Engineering the Rod of Asclepius – A Biochemical Investigation of Snake Venom Components and their Application as Potential Cancer Treatments

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Abstract

In the wild, venom is crucial to many snakes' success as predators. While antivenin research focuses on combatting venoms' abilities to disrupt physiological processes, new studies are attempting to manipulate these same abilities into anticancer therapies. Given the diversity of neurotoxins, hemotoxins, cytotoxins, and others, every new discovery and development within snake venom research adds to the knowledge base and broadens applicational opportunities. Cancer-related venom research isolates various components, manipulates their interaction with target cancer cell lines, and evaluates how their natural biochemical activity counteracts mechanisms that are integral to tumor development. Several more promising components, namely disintegrins, lectins, oxidases, and phospholipases, have emerged. Summarizing and highlighting recent research of these key components can serve as a springboard for future venom-derived antitumor medicines.

Keywords: venom, cancer, apoptosis, integrin, angiogenesis, disintegrin, L-amino oxidase, lectin, phospholipase

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Differing from the Hermes' caduceus in that it features a single serpent - rather than two - entwined about a staff, the Rod of Asclepius is an ancient symbol of medicine. Asclepius (Figure 1), born of Apollo and one of the four gods to whom the Hippocratic Oath is dedicated, is the patron Greek god of medicine and healing (Shetty, Shetty, & Dsouza, 2014). Though snakes and mankind have had complicated interactions since the Fall, the idea of the serpent around a staff has been symbolic of medicine for over 2400 years, dating back to the ancient Egyptians and Greeks and leading into the Renaissance period (Antoniou, Antoniou, Learney, Granderath, & Antoniou, 2011). There are also biblical grounds for the symbol, stemming from Hebraic tradition and integrating into the Christian faith. At its earliest reference in the book of Numbers, the Israelites complained against God and were plagued by venomous snakes, but God showed grace by commanding Moses to make a bronze serpent and raise it up on a staff. Those who were bitten could look upon the bronze serpent and be healed (Numbers 21: 4-8, New International Version). The biblical foundation for snake-staff symbol is continued as Jesus declares, "Just as Moses lifted up the snake in the wilderness, so the Son of Man must be lifted up, that everyone who believes may have eternal life in him" (John 3:14-15). The symbol continues to represent

Figure 1. Statue of Asclepius, God of Medicine, with his staff. From *Asclepius*, Britannica ImageQuest, https://quest.eb.com/search/asclepius/1/300_169696/Asclepius/more. Copyright by Universal Images Group.

medicine and healing today, such as in the logos of ambulances, hospitals, and the World Health Organization (Figure 2) (Shetty et al., 2014).

Figure 2. Official WHO logo (Global Cancer Observatory, 2013).

The literal realization of the Rod of Asclepius (using snake venom to produce medicine) has been part of scientific history for centuries, and the idea flourishes today. The development of medicine from animal venoms and toxins began with folk medicine, but some of modern medicine's recent toxin-derived drugs are antivenins. Due to the pressure of an increasingly health conscious world, drug developers have sought out natural resources for anticancer drugs. The biodiversity of animal venoms and toxins offers a wide range of structural templates for therapeutic agents, and certain venoms from snakes have active components that show anticancer activity (Gomes et al., 2010).

Pertinent Biochemical Characteristics of Cancer

Cancer is a chronic degenerative disease whose chief characteristic is uncontrolled cell proliferation, causing formation of tumors and possible metastasis (Calderon et al., 2014). Whether by personal suffering or that of a loved one or friend, cancer has affected most individuals around the globe. It is the second leading cause of death in modern countries, and GLOBOCAN (2013) reports indicate that 14.1 million new cases of cancer and 8.2 million cancer-related deaths occurred worldwide in 2012 alone. Pancreatic, lung, breast, ovarian, and hepatic cancers are some of the most common types of cancer today (Torre et al., 2015). In today's society, increasingly prominent risk factors such as obesity and pollution in both modern and third-world countries alike raise the worldwide morbidity rate each year (Global Cancer Observatory, 2013). As progressing research scrambles to combat the mounting cancer cases and find a

cure, comparative studies of normal and cancerous biology have revealed three cancer characteristics that can be specifically targeted by snake venom components: integrin malfunction, apoptosis inhibition, and angiogenesis.

Integrins: Cell Growth, Progression, and Metastasis

One of the most important aspects of cancer pathology is tumor cell development. A family of proteins called integrins are the key determinants in cell growth and progression. The molecular basis of tumor ontogenesis involves (dis)functional mutations of integrins, leading to adverse effects that trickle down into the processes they control: regulation of cell shape, cell survival rate, DNA transcription, and more (Figure 3) (Arruda Macedo, Fox, & de Souza Castro, 2015). Especially significant cancer-related processes coordinated by various types of integrins include cellular migration and invasion (Arruda Macedo et al., 2015). Malignant tumors are also characterized by their anchorage-independent growth and metastasis formation (Eble & Haier, 2006). They require cell-to-cell or cellular matrix attachment before they can proceed through the growth cycle (Eble & Haier, 2006). Integrins can mask themselves as oncogenes and tumor suppressor genes before transforming biologically healthy cells into tumor cells (Eble & Haier, 2006).

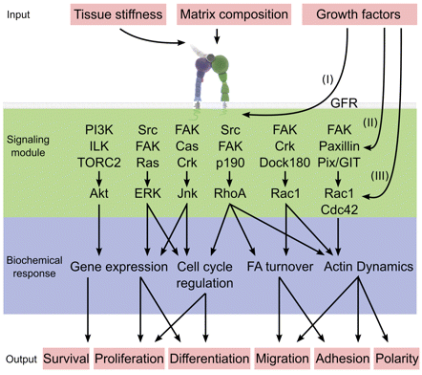

Figure 3. Downstream cascade after integrin activation (Legate, Wickström, and Fässler, 2009).

According to Huveneers, Truong, and Danen (2007), some types of integrins are membrane receptors that mediate adhesion of cells to other cells and within the cellular matrix. Properly functioning integrins maintain an appropriate level of cell adhesion and migration as they respond to growth factors, cytokines, and other genetically modulated signals for cell progression (Figure 4) (Huveneers et al., 2007). These regulatory functions of transmembrane integrins are the basis of their important roles in cell survival, proliferation, and differentiation (Huveneers et al., 2007). Comprised of natural integrin antagonists, many snake venom components such as disintegrins, peptides, and antibodies combat this aspect of cancerous growth (Huveneers et al., 2007).

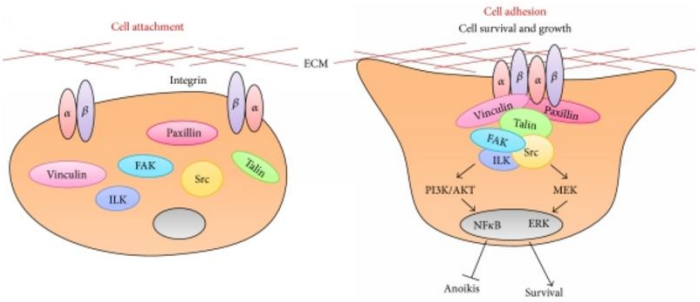

Figure 4. Integrin-mediated cell adhesion (Lee, Choi, Cha, & Huang, 2015).

Apoptosis: Programmed Cell Death

In normally functioning tissues, apoptosis is induced cell death that maintains cellular health and activity (Figure 5). After DNase is cleaved between its nucleosome links, apoptosis involves rapid phagocytosis and digestion of specified cells (Kerr, Winterford, & Harmon, 1994). It is suppressed at the genetic and ribosomal levels by RNases and protein synthesis inhibitors. Malignant tumors are characterized by their oncogenic ability to spontaneously cause

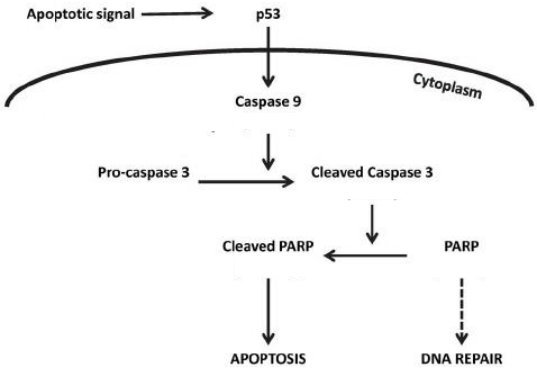

Figure 5. Mechanism of apoptosis: p53 and caspase activity (Malhotra, Zaidi, Kosovec, & Kasi, 2013).

apoptosis in normal cells or in cells suffering from radiation or chemotherapy (Kerr et al., 1994). Cancerous cells are also successful at suppressing their own apoptosis; cancer treatment can thus be rendered ineffective if a tumor induces and suppresses apoptosis effectively (Kerr et al., 1994).

Implications of apoptosis suppression include the effective lengthening of a cell's lifespan, allowing the cell to expand without dividing and aggregate with other similarly enlarged cells. At a genetic level, transcribed and synthesized growth factors and apoptosis-inducing checkpoint regulators can be destroyed or ignored when apoptosis is suppressed (Reed, 1999). If a malignant cell progresses and expands unchecked, it can accumulate genetic mutations that foster its ability to survive with less oxygen/nutrients, overcome natural immune responses, and resist anticancer drugs (Reed, 1999). Current cancer therapies focus on de-suppression and even upregulation of apoptosis in tumors, and cytotoxic components of snake venoms have mechanisms which are naturally efficient at this task.

Angiogenesis and CPA: Neovascularization and Procoagulation

In normal cells, new blood vessels are formed due to a process called angiogenesis. During their initial formation, tumors are able to survive by "sharing" the nutrients supplied by normal blood vessels. When their abnormally accelerated growth outpaces the regular supply of nutrients, they are able to create their own mini vascular system (Figure 6), creating an

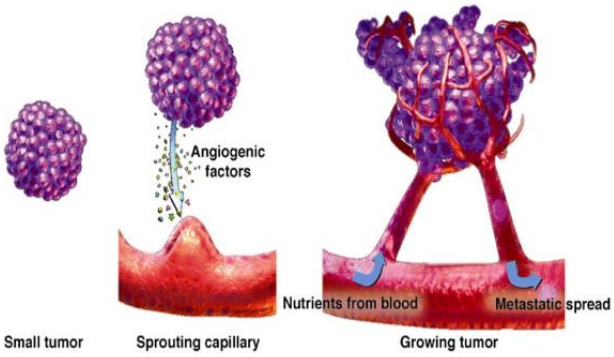

Figure 6. Tumor angiogenesis (Siemann, 2002).

intratumor network of constituent blood vessels (Ziyad & Iruela-Arispe, 2011). Research by Yeh, Peng, Tang, and Huang (2001) demonstrated that the mechanism of angiogenesis involves appropriately regulated reproduction, migration, and differentiation of endothelial cells in the cardiovascular system. Tumors combine highly interactive cell species, including fibroblasts and endothelial cells (Yeh et al., 2001). Such processes are controlled by angiogenic factors and extracellular integrins, but tumorigenic mutations of these proteins aid in unregulated angiogenesis, allowing tumor cells to develop their own blood vessels by which to siphon extra nutrients, growth factors, etc. (Yeh et al., 2001).

In addition to creating their own vascular network, tumor cells possess procoagulant factors that cause thrombosis, a key complication that further decreases a cancer patient's survival rate while ensuring tumor growth (Rickles & Falanga, 2001). Procoagulant mutations of cancerous oncogenes interfere with the normal processes of platelet interaction, fibrinolysis (prevention of clots), and regulation of vascular endothelium (Rickles & Falanga, 2001). Cancer procoagulant A (CPA) is a protease which deposits fibrin, a protein which impedes blood flow, and activating factor X, which has an important role in vascular thrombosis and initiation of coagulation (Gordon, Franks, & Lewis, 1975). Angiogenesis at an upregulated level allows nutrient-receptive tumors to survive, grow, and metastasize. Procoagulation of normal blood vessels simultaneously inhibits the survival of healthy tissues (Swenson, Ramu, & Markland, 2007).

Snake Venoms as Potential Cancer Therapies

While research on many types of animal venoms has yielded successful anticancer results, snake venoms have an increased natural ability to cause large amounts

of tissue damage, and as such they show the most potential for imminent development into clinically-applicable cancer therapies. Researchers such as Calderon and his team (2014) have successfully demonstrated anti-expansion and anti-aggregation factors in snake venoms by using *in vivo* mouse tumors. Both crude venom and its isolated components have shown antitumor mechanisms that are promising for clinical trials (Calderon et al., 2014). In demonstrating their ability to disrupt cell adhesion and aggregation, induce apoptosis, prevent angiogenesis, and otherwise return cancer biology to a normal, healthy state, such enzymes as disintegrins, L-amino acid oxidases (LAAOs), lectins, and phospholipases A_2 (PLA_2s) have emerged as significant anticancer venom components (Calderon et al., 2014).

Disintegrins

Disintegrins are among the most wide-ranging and broadly applicable components of snake venoms. Walsh and Marcinkiewicz (2011) explained how snake disintegrins have been classified into three functional families: RGD (Arg-Gly-Asp), MLD (Met-Leu-Asp), and KTS (Lys-Thr-Ser). The letter motifs represent specific amino acid sequences in the structure of each disintegrins (Walsh & Marcinkiewicz, 2011). Because various amino acids exhibit differing molecular charges and properties, the unique peptide structure of each disintegrin makes it specifically fine-tuned to target its respective integrin-dependent processes (Walsh & Marcinkiewicz, 2011).

Derivation and Isolation

All venom disintegrins are found in five snake families: Viperidae, Crotalidae, Atractaspididae, Elapidae, and Colubridae (Arruda Macedo et al., 2015). RGD-disintegrins are the most heavily researched and largest family, with derivations from all

five snake families (Walsh & Marcinkiewicz, 2011). Disintegrins that fall under the RGD category display the most variety in structure, function, and integrin targets; the Arg-Gly-Asp hairpin sequence is the only predominating factor that unifies them (Walsh & Marcinkiewicz, 2011). MLD-disintegrins are found exclusively in Viperidae, and their structures enable them to inhibit leukocyte integrins and improper adhesion of the cells they regulate (Walsh & Marcinkiewicz, 2011). They make up the most abundant disintegrin family, with the most investigated of MLD-disintegrins being from *Vipera lebetina* (Walsh & Marcinkiewicz, 2011). The first KTS-disintegrin was discovered by reverse phase chromatography of *Vipera lebetina obtusa* venom, and it was named obtustatin (Walsh & Marcinkiewicz, 2011). KTS-disintegrins are among the most selective, targeting a much smaller range of integrins with great specificity (Walsh & Marcinkiewicz, 2011). As proteins with low molecular weights, disintegrins are isolated by chromatography, as in Sánchez' research (2006). It is their unique charge, molecular size, and other properties that determine which method of chromatography is most efficient: reverse phase, size exclusion, or anion exchange. Once the proteins have been purified, they are categorized according to mass spectrometry and experimental proteolytic activity (Sánchez et al., 2006).

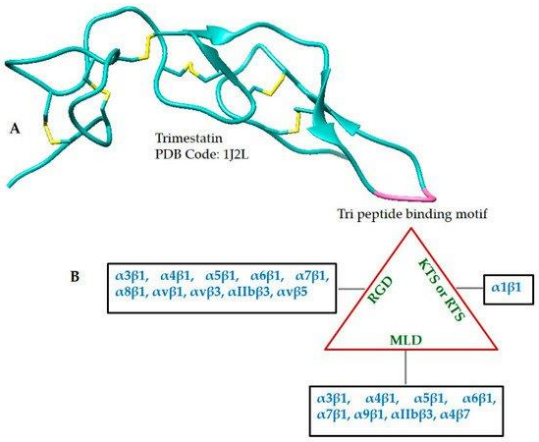

Figure 7. Deciphering disintegrins: motifs within the hairpin turn (pink) and their corresponding targets (Munawar, Ali, Akrem, & Betzel, 2018).

Biochemical Activity as Tumor Antagonists

The three disintegrin classes interact with their own sets of integrins. This specific pairing of inhibitor and target allows scientists to choose the disintegrin most suited to combat tumorigenicity in one specific area or cancer type. As an example of disintegrin-integrin pairing, RGD-disintegrins block the activity of integrins α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, αvβ1, αvβ3, αIIbβ3, and αvβ5 (Calderon et al., 2014). Contrary to their monomeric counterparts, MLD-motif disintegrins are heterodimeric, binding and inhibiting integrins α3β1, α4β1, α5β1, α6β1, α7β1, α9β1, αIIbβ3, and α4β7 (Calderon et al., 2014). As is expected of the most specific disintegrin class, only integrin α1β1 is affected by KTS-disintegrins (Calderon et al., 2014). Despite their specific targets, all disintegrins use the same general mechanisms to combat tumorigenicity.

One of the most central means by which disintegrins prevent cancer progression is by induced cell detachment. Disintegrins work in the extracellular matrix (ECM), and tumor cells that cannot adhere to the host cell network suffer severely limited nutrient supply and space for growth (Calderon et al., 2014). Because integrins also regulate cell checkpoint receptors and chemicals, such as cytokines, inhibition of their function renders the affected cell unviable. Unviable cancer cells are then phagocytized and disposed. Another mechanism by which disintegrins halt tumors is by downregulating proliferation of infected cells (Calderon et al., 2014). Without a means to grow and reproduce, the metastasis and progression of tumors is

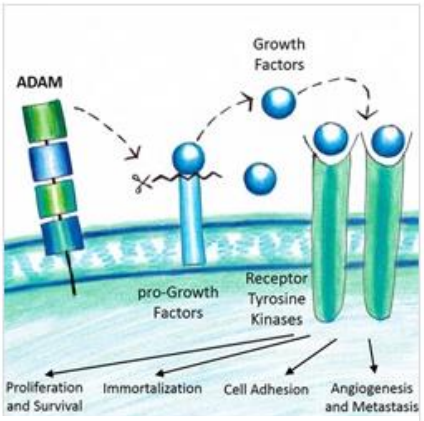

Figure 8. Disintegrin target: anti-ADAM disintegrins would halt all processes regulated by ADAM, including proliferation, metastasis, etc. (Horiuchi et al., 2003).

stunted. Lastly, disintegrins especially high in disulfide bridges can be manipulated to target the growth factors of cancerous vascular endothelium, stopping angiogenesis and severing shunted vascular networks (Swenson et al., 2007). If disintegrins can simultaneously cut tumor cells off from their manufactured supply artery and prevent adhesion (and therefore nutrient uptake) to healthy cells, the cancer cells will have no means by which to grow, expand, invade, and migrate throughout the body.

Current Experimental Trials

Recent experiments and research have focused on various disintegrins' abilities to successfully target and mitigate abnormal integrin function across several types of cancer. Scientists successfully observed and recorded one disintegrin, *rhodostomin*, as it inhibited basic fibroblast growth factor (bFGF), a signaling molecule necessary for angiogenesis in rodent melanoma (Yeh et al., 2001). Yeh's research team (2001) injected mice with a subcutaneous tumor and then treated them with *rhodostomin*. The disintegrin successfully prolonged survival by inhibiting neovascularization (angiogenesis) and by blocking tumor cell migration and invasion of normal cells (Yeh et al., 2001). Research by Lucena et al. (2015) demonstrated that the disintegrins *mojastin* and *viridistatin* efficiently inhibited tumor metastasis in human pancreatic carcinoma; rates of successful inhibition increased with correlating dose increases (2015). The two recombinant disintegrins also downregulated carcinoma proliferation while inducing its apoptosis, a result of significance because pancreatic cancer is often immune to chemotherapy methods (Lucena et al., 2015).

A disintegrin called *contortrostatin* (CN) has been through numerous trials and has successfully prevented tumorigenicity of many different types of cancer, attracting

notable interest in initial disintegrin cancer research (Golubkov, Hawes, & Markland, 2003). Derived from *Agkistrodon contortrix* (a southern copperhead), CN has successfully inhibited migration and invasion of cancerous human umbilical vein cells (HUVEC) in research by Golubkov (2003) and others. *Contortrostatin* disrupted cancer progression in HUVEC by four mechanisms: blockading integrin-dependent cytoskeleton actin filaments (internal cell structures), altering intercellular cadherin (allows cells to attach to one another), downregulating focal adhesion kinase (manufactures adherins), and inhibiting tyrosine phosphorylation (an important signal transduction regulator) (Golubkov et al., 2003). Beyond its success against malignant HUVEC, CN also inhibited angiogenesis in cancer-inoculated mice (Golubkov, Hawes, & Markland, 2003). Markland and his associates performed experiments in 2001 which observed CN hindering human ovarian adenocarcinoma. By blocking adhesion to extracellular proteins and creating a fake basement membrane through which invasive cells could not penetrate, CN prevented aggregation and migration of an ovarian cancer tumor *in vitro* (Markland et al., 2001). Studies with melanoma (skin cancer) indicate that CN uses similar mechanisms to prevent its metastasis: melanoma cells are blocked from adhering to extracellular proteins, more specifically collagen, fibronectin, and vitronectin (Trikha, De Clerck, & Markland, 1994). It is important to note that inhibitory effects are enhanced with increased experimental doses of CN, without harm to surrounding normal cells (Trikha et al., 1994).

In an experiment involving the inoculation of mice with metastatic human mammary cancer, Swenson et al. found that unlike ovarian, skin, and HUVEC cancers, breast cancer did not respond well to clinical trials of intratumor CN injections (2004).

While intratumor injections of CN were met with adverse effects, the researchers observed that a liposome-CN recombinant administered intravenously induced antiangiogenic activity successfully (2004). Researchers suggest that because of these results, LCN could serve as the more advantageous alternative to intratumor CN in all cases for the following reasons: LCN circulates more effectively and conservatively through the bloodstream, LCN is passively taken up into the tumor (rather than forced injection), LCN causes no platelet immune response, and LCN is not tagged as a foreign substance by the immune system in other parts of the body (Swenson et al., 2004).

Relevant Drugs on the Market

Clinical drugs have yet to be produced from pure isolations of snake venom disintegrins, but researchers have developed several peptidomimetics which mimic the structure and function of disintegrins, two of which are readily available in clinical sectors. Tirofiban (marketed by Aggrastat) mirrors the Arg and Asp interaction of RGD-disintegrins, allowing it to bind to αIIbβ3 and αvβ3 (Arruda Macedo et al., 2015). Tirofiban combats the procoagulant activity of GPIIb-IIIa, instead promoting platelet adhesion and vascular cell health (Arruda Macedo et al., 2015). As an FDA-approved anticoagulant, it is currently used as a clinical treatment of myocardial infarction (heart attack) and refractory ischemia (chest pain due to inadequate blood supply to the cardiac muscles) (Arruda Macedo et al., 2015). Eptifibatide markets Integrilin, which was founded upon screenings of sixty-two different snake venoms (Arruda Macedo et al., 2015). Barbourin is the main foundation for Integrilin, which mimics the function of the KGD-disintegrin of *Sistrurus miliarus barbouri* and binds exclusively to αIIb3 integrin (Arruda Macedo et al., 2015). Integrilin is FDA-approved for treatment of acute coronary

syndromes (Arruda Macedo et al., 2015). Though current disintegrin-based drugs on the market are not clinically used as cancer therapies, each successful trial and manipulation of the biochemistry of disintegrin is one step closer to its clinical use to combat tumorigenicity.

Future Research: Opportunities and Limitations

Future research of disintegrins as potential cancer therapies is met with both opportunities and road blocks. Cancer operates by complex mechanisms that are consistently changing with environmental factors, biological mutations, and population dynamics. This every-changing complexity therefore presents the challenge of discovering and isolating the exact function for any given integrin and its disintegrin counterpart. While this is a daunting task, the same complexity allows for specificity in attacks on cancer. Unlike chemotherapy or radiation, which produce widespread damage to cancerous and healthy cells alike, development of therapies based in integrin-disintegrin pairs will target tumor cells with fewer adverse effects to healthy cells (Arruda Macedo et al., 2015).

Another problem that hinders the progress of disintegrin-based cancer treatments is the limited supply of natural resources and the instability and immunogenicity of chemically engineered mimics (Kim et al., 2003). As with any natural resource, snake venom is in limited supply, and ethics of animal care make harvesting snake venom and experimental trails increasingly difficult. On the other hand, synthetic inventions that mirror the structures and functions of natural disintegrins are still unstable and can provoke immune responses in healthy cells (allergies) (Kim et al., 2003). This is the case with many peptide drugs, but scientists such as Kim et al. are experimenting with the

fusion of disintegrin-coding genes into cationic liposomes (2003). This process circumvents immune responses due to a peptide foundation, and the resulting genetically-modified liposomes can introduce and express disintegrin genes once they are injected into a tumor (Kim et al., 2003).

As a last resort, researchers are isolating and manipulating antibodies that have very similar functions to disintegrins, but trials thus far have proven that disintegrins are a better alternative to antibodies (Leong-Poi, Christiansen, Klibanov, Kaul, & Lindner, 2003). Disintegrins decay at faster rates, a desirable characteristic in that after injection and action, disintegrins will not remain in the body indefinitely (with unknown effects) (Leong-Poi et al., 2003). A second advantage of disintegrins is their controllability. At any point in time, after the disintegrin has been injected and has inhibited its target, it can be inhibited itself in case upregulation of integrins is abruptly necessary (Leong-Poi et al., 2003). Lastly, disintegrins are both cheaper and are more readily available than antibodies (Leong-Poi et al., 2003).

L-Amino Acid Oxidases

Dimeric flavoenzymes called L-amino acid oxidases exist within many diverse biological kingdoms such as fungi, bacteria, algae, and snakes (Calderon et al., 2014). From a biochemical standpoint, LAAOs form alpha-keto acids, hydrogen peroxide, and ammonia by deaminating L-amino acids in aerobic conditions (Calderon et al., 2014). Because these chemical byproducts can be harmful depending on where they are deposited, LAAOs are often classified by their location-dependent function (Calderon et al., 2014). Some categories of LAAOs are organized by their substrate preference (must

be aerobic), cytotoxicity, induction of apoptosis, platelet effects, or bactericidal mechanisms (Calderon et al., 2014).

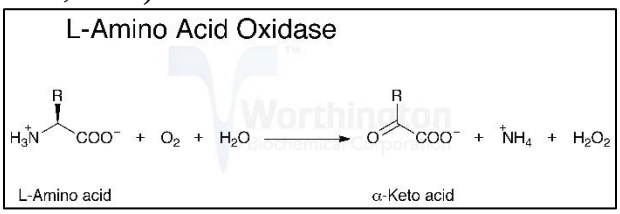

Figure 9. Biochemical reaction of LAOO to alpha-keto acid, hydrogen peroxide, and ammonia (Worthington Biochemical Corporation, 2019).

Derivation and Isolation

Snake venom L-amino acid oxidases (svLAAOs) can be isolated from all snake families, but Viperidae, Crotalidae, and Elapidae venoms are richest in LAAOs, which interestingly give venoms their characteristic yellow color (Guo, Liu, Yao, Zhang, & Sun, 2012). As in research by Toyama's team (2006), most svLAAOs are isolated by molecular exclusion and ion exchange chromatography. Purification and identification are accomplished by reverse phase chromatography, gel electrophoresis, and spectrophotometric analysis of amydolitic activity (ability to cleave amide groups) (Toyama et al., 2006).

Biochemical Activity as Tumor Antagonists

In their natural application, LAAOs are extremely cytotoxic, first and foremost causing necrosis (Calderon et al., 2014). There are a few mechanisms by which varying svLAAOs induce apoptosis. At initial exposure, LAAOs cause hypotension (low blood pressure) and upregulate the production of hydrogen peroxide in the cell (Calderon et al., 2014). Hydrogen peroxide breaks down cell walls and membranes, causing cell lysis and death. svLAAOs that are manipulated and paired with targeting nanoparticles can therefore be injected into tumor cells to lyse their membranes, with negligible damage to

surrounding normal cells (Calderon et al., 2014). In addition to the production of excess hydrogen peroxide, LAAOs can interfere with intra- and intercellular membrane receptors, stunting cell communication (Costa, Burin, Menaldo, de Castro, & Sampaio, 2014). This communication breach can cause an immune response to tag the affected cell as foreign and malfunctional, inducing apoptosis (Costa et al., 2014). Induction of apoptosis can also stem from premature activation of caspases 3 and 9, which are parts of the mitochondrial pathway of anti-apoptotic and death receptors. As oxidases, these flavoenzymes produce reactive oxygen species that activate caspases (Guo et al., 2012). Once the caspases are activated, cytochrome c is released, apoptosis-inducing ligands are synthesized, and vital proteins are cleaved, leading to cell death (Guo et al., 2012).

Though their main function is to upregulate cellular apoptosis, LAAOs also have platelet aggregation and cell cycle arrest capabilities. Experimental results have been lacking in consistency and agreement, and svLAAOs have been observed to both induce platelet aggregation and inhibit it, depending on LAAO isoforms, cellular location, and types of platelets involved (mouse vs human, washed vs platelet rich plasma) (Sakurai et al., 2001). Induction of aggregation can theoretically be used to target tumors' neovascular systems to stop the flow of nutrients and oxygen to cancerous cells. On the other hand, aggregation-inhibiting isoforms can be injected into cells suffering from cancer-related procoagulant effects, therefore reducing thrombosis-related cancer complications (Sakurai et al., 2001). Concerning the arrest of cell growth, it has been observed that svLAAOs interfere with thymidine uptake into the cell (Ahn, Lee, & Kim, 1997). Thymidine is an important component in DNA and interrupting its incorporation into the cell genome can halt cell progression through the S phase of growth,

discontinuing progress to cell growth and proliferation (Ahn et al., 1997). This prevents tumor proliferation and expansion.

Current Experimental Trials

As with disintegrins, different svLAAO variants produce antitumor results consistent with their isoforms, molecular weights, peptide structures, and other defining properties (Mukherjee, Saviola, Burns, & Mackessy, 2015). In research by Mukherjee et al., one LAOO called *rusvinoxidase* (harvested from the venom of *Daboia russelli russelli*) successfully induced apoptosis in breast cancer cells by corrupting cell morphology, activating DNases, destabilizing membrane proteins, and initiating cell shrinkage (2015). Even after freezing and thawing, *rusvinoxidase* activated caspase-8 and the release of cytochrome c to activate caspase-9, while simultaneously downregulating anti-apoptotic proteins Bcl-XL (Mukherjee et al., 2015). Activity of *rusvinoxidase* is both time and dose-dependent, but trials indicate that up to 4 mg/kg is non-toxic to normal mouse cells, encouraging prospective tests on cancer-affected human cells (Mukherjee et al., 2015).

Venom extraction from another snake, *Agkistrodon acutus*, yielded another svLAAO: ACTX-8 (Zhang & Wei, 2007). ACTX-8 induced apoptosis of Hela cervical cancer cells by DNA fragmentation and caspase induction (Zhang & Wei, 2007). According to Zhang and Wei, one unique aspect of ACTX-8 is its ability to cause anti-apoptotic proteins to move from the cellular matrix to the mitochondria, where they are bound to dissociating enzymes and broken down (2007). The production of reactive oxygen species interferes with antioxidant-sensitive mechanisms in the mitochondria, further fostering cell failure and death (Zhang & Wei, 2007). The specific method of

enzyme translocation is still unknown, but further research may introduce anti-apoptotic inhibition as a new mechanism of cancer therapy where traditional mechanisms are unsuccessful.

A third cancer proven to suffer apoptosis induction in the presence of svLAAOs is prostate cancer. In research by Tan et al., *Crotalus mitchellii pyrrhus* venom was extracted, and Cmp-LAAO was isolated by chromatography (2017). Prostate cancer cells (LNCaP) suffered premature apoptosis and oxidative stress after injection of appropriate concentrations of Cmp-LAAO (Tan, Ler, Gunaratne, Bay, & Ponnampalam, 2017). Cmp-LAAO targeted activation of caspase-3 and 9, while production of hydrogen peroxide was a secondary yet harmful effect (Tan et al., 2017). Cmp-LAAO showed preferential specificity for cancer cells but did produce cytotoxic effects in normal cells at certain concentrations, so more research and trials must be developed before it is an adequate and safe prostate cancer therapy (Tan et al., 2017).

Future Research: Opportunities and Limitations

Though no drugs based on svLAAOs have been developed, their anti-tumor effects and abilities have not gone unnoticed. As studies within the past decade have yielded successful evidence of svLAAOs in combatting cancer, related research has earned respect and increased attention. Caspase activation, thymine blockage, membrane receptor instability, and cell membrane breakdown are some mechanisms of apoptosis induction which are unique to svLAAOs. Recent progresses have revealed that svLAAOs act preferentially against cancer cells before attacking mammalian cells in the same region (Guo et al., 2012). This should encourage research that helps streamline the targeting process, in order to eliminate any negative effects svLAAOs might induce in

healthy cells. At the same time, such efficient mechanisms that show marked preference for cancer cells over normal cells may cement the role of svLAAOs as foundational for synthetic anti-tumor mimic drugs and therapies. One disadvantage of svLAAOs is their high molecular masses, which makes them stand out as unusually large or dense glycoproteins and creates issues with immunogenicity and allergic reactions to derivative cancer treatments (Guo et al., 2012).

Lectins

Lectins are (glyco)protein subunits with carbohydrate binding sites. Though they can be found in several animal and vegetable species, snake lectins (snaclecs) have been proven to inhibit tumorigenicity in a few different cancer types (Calderon et al., 2014). Their key importance to cancer research involves their interaction with a tumor cell's surface (Calderon et al., 2014). Lectins are unique venom-derived cancer antagonists because their dimeric tertiary units can bind together and aggregate into quaternary structures stabilized by disulfide bonds (Doley & Kini, 2009). Lectins bind by two mechanisms, the first being a lock-and-key model where their subunits fit exactly with specific carbohydrates (Lu, Navdaev, Clemetson, & Clemetson, 2005). This specificity allows for the manipulation of lectins for tumor cell identification based on the carbohydrates, proteins, and other molecules on cell surfaces (Lu et al., 2005). Carbohydrates located on the cell membrane are especially important in cell-to-cell interactions. Thus, lectin-carb interactions heavily influence how abnormal cells are

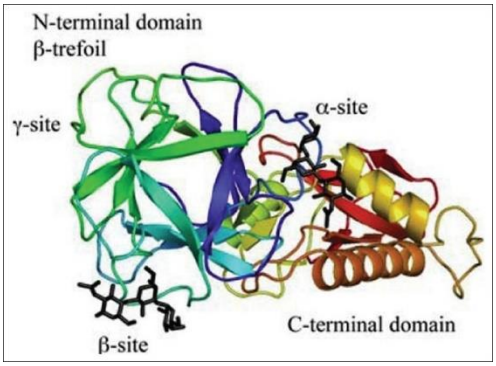

Figure 10. Lectin folding structure provides three specific binding sites: α, β, and γ (Kapoor, Vaidya, Kaur, & Jain, 2014).

tagged and their extracellular binding is regulated (Lu et al., 2005). Lectins also bind via electrostatic interactions in which the charges and properties of amino acids in their primary peptide structures determine binding or repulsion (Lu et al., 2005). With slight genetic mutations or synthetic manipulation, lectins can undergo structural changes which allow multiple binding sites or selectivity for a greater range of cell proteins/receptors (Lu et al., 2005).

Derivation and Isolation

Snaclecs typically fall under two classes: C-type lectins (CTLs) and C-type lectin related proteins (CLRPs). They are found in all snake families, and they comprise a majority of venoms within the Viperidae and Colubridae families (Doley & Kini, 2009). As with other components of snake venom, lectins are purified from crude venom by chromatography, specifically affinity and get filtration chromatography (Nunes et al., 2011). Fluorescence emission spectra are used to determine the tryptophan peptide residues present in the various lectins, while circular dichromism reveals their secondary structures (α-helix or β-sheet) (Nunes et al., 2011).

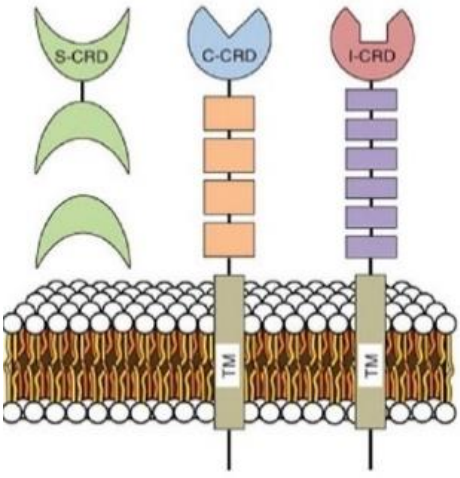

Figure 11. Lectin shape determines lock-and-key fit (Kapoor et al., 2014).

Biochemical Activity as Tumor Antagonists

Lectins exhibit antitumor activity in a few ways. Like disintegrins, some lectins from specific snake families block the progression of integrin-dependent growth processes, but lectin also works uniquely against the actin dis(assembly) and therefore halts cell proliferation (Calderon et al., 2014). Other lectins prevent adhesion within the extracellular matrix, fighting tumor cells for intercellular binding sites and immobilizing weakly adhered cancer cell lines (Calderon et al., 2014). Activity specific to C-type lectins includes induction of red blood cell agglutination and platelet aggregation (Doley & Kini, 2009). CLRPs differ from CLTS in that they cannot bind to carbohydrates, but instead have only hemostatic mechanisms (Doley & Kini, 2009). As do disintegrins and LAAOs, CLRPs vary in their structure and their consequential functions, varying from platelet aggregation/activation to anti- or procoagulant factor binding (Doley & Kini, 2009). Many CLRPs disturb the coagulation process by targeting and inactivating thrombin, diminishing clot-related cancer complications (Doley & Kini, 2009).

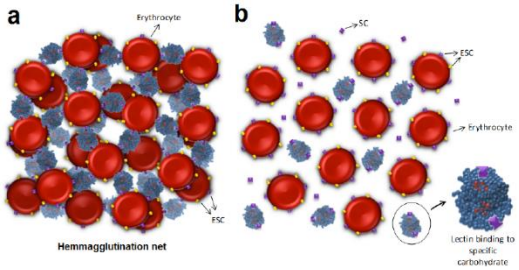

Figure 12. Different lectins, based on genetic function and carbohydrate specificity, can induce or inhibit coagulators (Santos et al., 2013).

Current Experimental Trials

Lectins reduce cancer patients' cardiovascular complications by their variety of coagulant activity, whether excitatory or inhibitory, but their hindrance of cell proliferation is also important in cancerous cells themselves. Experimental trials with BJcuL from *Bothrops jararacussu* have supported this lectin's inhibitory effect on human renal and pancreatic cancer cell lines (de Carvalho, Schmitmeier, Novello, & Markland,

2001). BJcuL also binds to intercellular receptors on the membranes of human ovarian and breast cancer cells, effectively detaching the cell from the extracellular matrix as BJcuL concentration was raised (Doley & Kini, 2009). Detaching cancer cells cuts off pathways of communication and nutrient uptake, inhibiting growth of tumor cells and endothelial cells (needed for angiogenesis) (Doley & Kini, 2009). Other studies and manipulations of BJcuL have yielded positive agglutination results: the lectin binds to lactose moieties and causes erythrocytes to agglutinate (de Carvalho et al., 2001). By decreasing cell adhesion and proliferation and increasing agglutination, BJcuL decreases the viability of tumor cells in tested human and bovine cancer lines.

Botrocetin and *bitiscetin* are two well-studied CTLs which bind to GPIb receptors on platelets and cause agglutination. By recreating crystallography pictures of the two lectins, Lu et al. and other research teams have determined that specific binding of lectin subunits to their matching GPIb domains enhances lectin-receptor binding efficiency, which is significantly more advantageous than forcing conformational changes as do some enzyme-substrate complexes (2005).

Komori et al. studied a unique lectin called A lectin, from *Agkistrodon piscivorus piscivorus* (the water moccasin), which belongs to the C-type lectin related protein family (1999). The disulfide-linked monomers allow this lectin to agglutinate erythrocytes in human ad rabbit cancer cells (Komori, Nikai, Tohkai, & Sugihara, 1999).

Future Research: Opportunities and Limitations

Present studies have established the role of snaclecs in preventing tumor cell proliferation and varying platelet aggregation. While these roles and effects have been observed and replicated, the mechanisms by which lectins accomplish their functions

remain unknown. Lectins are one of the lesser-studied snake venom components, so more studies are needed before research can confirm their apoptotic, anti-angiogenic, anti-proliferation, and cardiovascular effects in different cancer types and different environmental conditions (de Carvalho et al., 2001).

Carbohydrate recognition is the basis for the targeting efficiency seen in lectin-based trials. As mentioned earlier, structural changes of lectins can affect how they interact with receptors, integrins, and other cell features important to cancer biology. Studies today focus on establishing correct models of lectin varieties and understanding the carbohydrate-lectin pairing mechanisms (Nunes et al., 2012). Once these aspects are better understood, cancer therapies which use lectins can streamline the efficiency of the lectins' electrochemical/shape-fit mechanism. Paired with targeting agents with even higher specificity, lectins in all their variety can be used for specific cancer types without risk of harm to healthy cells (Nunes et al., 2012). The ever-changing dynamics of oncology and genetic mutations of various cancer types increase therapy immunogenicity, or the ability of cancer cells to tag drugs as foreign entities for destruction (Nunes et al., 2012). Gene distribution among cancer cells helps them evoke immune responses to treatments such as chemotherapy; these immune responses attack and eliminate the medicines before they can destroy the cancer (Nunes et al., 2012). New drugs based on natural components such as snaclecs can reveal more effective, resilient, and longer-lasting cancer treatments.

Phospholipases A_2

Phospholipases A2 are clinically important today for their role in inflammatory diseases and lipid catabolism. They are characterized based on their amino acid

sequences and other biochemical characteristics. Snake venom phospholipases A2 (svPLA2) are divided into three families: acidic, basic, and peptide homologues (Calderon et al., 2014).

Classification and Derivation

svPLA2s are present in all snake venoms, but those derived from the Viperidae family are most commonly used for cancer research; their mechanisms are therefore the most understood and applicable to future cancer therapies (Higuchi et al., 2007). Gel filtration, ion exchange, and reverse phase chromatography are used in the purification of these phospholipases (Higuchi et al., 2007). Once isolated, the PLA2 activity is best measured by a spectrofluorometer, which measures and records substrate fluorescence at a specific wavelength (Higuchi et al., 2007).

Biochemical Activity as Tumor Antagonists

While apoptosis, integrins, and angiogenesis are focal components of cancer, another minor mechanism by which cancer invades and migrates through the body is by altered lipid synthesis and downregulated lipogenesis. svPLA2s can mitigate these lipid synthesis changes by altering cell membrane metabolism (Calderon et al, 2014). Such alterations affect microtubules ad focal adhesions between cells, as with the PLA2 of *Macrovipera lebetina transmediterranea* (Calderon et al., 2014).

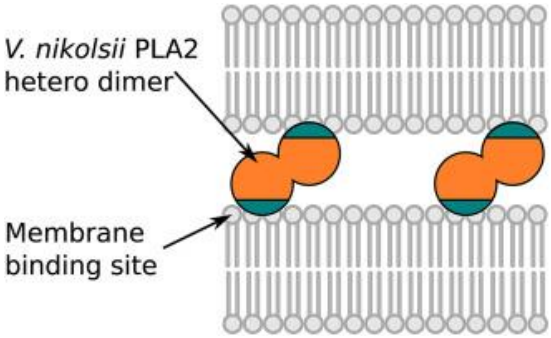

Figure 13. Binding of snake PLA2 to lipid membrane (Alekseeva et al. 2017).

PLA2s destabilize the lipid bilayer of the cell membrane. In the presence of calcium, phospholipid bonds are hydrolyzed to produce free fatty acids, molecules which cannot be incorporated into the lipid bilayer until they are rebonded (Vyas, Brahmbhatt, Bhatt, & Parmar, 2013). Such hydrolysis of the lipid bilayer weakens the integrity of the cell membrane, allowing for the release of bioactive products, cell shrinkage, and problems of cell adhesion (Vyas et al., 2013).

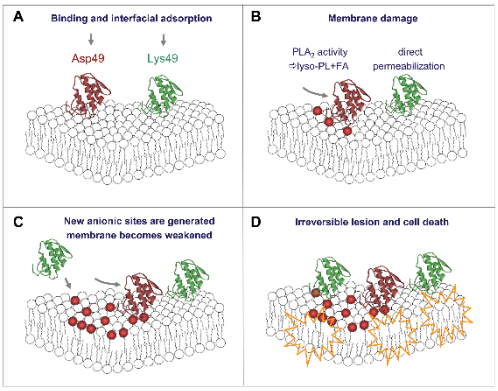

Figure 14. PLA2s attach, weaken, and lesion lipid bonds in the cell membrane (Mora-Obando, Fernández, Montecucco, Gutiérrez, and Lomonte, 2014).

By the primary means of lipid destabilization and membrane disruption, PLA2s employ mechanisms that combat the three focal components of cancer. They have been observed to reduce cancer cell viability by 50%, as cells without intact membranes cannot survive (Higuchi et al., 2007). Another type of svPLA2, though it did not interfere with platelet aggregation, did inhibit plasma clotting (Higuchi et al., 2007). Plasma cells injected with PLA2 suffered membrane breakdown, resulting in communication loss and tagging for apoptosis (Higuchi et al., 2007).

Current Experimental Trials

Studies observing svPLA2 interactions with cancer cells *in vivo* and *in vitro* are few and far between, as most PLA2 focus has been on anti-inflammatory uses. However, two emerging svPLA2s are VRCTC-310 and Lys49 PLA2. VRCTC-310 is a purified PLA2 from the venom of *Naja haje* (Costa et al., 1997). In their research (1997), VRCTC-310 significantly inhibited metastasis and induced apoptosis in patients after a 30-day treatment. Researchers found, however, that with this treatment of mice and

human cancer *in vivo*, side effects such as eosinophilia (abnormal white blood cell count) and palpebral ptosis (paralysis of the eye muscles) were experienced (Costa et al., 1997). Further studies will consider finding the components producing adverse reactions and isolating the lipid-lysing components of VRCTC-310 to create synthetic mimics.

In another study by Araya and Lomonte, Lys49 PLA2 was injected subcutaneously into five murine cancer cell lines: melanoma, mammary carcinoma, sarcoma, myeloma, and cancerous endothelial cells (2007). All five cancer cell lines showed marked lipid lysis by the peptides (Araya & Lomonte, 2007). Tumor growth was significantly stunted, and tumor recession proceeded until tumors were about 64% of their original size (Araya & Lomonte, 2007). Scientists especially praised these results because they were achieved by similar methods and doses as paclitaxel, an anticancer drug currently in clinical use (Araya & Lomonte, 2007). This similarity in results but with a novel source encourages research of svPLA2s as potential natural cancer therapies.

Future Research: Opportunities and Limitations

Research on PLA2s from snake venoms is still in its earlier stages. Unlike with disintegrins, LAOOs, and lectins, PLA2s are difficult to extract. While successfully isolated samples have demonstrated the promising nature of PLA2s to destabilize the lipid membranes of cancer cells, scientists are still struggling to pair snake PLA2s with agents that help them differentiate between normal and cancerous cells. Disintegrins, LAOOs, and lectins remain at the forefront of venom-based cancer treatment developments and experiments, but any discovery and breakthrough with PLA2s introduces for yet another mechanism by which cancer can be treated when other methods fail.

Conclusion

Because cancer is one of the most common and feared diseases, it is imperative that scientific research develops cancer treatments quickly and effectively. Chemotherapy and radiation are the two traditional treatments, and they focus on the undifferentiating killing of cancerous tissues, even if normal cells are affected too. Research on snake venoms and their components has produced some promising results as experiments have put disintegrins, L-amino acid oxidases, lectins, and phospholipases to the test. Recent research continues to reveal fascinating and practical details about cytotoxicity and the mechanisms of inhibiting tumorigenicity.

Rather than attacking all regionally-affected cells, specific aspects of cancer progression are now becoming targets, including increased growth and proliferation, avoidance of apoptosis, and angiogenesis. These overarching characteristics of cancer cells lead to invasion, metastasis, and interruption of normal cell function, but components of snake venom have been found to directly hinder such activity. Now the invention of new treatments is expanding to include factors that reduce cell adhesion, halt neovascularization, destabilize cell membranes, and activate caspase-mediated apoptosis.

This thesis discussed four significant components of snake venom: disintegrins, L-amino acid oxidases, lectins, and phospholipases. General isolation and derivation of these components was described, and their biological effects on cancer cells were explained. Lastly, current research of each target-specific component and its effects was summarized to provide background for the leading opportunities and problems with future experimental directions.

Finding weaknesses in the mechanisms of tumor progression starts with isolating the compounds that inhibit these focal processes, and scientists have found potential solutions in snake venom components. Further trials may pair the natural abilities of these components with synthetic manipulation to inhibit angiogenesis, induce apoptosis, inhibit proliferation, etc. Such trials and their success are essential to the search for new drugs and cancer inhibitors that combat the wide variety of cancer mechanisms. With the development of cancer treatments using snake venom components, one of many manifestations of the Rod of Asclepius will be realized.

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