157 of the 212 patients (74.1%) had stage IV cancer. Of the patients who took part in the study, 182 were smokers (85.5%). The data on the patients involved in this cohort study solidify the link between carcinogenic exposure, such as long-term smoking habits, and adenocarcinoma of the lung (Kukulj et al. 2014).

In a separate study conducted by Yang et al. from 1986 to 1998, approximately 38,000 women between the ages of 55 and
69, living in Iowa, were monitored with the intent of linking the risk factor of smoking to the development of lung cancer. This research study followed the women for 13 years. Of the 38,006 women in this study, 34% were former or current smokers at the beginning of the study. Of the entire cohort, 234 of the women developed adenocarcinoma of the lung. Published in 2002, this study also draws a very strong conclusion that smoking, current or past, can increase the risk of developing adenocarcinoma of the lung (Yang et al. 2002).

The treatment for adenocarcinoma is usually surgery, in combination with chemotherapy (American Cancer Society 2017). An efficient and safe treatment method that would both eradicate the cancer and not harm the patient is highly sought after.

**The Dangers of Chemotherapy**

Chemotherapy is a well-known cancer treatment method. It is known to be efficient in killing cancerous cells; however, this treatment method is also very effective at killing the healthy tissue and cells surrounding the cancer. According to the American Thoracic Society (2014), after a non-small cell lung cancer progresses to stage III, chemotherapy is a strongly considered treatment method. Chemotherapy causes side effects that are degenerative to the quality of life of the patient, including, but not limited to, fatigue, hair loss, nausea and vomiting, anemia, increased risk of infection, kidney damage, and nerve damage (American Thoracic Society 2014). The most concerning risk from chemotherapy is the possibility of infection, which could prove deadly. Chemotherapy kills the cancer cells in the body that grow quickly, but it also kills the cells that naturally grow quickly, such as hair cells, nail cells, and white blood cells (Centers for Disease Control and Prevention [CDC] 2017). With low white blood cell counts, a patient undergoing chemotherapy is susceptible to infection and even sepsis (CDC 2017). The efficiency, effectiveness, and safety of chemotherapy call into question whether chemotherapy should remain one of the primary treatment methods for distinct types of lung cancer, including adenocarcinoma. There has been exploration into other treatment methods, such as targeted therapy. The
possibility of using antimicrobial peptides (AMPs) as a safe, efficient treatment specifically for adenocarcinoma would bring us one step closer to understanding the cure for this type of cancer.

**Antimicrobial Peptides**

AMPs are found in plants, animals, and other organisms. AMPs are characterized as both cationic and amphipathic (Schweizer 2009). Recent advances in research have encouraged the use of AMPs as targeted therapy to combat especially resistant bacterial strains. The success of these targeted therapies is consistent with interaction with the cell membrane. Antimicrobial peptides that kill bacteria through membrane interaction alone are more likely to kill bacteria without giving them a chance to become resistant (Riedl et al. 2011). To do this, the membrane of the bacterial cell must be penetrated. Models summarizing how activity may take place are based both on the known characteristics of the bacterial cell and the peptide. The significant differ-

**Figure 1.** Visual comparison of the components of the cell membrane of a healthy mammalian cell and a bacterial cell. Antimicrobial peptides have more interaction with the negative bacterial cell membranes due to charge. The healthy mammalian cell membrane is neutral (Riedl et al. 2011).
ence between the bacterial cells and the healthy mammalian cells is found in the charge of the membrane. Both gram-positive and gram-negative bacteria have an overall negatively charged membrane. Bacteria gain their charge due to the molecules found on the outside of the membrane. Bacterial cells are anionic due to the acidic phospholipids found on the membrane. Healthy mammalian cells, on the other hand, are overall neutral, due to the presence of both cationic and anionic phospholipids, giving them a zwitterionic character. Figure 1 (above) shows the composition of the membrane of the cancer cell and bacterial cell comparatively (Riedl et al. 2011). Because of the similarity, the mechanism by which the membrane disruption takes place and induces cell death is also thought to be similar.

Potential Mechanisms

There are four commonly cited models that seek to summarize the permeation of the bacterial or cancer cell membrane. One of the possible mechanisms of the antibacterial characteristic of AMPs is the invasion of the bacterial membrane after contact is made, causing cell lysis and eventually cell death. Proposed mechanisms for the interaction that cause cell lysis are the barrel-stave model, the toroidal model (also known as worm-hole), and the carpet model. All are visually represented in Figure 2 (Giuliani et al. 2008). The product of all the models is the creation of a pore in the plasma membrane in the case of bacteria, or the cell membrane in the case of a mammalian cancer cell. In the barrel-stave model, the peptide selectively attaches to the membrane due to the attractive charges of the peptide (positive) and the membrane (negative). Multiple molecules of the peptide group together to create a channel, lined with peptide, within the membrane. The hydrophobic region of the peptide faces out, towards the hydrophobic regions of the lipid bilayer (Brogden 2005). Similarly, in the toroidal model, the peptide creates a pore, but the lipid bilayer curves around so that the hydrophilic heads are facing out where the lipid bilayer meets the hydrophobic side of the peptide (Brogden 2005). In the carpet model, the peptide forms a layer over the membrane and breaks the membrane down, causing cell
lysis (Brogden 2005). This is sometimes also referred to as the "detergent model" because the peptide breaks down the membrane like a detergent.

**Tachyplesin-1**

Tachyplesin-1 (TP-1) is a β-hairpin cationic antimicrobial peptide isolated from the hemocyte debris of the Japanese horseshoe crab, *Tachypleus tridentatus* (Nakamura et al. 1988). TP-1’s unaltered sequence is 17 amino acids in length (see Figure 3; Nakamura et al. 1988). Using the single character symbol for each amino acid, the sequence is as follows: KWCRFRVCGICYRRCR. The peptide has been found to exhibit antimicrobial properties. The increasing resistance of bacterial strains to antibiotics has caused an interest in antimicrobial peptides, such as TP-1, as a solution (Edwards et al. 2017).

TP-1 exhibits membrane disruptive activity in both gram-negative and gram-positive bacteria as well as fungi (Edwards et
Previous studies have shown that TP-1 caused rapid cell death to *E. coli* and *S. aureus*. In a study conducted by Hong *et al.* (2015), *E. coli* treated for 60 minutes with the peptide had a survival rate of 27%, while *S. aureus* being treated for 30 minutes had a 10% survival rate. The antimicrobial properties of TP-1 are substantiated by its efficiency in killing bacterial strains, but how exactly does this take place? In the same study, the peptide was dyed with fluorescein isothiocyanate (FITC) and visualized with laser confocal scanning microscopy to determine the process of TP-1. Hong *et al.* (2015) wrote the following summarizing the process of TP-1 causing cell death in *E. coli* and *S. aureus*:

> FITC-labeled tachyplesin I (10 μg/mL) penetrated the cell membrane and accumulated in the cytoplasm immediately after being added to the *E. coli* and *S. aureus* cell cultures. In *E. coli*, tachyplesin I later accumulated at the head of the cytoplasm and caused cell body transformation and also accumulated at a hole in the cytoplasm. In *S. aureus*, no cell membrane disruption was observed following treatment with 10 μg/mL of tachyplesin I, the same phenomenon was also observed following treatment with 20 g/mL of tachyplesin I. The mode of action of tachyplesin I on *E. coli* was determined to be dose-dependent. Membrane disruption was clearly visible in *E. coli* after treatment with 20 g/mL of tachyplesin I for 3 min and 40 g/mL in no time. These results indicate that the primary target of tachyplesin I is the cell membrane and cytoplasm of *E. coli* and *S. aureus* and that it has a different killing mechanism for each bacterium. (p. 71)

To determine which amino acid(s) were responsible for the activity, analogs of the sequence were tested in a separate study (Edwards *et al.* 2017). In each analog, each amino acid was replaced with alanine, one at a time, and additional analogs were made where a cysteine was replaced with alanine. The experiment tested a total of 32 analogs. All of the peptide analogs, despite their rearrangement, retained their cationic charge. By altering and rearranging the sequence of some analogs, Edwards *et al.* (2017)
found that they were able to increase and decrease the activity of the peptide analogs relative to TP-1. The analogs with the highest efficiencies were tested for toxicity against human embryonic kidney cells (HEK293) and human red blood cells (RBCs). Determining if these analogs were harmful to healthy cells—causing cell lysis—is a crucial step in determining whether the peptide could even be considered an option for human treatment methods. The experiment found that, while the antimicrobial activity was efficient, the hemolytic activity was often too high to be considered a viable treatment for humans, whereas when the concentration was decreased, there was little to no activity (Edwards et al. 2017).

As anticancer agents, peptides with anticancer activity also initially rely on membrane interactions to induce apoptosis (Chen et al. 2001). Chen et al. (2001) studied the effects of RGD-Tachyplesin, where amino acids are added at the N-terminus, on TSU prostate cancer cells. They found that the disruption of the mitochondria causes the activation of an apoptotic cascade. Overall, this study demonstrated the ability of RGD-Tachyplesin to inhibit tumor growth and damage the cell membranes of cancer cells to induce apoptosis. To explore further the abilities of TP-1, previous studies have focused on the effects on the cell cycle. A study conducted by Li et al. (2003) analyzed the effects of TP-1 on the regulation of the cell cycle in human heptocarcinoma cells (SMMC-7721). Their experiment demonstrated that TP-1 can arrest cells at the G0/G1 phase. P53 and p16 protein levels were also found to be significantly lower in the heptocarcinoma cells that had been treated with TP-1. The proteins p53 and p16 both interact with tumor suppressor pathways (Beauséjour et al. 2003). When these proteins are mutated, the affected cells are more susceptible to becoming cancerous (Figure 3).

**Cysteine Deleted Tachyplesin (CDT)**

Cysteine deleted tachyplesin (CDT) is the analog of TP-1 with the four cysteines completely removed from the sequence. In doing this, the peptide is still β-hairpin in shape despite elimination of the disulfide bonds (Ramamoorthy et al. 2006). CDT is 13 amino acids in length with the following sequence: KWFRVYRGKYRRR. The activity of CDT was studied by Saravanan et al. (2012), and the study concluded that the bacterial activity, like
TP-1, is due to the selectivity for the negatively charged lipids. CDT successfully neutralized the LPS and “disrupted the permeability barrier of the outer membrane” (Saravanan et al. 2012 p. 1613). CDT succeeds in other areas of efficiency where TP-1 was lacking as well. By running a hemolysis assay comparing TP-1 and CDT, it was concluded that CDT did not cause cell lysis in concentrations less than 200 μg/mL, while TP-1 caused 10% of hemolysis at concentrations of 100 μg/mL and 25% at concentrations of 150 μg/mL (Ramamoorthy et al. 2006). This makes CDT much more promising as a pharmaceutical, due to the improved therapeutic index.

As an anticancer agent, CDT was found to interact with heparan sulfate and chondroitin sulfate, anionic glycosaminoglycans found on the surface of the cancer cell membrane (Fadnes et al. 2009). Fadnes et al. (2009) found that lymphoma cells were less vulnerable to other cationic peptides (lactoferricin) if they expressed heparin sulfate on the cell surface. Their study concluded that, although some negatively charged molecules help with selectivity, molecular components such as heparan sulfate and chondroitin sulfate on the membrane of a cancer cell can inhibit peptide activity as well (Fadnes et al. 2009).
Wood et al. (2014) conducted a study of CDT and its analogs that analyzed the hemolytic activity as well as the antimicrobial effects of CDT with the removal of arginine residues from the C-terminus, to determine how many were necessary for activity. As arginine was systematically removed from the C-terminus, the activity against *E. coli* and *S. aureus* increased, but only CDT-desRR resulted in virtually no hemolysis. The removal of a third arginine residue caused a significant increase in hemolysis and a decrease in antimicrobial activity. CDT required 67 μM to cause less than 1% hemolysis where CDTdesRR required 650 μM to have the same hemolytic effect (Figure 4). These findings are important because they qualify CDT as a possible therapeutic treatment for cancer; the hemolysis must be practically non-existent to be considered safe. If CDT and its analogs had resulted in elevated levels of hemolysis, then they would be dangerous and potentially deadly to humans.

Evans *et al.* (2017) tested four separate peptides against adenocarcinoma human alveolar basal epithelial cell line (A549), *E. coli* and *S. aureus*; CDT, D-CDT (where all amino acids have the D-configuration), reverse CDT (where the amino acid sequence was “backwards” relative to CDT), and reverse D-CDT. Their findings showed that D-CDT was more selective for the negatively charged cells than the other peptide analogs. In addition, D-CDT was also showed the most activity with the least amount of hemolytic activity. D-CDT had an IC50 of 9.813 μM against A549 lung cancer cells, the most efficient of the analog they tested. D-CDT’s ability to exhibit cytotoxicity against the ad-

![Figure 4. Molecular structure of CDT (pepbank.mgh.harvard.edu)](pepbank.mgh.harvard.edu)
enocarcinoma cells substantiates its classification as a peptide that shows activity against microbial and cancer cells, but lacks activity against healthy mammalian cells.

The Hyaluronan Binding Sequence and Cancer

Hyaluronan (HA) was discovered in 1934 by Karl Meyer and John Palmer. One member of a bigger system of molecules, receptors, and synthases that allow cancer cells to interact with the environment around them (Simoni et al. 2002), HA is a glycosaminoglycan made up of repeating units of D-glucuronic acid and N-acetylglucosamine (see Figure 5 below; Necas et al. 2008). To survive, cancer cells must have the ability to adhere to the matrix (Delpech et al. 1997). Tumor cells contain HA, which induces cell growth, migration, and the ability to metastasize (Misra et al. 2015). HA is produced by enzymes called hyaluronan synthases and is degraded by enzymes called hyaluronidases (Kultii et al. 2012).

HA protects tumors from attacks from the immune system (Misra et al. 2015). Focusing on the mechanism and cell receptors that aid the relationship between HA and tumor growth could be the key to defining new treatment methods for cancer. By disrupting the protective environment of the tumor cell, the effectiveness of the anti-cancer properties of the CDT may be enhanced. Evans et al. (2017) tested and compared the efficiency of CDT analogues in the presence of hyaluronidase, which degrades HA and chondroitin sulfate. As discussed above, chondroitin sulfate inhibits cationic peptide activity (Fadnes et al. 2009). D-CDT lost its activity when

![Figure 5](image_url). The chemical structure of Hyaluronan (Necas et al. 2008).
the cells were treated with hyaluronidase before the peptide, suggesting that HA somehow plays a role in the anti-cancer mechanism of D-CDT. In all, the study ruled out apoptosis as a mechanism, unlike RGD-Tachyplesin used, but retained the dependence on the HA interaction as a possibility (Evans et al. 2017).

The CD44 receptor is characterized by its interaction with HA, along with other receptors such as LYVE-1, TLR-4, and RHAMM (Matthaiolampakis et al. 2015; Litwinuik et al. 2016; see Figure 6 below). The binding sequence B(X7)B is the HA binding sequence, which allows peptides to interact with receptor domains (Yang et al. 1994). B represents a basic amino acid, arginine or lysine, and X7 represents any other amino acid (Yang et al. 1994.). This binding sequence can be found within the sequence of CDT (RVYRGIVYRR). This is similar to the sequence that Wood et al. (2014) found to be effective against bacteria but did not induce hemolysis. The mechanism to kill cancer cells could therefore be increased, due to the binding sequence of HA found in CDT interacting with receptors found on the membrane of the cancer cell.

Looking forward, testing the shorted internal part of CDT, which only contains this binding sequence, against cancer cells in both the natural L-form and D-form may exhibit increased activity against A549 lung cancer cells. Shortening the sequence of the pep-

![Figure 6. Receptors that interact with Hyaluronan (Litwinuik et al. 2016.)](image-url)
tide makes it more deliverable, less immunogenic, and both easier and cheaper to synthesize. The D-form affords some protection from natural proteases, which only recognize L-amino acids, and gives it a longer half-life in addition to unique conformational properties.

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