A NOVEL TREATMENT FOR DIABETIC FOOT ULCERS

A MECHANISTIC ANALYSIS ON TRANSDERMAL DOXYCYCLINE:
A NOVEL TREATMENT FOR DIABETIC FOOT ULCERS
By
SIMONA GABRIELE, B.Sc. Honours

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ABSTRACT

Tetracycline molecules including doxycycline (DOX), consist of a group of broad-spectrum antibiotics. In addition, tetracyclines inhibit matrix metalloproteinase (MMPs) that contribute to tissue remodeling, inflammation, angiogenesis and are over-expressed in certain pathologies - such as Alzheimer's disease, metastasis and diabetic foot ulcers (DFUs). Tetracyclines are hypothesized to inhibit MMPs through the chelation and sequestration of catalytic divalent ions such zinc and calcium. This inhibitory duality may be beneficial in pathologies that are characterized by MMP over-expression and prone to infection, such as DFUs. Compared to oral administration, topical DOX is an attractive route of administration for chronic wound healing as it may minimize the risks: associated antibiotic resistance; is being targeted directly to the wound bed. However, DOX is notoriously unstable in aqueous solution and common topical formulations. Liquid chromatography and mass spectrometry (LCMS) were employed to monitor stability using an in vitro MMP assay and an applicable E. coli anti-bacterial assay was assessed to quantify drug activity. 2 % (w/w) topical DOX demonstrated an acceptable stability 30 day when stored at 4 ºC. DOX inhibited MMP9 activity with an IC50 value of 48.27 μM. With respect to anti-bacterial activity, using cultured BL21 E.Coli and quantification of drug activity as an expression of colony forming units (CFUs) successfully reproduced the antimicrobial IC50 of doxycycline as 4.3 μM. Transdermal DOX has the potential to improve standard of care for DFUs, quality of life for the patient and reduce costs to the healthcare system.

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LIST OF FIGURES

LIST OF ABBREVIATIONS

aa- tRNA	Aminoacyl- transfer ribonucleic
β -HPCD	Beta- hydroxypropyl- cyclodextrin
CA	Cysteine array
CE	Capillary electrophoresis
CFUs	Colony forming units
CMT	Chemically modified tetracycline
Cys73	Cysteine73 residue
DOX	Doxycycline, doxycycline hyclate
DFU	Diabetic foot ulcer
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ESI	Electrospray ionization
Ex/Em	Excitation/emission
F	Fibronectin repeats
FGF	Fibroblast growth factor
FRET	Fluorescence resonance energy transfer
Fu	Furin-like serine proteinases
GI	Gastrointestinal
GPI	Glycosylphosphatidylinositol
HCl	Hydrochloride

HGF	Hepatocyte growth factor
HPLC	High pressure liquid chromatography
IC50	Inhibitory concentration at 50%
IL-1 β	Interleukin 1 beta
Ig-Like	Immunoglobulin-like
KGF	Keratinocyte growth factor
LB	Lysogeny broth
LCMS	Liquid Chromatography Mass Spectrometry
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
MeOH:H_2O	Methanol: water
MgCl$_2$	Magnesium Dichloride
M.HCl	Methacycline Hydrochloride
MMP	Matrix metalloproteinase
MMP-2	Gelatinase A
MMP-9	Gelatinase B
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NaOH	Sodium hydroxide
NF-kβ	Nuclear factor kappa beta
NNGH	MMP9 assay kit inhibitor
NO	Nitric oxide

QTRAP	Hybrid triple quadrupole/ linear ion trap
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSD	Relative standard deviation
SA- N	Terminal signal anchor
SEM	Standard error of the mean
TGF- β	Transforming growth factor beta
TIMPs	Tissue inhibitors of metalloproteinases
TM	Transmembrane domain
TNF- α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
Vn	Vitronectin-like insert
W/w	Weight per weight
Zn^{2+}	Zinc ion

INTRODUCTION

1.0 CHRONIC WOUNDS- DIABETIC FOOT ULCERS

1.1 Diabetes

Diabetes is a metabolic disease, which results from reduced insulin production or insulin resistance. Insulin is instrumental in facilitating sugar being removed from the blood and used as energy within the body's cells (Dali-Youcef et al. 2013). The disturbance or inefficient use of insulin causes the blood glucose levels to rise, which in turn results in cells being deprived of energy. This disruption and deprivation of energy causes downstream effects on major body systems; these effects are exacerbated if blood sugar levels are not controlled. In 2015, The Canadian Diabetes Association (2015) estimated that the prevalence of diabetes in Canada is approximately 3.4 million (9.3% of the total population). The Canadian Institute for Health Information (2013) stated that Canadians with diabetes possess a 15-25% chance of developing chronic impairment of healing in acute wounds, specifically, diabetic foot ulcers (DFUs) (Singh, Armstrong, and Lipsky 2005). The severity and staging of DFUs is based on the depth of the tissue, osseous involvement, infection and ischemia (Figure 1). The Canadian Diabetes Association reported a common complication with DFUs is amputation, which has a staggering prevalence of 84% of individuals with DFUs. Individuals with DFUs are 20 times more likely to be admitted to hospital for amputation related treatment than other

Canadians. Diabetic foot lesions are accountable for more hospitalizations than any other complication related to diabetes. The factors that impact ulcer formation and that contribute to impair healing in individuals with diabetes are crucial to understand when considering treatments for DFUs.

Stage	Grade			
	0	**I**	**II**	**III**
A (no infection or ischemia)	Pre- or post-ulcerative lesion completely epithelialized	Superficial wound not involving tendon, capsule, or bone	Wound penetrating to tendon or capsule	Wound penetrating to bone or joint
B	Infection	Infection	Infection	Infection
C	Ischemia	Ischemia	Ischemia	Ischemia
D	Infection and ischemia	Infection and ischemia	Infection and ischemia	Infection and ischemia

University of Texas Diabetic Wound Classification System

Fig. 1 University of Texas Diabetic Wound Classification System: This chart shows the classification system used to determine the severity of the DFU in relation to depth of the tissue, osseous involvement, infection and ischemia. Adapted by Ince *et al.,* 2008.

1.2 Cause of Ulceration

Diabetes leads to changes to blood vessels and nerves, and these neuropathic and vascular complications of the diabetes contribute to DFU formation. Peripheral neuropathy that results from diabetes causes reduced or loss of feeling in the lower extremities (Chiles et al. 2014). As a result, any injury or pressure that maybe inflicted on the lower extremities can go unnoticed due to the loss of sensation. This compromise of the sensory feedback warning system may lead to injury of the skin and the formation of wounds (Dobretsov, Romanovsky, and Stimers 2007). Vascular disease that occurs as a complication of diabetes may also contribute to the formation of DFUs (Rathur and Boulton 2007). The lack of blood flow and oxygen to tissues because of narrowed or blocked arteries may lead to ulceration and may directly impact the wound healing process, resulting in chronic non-healing wounds. The formation of new blood vessels and the supply of oxygen to the tissues is a key component in remodeling of the tissue and in the re-epithelialization of that in normal wound healing.
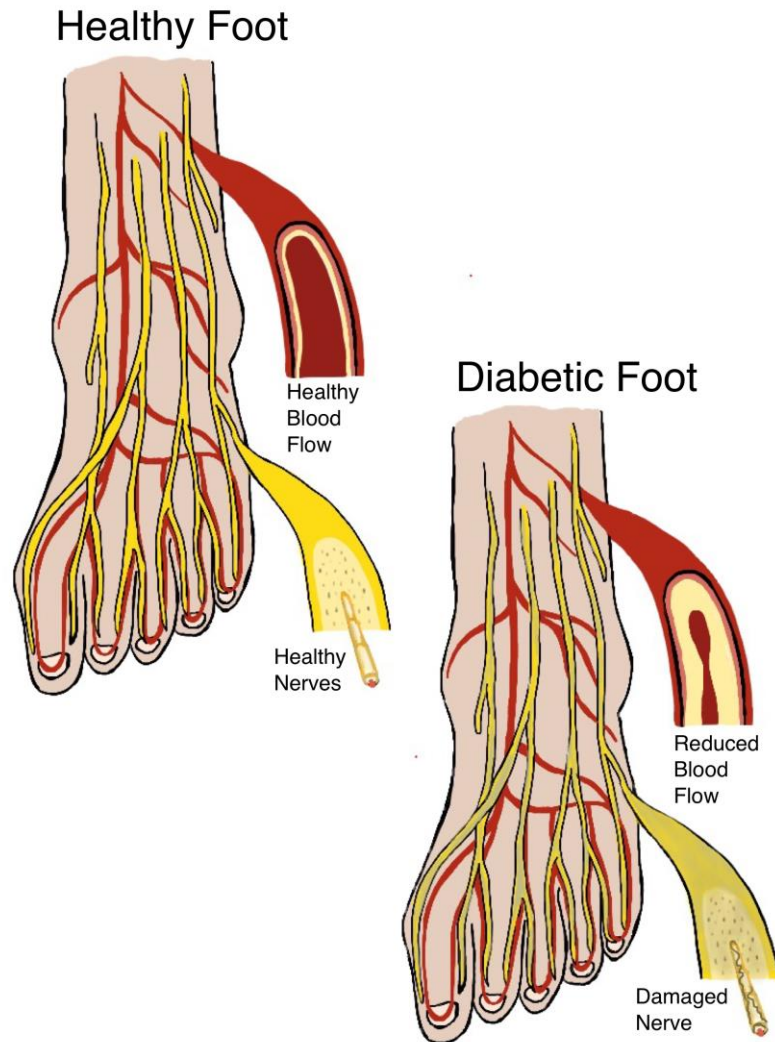
Fig. 2- Cause of Ulceration- Healthy Foot Vs. Diabetic Foot: The healthy foot has healthy vasculature and blood flow alongside healthy nerves with feeling and sensation. In contrast the diabetic foot has reduced blood flow and poor vasculature alongside peripheral neuropathy making this foot at risk for the development of DFUs. Figure modified from Chiles et al. 2014.

1.3 Wound Healing

The skin is a mechanical barrier to the external environment aiding in protection of the internal body. Once this barrier has been breached, many processes ensue in attempt to restore homeostasis and to heal the defect. Wound healing is a multifaceted process involving four phases working in conjunction; homeostasis, inflammation, proliferation and remodeling (Diegelmann and Evans 2004). A cellular immune response is triggered by the injury and initiate downstream signaling, activation and respective responses (Lawrence and Diegelmann 1994). This process results in activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets (Harold Brem and Tomic-Canic 2007).

Homeostasis is the initial phase which involves the recruitment of platelets into the wound to stop the loss of blood and to fill the wound with blood clot. The platelets adhere to the sub-endothelial surface of the ruptured blood vessel's epithelial wall. The inflammatory response is initiated once the mechanical barrier has been breached and factors released from the activated platelets contribute to this. In this process a cascade of neutrophils, lymphocytes, macrophages, enter the wound to defend the site from infectious or damaging agents and to clear debris. This process ensures that damaged cells, pathogens and bacteria are removed from the wound site. During the proliferative stage the wound is rebuilt with new tissue made of collagen and the extracellular matrix (ECM) and myofibroblasts cause the wound to contract (Marshall et al. 2015). A network of blood vessels is also constructed during this stage as part of the formation of healthy

granulation tissue. Angiogenesis is critical for the wound healing process as the new capillaries supply the wound with oxygen. During maturation, the accumulated of collagenous and non-collagenous proteins of the extra cellular matrix (ECM), are remodeled via matrix metalloproteinases (MMPs) (Lazarus et al. 1994; Lohmann et al. 2004). The collagen fibers are reorganized from being laid out in the previous phase and through maturation the new tissue progressively gains strength and flexibility. The inhibition of MMPs are also instrumental in successful healing of the wound with cell migration, angiogenesis and tissue remodeling, however, their regulation and inhibition are important to prevent continued breakdown of tissue and consequent stalling of the wound healing process. The process of wound healing is also influenced by many variables including age, physical condition, location of the wound, cause of the injury and co-morbidities. The failure to progress in the stages of wound healing can lead to chronic wounds which are commonly seen in diabetes, chronic venous disease, infection and other metabolic deficiencies.
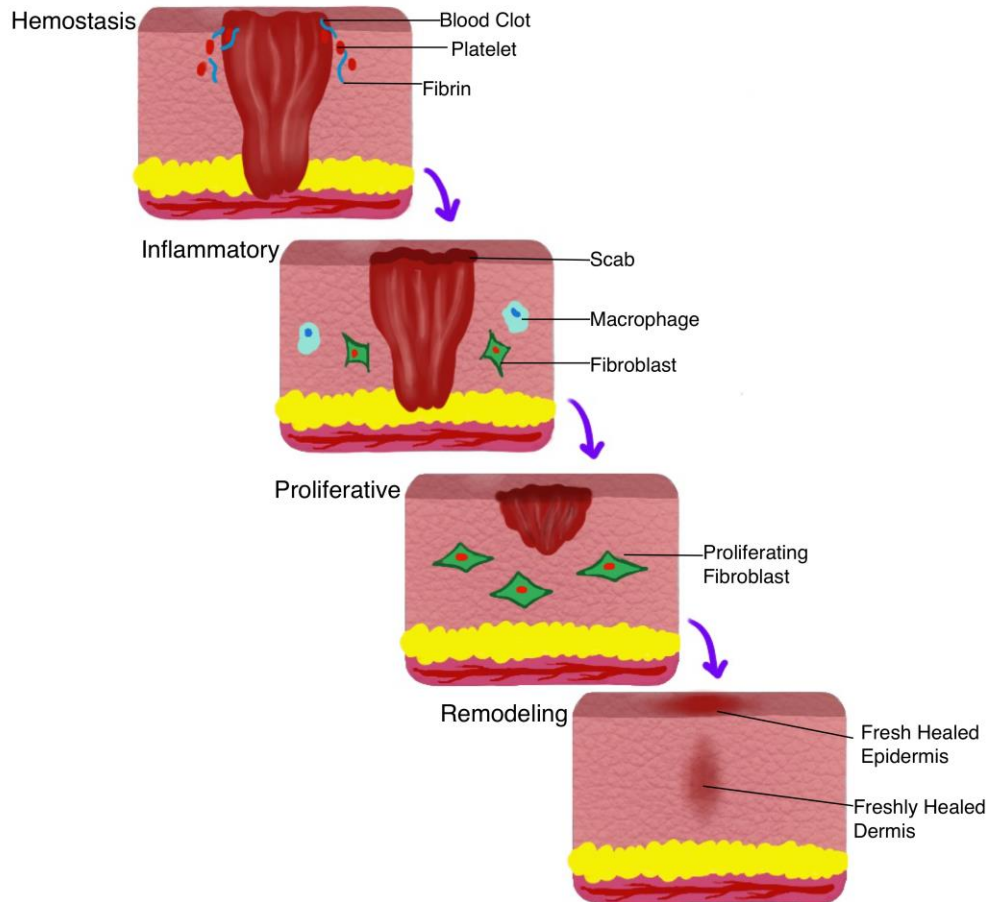
Fig. 3 - Phases of Normal Wound Healing: Hemeostasis, Inflammatory, Proliferative, Remodeling. Hemostasis begins when the mechanical barrier is breach initiating the innate immune system and ensuing coagulation. The inflammatory stage involves recruitment of neutrophils and macrophages to clear the bacteria and debris. The proliferative phase involves the formation of granulation tissue through a network of blood vessels alongside epithelialization. The final phase is the reorganization of the collagen fibers that were laid down in the previous phase. Overtime and through remodeling the new tissue regains flexibility and strength. Figure modified from Maynard 2015.

1.4 Impaired Wound Healing in Diabetics

Chronic wounds exhibit a disrupted repair process in which these wounds fail to progress through the stages of wound healing. There are a multitude of factors that have been shown to be involved in the pathogenesis of DFUs. These include impairments in growth factor production (Galkowska et al. 2006), macrophage function (Maruyama et al. 2007), collagen accumulation, angiogenesis and granulation tissue formation (Falanga 2005; Galiano et al. 2004), keratinocyte and fibroblast migration and proliferation (Gibran et al. 2002) and accumulation of ECM components, which involves the activation and inactivation of MMPs throughout the wound healing phases (R. Lobmann et al. 2002). The tissue damage that results from this abnormal wound healing, also causes the wound to remain in a prolonged and heightened inflammatory state.

This stage of wound healing is characterized by excessive neutrophil infiltration, which perpetuates the cycle of chronic inflammation, as these cells produce an abundance of ROS directly damaging the ECM and cell membrane (Demidova-Rice, Hamblin, and Herman 2012). Further neutrophils release proteases and MMPs, which are involved in the degradation and inactivation of ECM. The levels of proteases can be up to 60 times higher in chronic wounds than in acute healing wounds, thus playing a vital role in perpetuating the inflammatory stage and preventing wound closure (Woo, Ayello, and Sibbald 2007). The proteases degrade multiple growth factors, receptors and ECM proteins that are vital to wound healing (Ralf Lobmann et al. 2006; Neely et al. n.d.; Sibbald and Woo 2008). Growth factor production is increased in chronic wounds;

however, their bioavailability is decreased because of degradation of the growth factors and their receptors. These wounds also have elevated levels of pro-inflammatory cytokines, IL-1β and TNF-α, which are produced by neutrophils and macrophages, which lead to increased levels of MMPs (Mast and Schultz 1996; Nath and Gulati 1998; Trengove et al. 1999). The presence of these cytokines influence the levels of TIMPs, which are also increased, but the relative proportion of MMPs and TIMPs varies. Therefore, this imbalance facilitates degradation of the ECM, collagen synthesis, cell migration and reduces fibroblast proliferation (Mast and Schultz 1996). The ECM breakdown products further promote inflammation and perpetuate the impaired healing state.

Local tissue hypoxia, lack of oxygen to the tissue, contributes to the impaired the wound healing process as the inflammatory cascades are perpetuated. The balance between ROS and antioxidants such as nitric oxide (NO) are disrupted and altered in chronic wounds (Toledo-Pereyra, Lopez-Neblina, and Toledo 2004). NO is responsible for inactivating NF-kB, an important modulator in the inflammatory process. ROS causes oxidative damage and also activates signal transduction pathways that enhance the expression of MMPs and inflammatory cytokines (Peschen et al. 1999). Hypoxia enhances inflammation as well as impeding re-epithelization, proliferation, synthesis of collagen and remodeling (Schreml et al. 2010). Angiogenesis is also impaired in chronic wounds and therefore the microvascular network required to form healthy granulation tissue does not form, thereby contributing to impaired healing. The activation of angiogenesis is a part of the selective remodeling of the ECM and basement membrane

that occurs prior to the formation of new blood vessels (Egeblad and Werb 2002). Further tissue remodeling requires proteolytic systems, specifically MMPs which are involved in processing and generating angiogenic regulatory molecules, which in turn activates growth factors and cytokines. A combination of these factors prevents the tissue from forming granulation tissue, remodeling and closing as seen in normal wound healing. Diabetics experience an impairment in the healing of acute wounds, which is contributed to by an increase in the expression and activation of MMPs (Brem and Tomic-Canic 2007).

2.0 MATRIX METALLOPROTEINASES

2.1 Matrix Metalloproteinases

Matrix Metalloproteinases are calcium-dependent zinc-containing enzymes that facilitate biological reactions that act on ECM degradation. The substrate binds with the enzyme's active site within the catalytic domain forming an enzyme-substrate complex. The enzyme substrate usually ECM (collagen, gelatin, proteoglycan) are cut up into pieces via MMPs. The MMP family possesses homologous conserved domains, which include a pro-peptide domain that contains cysteine residue and a catalytic domain that contains the zinc binding site (Van Wart and Birkedal-Hansent 1990). MMPs can be subdivided into seven groups characterized by domain organization, additional insertions and substrate binding. The various subfamilies include: collagenases, gelatinases, stromelysins, matrilysins, metalloelastases, membrane type MMPs and other MMPs (Caley, Martins, and O'Toole 2013). MMPs are heavily involved in all the wound healing phases and in turn the success of wound closure. MMPs are elevated in chronic inflammation and impaired wound healing and add to perpetuating the chronic wound.

2.2 Expression, Regulation and Domains

MMPs are expressed minimally or at basal levels in homeostatic tissue. However, when tissue remodeling is warranted - MMPs are quickly activated by inflammatory and wound cells, regulated and expressed. The regulation of MMP expression and activity is mediated by various cells including: monocytes, lymphocytes, neutrophils and macrophages. MMP expression can be triggered through cell signaling via cytokines, hormones, ECM and other cell types (Caley, Martins, and O'Toole 2013). Specifically, these include epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor, tumor necrosis factor-α (TNF-α), keratinocyte growth factor (KGF), transforming growth factor-β (TGF-β), interleukins and interferons (Chen and Chen n.d.; June et al. 1998).

MMP activity is regulated through gene expression and MMPs synthesized then secreted in a latent, inactive form (pro-MMP). Further, MMP activity is controlled by enzymatic activation and specific inhibition. The catalytic mechanism of action in MMPs demands a zinc ion in the active site. In the inactive state, the cysteine[73] (Cys[73]) residue is reported to block the active site and is not freely accessible in the latent enzyme (Van Wart and Birkedal-Hansent 1990). The autolytic cleavage activation triggers the dissociation of Cys[73] from the zinc atom allowing the active site to be exposed. This dissociation is known as the cysteine switch, which is the mechanism of activation for all MMPs. The amino acid sequences around the cysteine residue and region of protein

chains that possess the zinc-binding ligands are highly conserved (Van Wart and Birkedal-Hansent 1990). Following activation, most MMPs are seen to have three distinct domains: N-terminal pro-peptide domain, C-terminal catalytic domain and hemopexin-like C-terminal domain. The other MMPs differ based on the addition and deletion of domains, leading to diverse functionality. The activation of MMP can also be controlled by the inhibition of protease activity; this is accomplished via plasma proteinase inhibitors. Furthermore, MMPs are regulated by tissue inhibitors of metalloproteinases (TIMPs) (Gomez et al. 1997). There are four TIMPs altogether (TIMP-1, TIMP-2, TIMP-3, TIMP-4); these protein inhibitors are able to control the proteolytic activity of all MMPs (Nagase, Visse, and Murphy 2006; Nuti and Tuccinardi 2007).
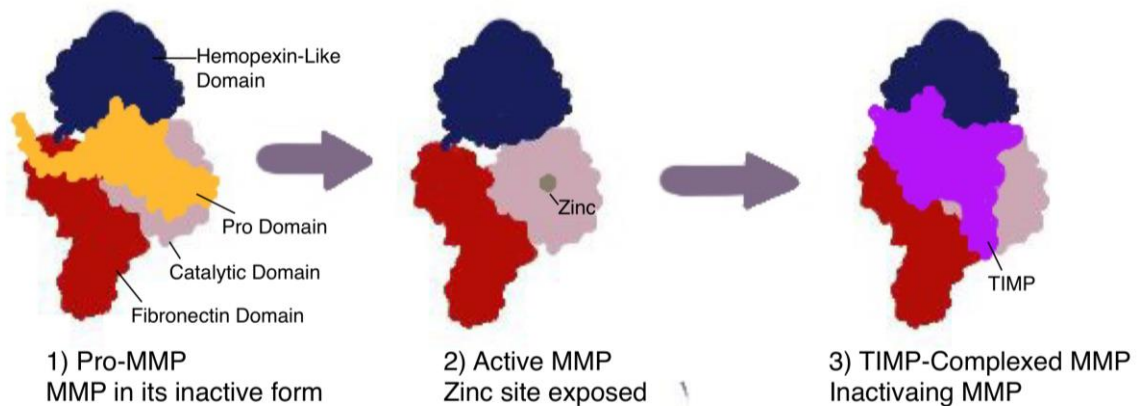
Fig. 4 - Matrix Metalloproteinase Structures and Forms: This diagram depicts MMP-2 (Gelatinase A) specifically. MMPs exist in three forms (1) the pro-MMPs which is in the inactive active, the pro-domain (red complex) covers and inactivates the catalytic domain (yellow); (2) active MMPs, with the catalytic domain exposed and revealing the zinc ion, the MMP is able to cleave substrates; (3) TIMP-complexed MMPs which is in the inactive state, TIMPS (purple complex) regulate and inactivate MMPs. Figure modified from Nguyen, Mobashery, and Chang 2016.
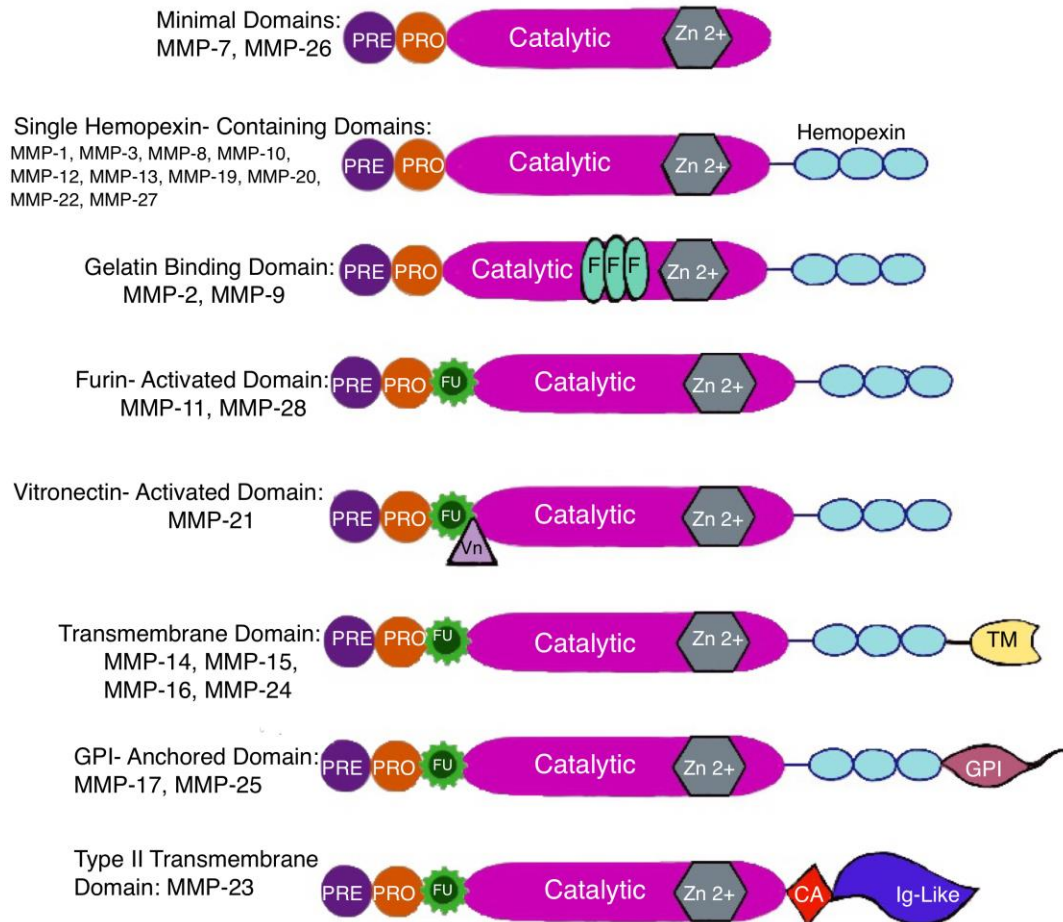
Fig. 5 - Matrix Metalloproteinase subfamilies and domains: MMPs are made up of eight different subgroups divided based on structural similarities. The domains include Pre: signal sequence, Pro: pro-peptide, Catalytic: catalytic domain containing zinc (Zn^{2+}) ion, F: repeats of fibronectin, Fu: furin-like serine proteinases, Vn: vitronectin-like insert, TM: transmembrane domain, GPI: glycosylphosphatidylinositol, SA: N-terminal signal anchor, CA: cysteine array, Ig-Like: immunoglobulin-like. Figure from Nguyen, Mobashery, and Chang 2016.

2. 3 Involvement in Wound Healing

MMPs are involved in all stages of wound healing through adjustment of the wound matrix, permitting cell migration and tissue remodeling. Healthy skin is made up of ECM, blood vessel networks and fibroblasts within the matrix. Once this mechanical barrier has been breached - a wound or injury is established. In the stage of inflammation MMP-2 and MMP-9, the gelatinase subfamily of MMPs are present (Nguyen, Mobashery, and Chang 2016). In normal wound healing; inflammation is followed by angiogenesis which involves the formation of new blood vessels, which involves the upregulation of MMP-1, MMP-8, MMP-9 and MMP-13. These MMPs facilitate keratinocyte migration and aid re-epithelization over the wound bed. In chronic wounds, such as, DFUs, inflammation is prolonged as MMP-9 levels are increased which in turn decreases MMP-8 levels, which is harmful to ECM remodeling. The overexpression of MMP-9 prevents the wound from advancing through the phases of angiogenesis, remodeling and successful wound closure. Many studies focus on all protease activity in the wound fluid rather than subfamilies; MMP-9 was identified as the major MMP in chronic wound fluid (Rayment et al. 2008).
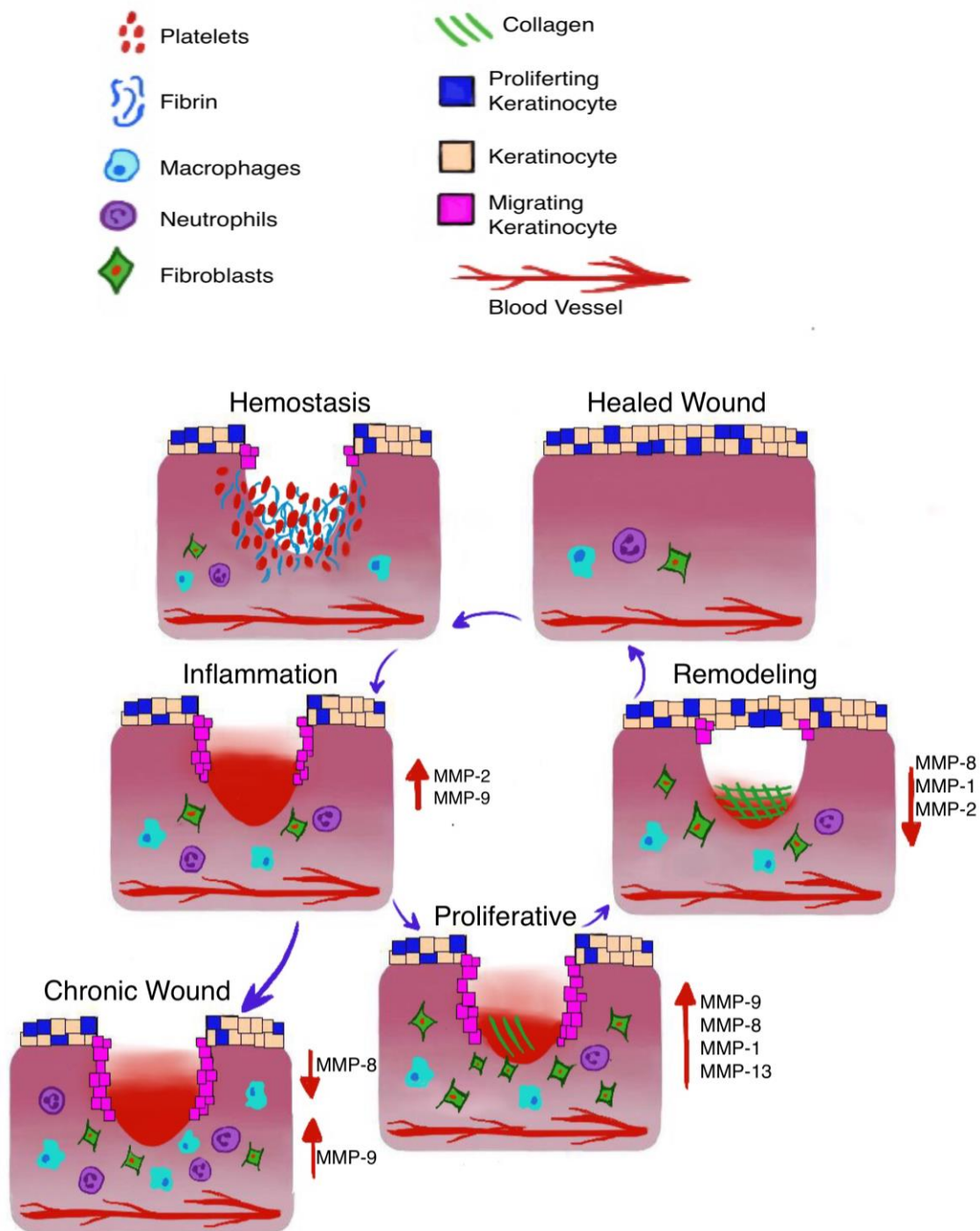
Fig. 6 - MMPs Involvement in Wound Healing and Impaired Wound Healing: The various subfamilies of MMPs are implicated in normal wound healing as well as impaired wound healing. The elevated levels of MMP-9 and decreased levels of MMP-2 during the inflammatory stage traps the wound in a chronic state delayed wound closure. Figure from Nguyen, Mobashery, and Chang 2016.

2.4 Gelatinase

Gelatin binding MMPs (MMP-2 and MMP-9) contain five distinct domains: pro-domain which is cleaved upon activation, gelatin binding domain consisting of three fibronectin repeats, catalytic domain containing zinc binding site, proline rich linker region and carboxyl terminal hemopexin-like domain (Rowsell et al. 2002). The fibronectin repeats are a unique domain in gelatin-binding MMP, which binds extracellular matrix components such as collagen, fibrin, and heparan sulfate proteoglycans. There is a highly structural homology in MMP-9 catalytic domains making it difficult for inhibitors to interact; MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) are very similar and their main differences are due to substrate specificity. Gelatinase MMPs are able to break down collagen more effectively than any other MMP subfamily and also cleave the basement membrane protein, collagen type IV (Parks 1999; Tarnuzzer and Schultz 1996).

MMP-9 is also known as gelatinase B and is a 92 kDa type IV collagenase. The elevated levels of MMP-9 seen in chronic non-healing wounds are due to increased inflammation. MMP-9 is expressed primarily by neutrophils and macrophages, both involved in the inflammatory response (Wysocki et al. 1999). The presence of elevated MMP-9 levels has been associated with poorly healing ulcers, specifically DFUs. There is evidence that suggest MMP-9 is expressed in chronic wounds, as MMP-9 activity levels in chronic wound fluid were found to be 25 times higher than normal wounds (Wysocki et al. 1999; Yager et al. 1996). In contrast, MMP-2 is decreased in chronic

wound fluid, suggesting a decrease in its production in fibroblasts as MMP-2 is derived from these cells (Cullen et al. 2002). MMP-9 presence is potentially correlated with ulcer's chronicity and may therefore play a role in preventing wounds from healing. MMPs are inhibited by TIMPs that bind to the catalytic domain of the enzyme, preventing substrates from attaching to the site (Henriet, Blavier, and Declerck 1999). TIMP-1 has selective binding with gelatinase B and is considered a its main inhibitor. The overexpression of MMP-9 and insufficient TIMP-1 to inactivate the enzymes may contribute to the failure of healing in chronic ulcers. There are treatments options that could potentially assist in closure of chronic wounds combatting these imbalances that perpetuate the inflammatory phase.

3.0 TREATMENT

3.1 Current Available Treatments and Standard of Care

DFUs require extensive preventative care including: patient education, appropriate footwear, regular foot care and pressure offloading dependent of the severity of the ulceration (Armstrong et al. 2010; Cavanagh and Bus 2010; Rathur and Boulton 2007). The current standard of care for acute foot wounds in diabetic patients includes debridement, pressure off-loading, infection management, and revascularization. This standard of care must be executed regularly and aggressively - to preserve healthy tissue and prevent the ulcers from worsening. This is a difficult task when the healing process is stagnant due to excess proteases and cell migration is absent (H Brem and Tomic-Canic 2007). The problem of amputation due to DFUs is very prevalent, impacting 1 in 4 patients even with the current treatment available (Hackethal 2014). Foot ulceration is a major ongoing problem in individuals with diabetes and is contributed to by unmet needs and a lack of validated advances in treatment. Significant new treatments for DFUs have not emerged in the past two decades and current treatment options are very expensive to the patient and the healthcare system (Hackethal 2014). Many diabetic wounds fail to heal with conventional therapy and the current standard of care, resulting in disability and decreased quality of life. The available advanced technologies for DFUs that have been shown to be effective are recombinant growth factors, autologous growth factors and bioengineered skin-tissue; these options are extremely costly (Shevchenko, James, and

James 2010). Therefore, there is a great need for novel, effective modalities of conquering this debilitating condition, DFUs.

3.2 Tetracyclines

Tetracyclines comprise a group of broad-spectrum antibiotics known for their effectiveness against Gram-positive and Gram-negative bacteria. Tetracycline's possess various mechanisms of action and functions which make this antibiotic a multifaceted therapeutic. The primary mechanism of action is reported to be the inhibition of protein synthesis through binding to the bacterial ribosome (Hash, Wishnick, and Miller 1964). Tetracyclines function to inhibit bacterial protein synthesis, interfering with ribosomal activity and obstructing messenger-RNA codon from reading the t-RNA anticodon. The bacterial ribosome binds to the 30S subunit with high-affinity alongside many other binding sites 30S and 50S subunits that bind with low-affinity (Tritton, 1977). The tetracycline binds to the ribosome subunit, initiating a conformational change as the drug allosterically inhibits the binding of aa-tRNA at the acceptor site, in turn preventing protein synthesis (Semenkov et al., 1982). Therefore, new amino acids are unable to be added to the developing polypeptide chain. Thus, the use of tetracyclines would be useful in ceasing gram-positive and gram-negative bacteria synthesis. The degree of inhibition is dependent on high concentrations of the tetracycline, as low concentrations of the antibiotic do not result in inhibition (Semenkov et al. 1982).
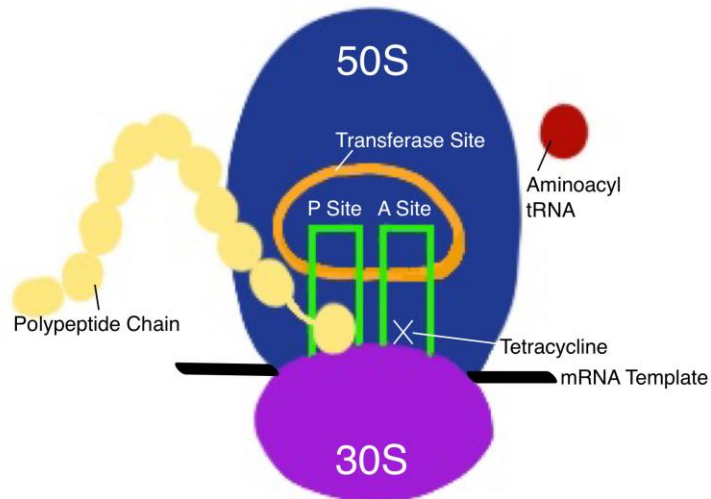
Fig. 7 – Tetracycline Inhibition of Bacterial Protein Synthesis: Tetracycline binds to the 30S unit and blocking tRNA from binding to the A site and inhibiting the synthesis of protein. Figure modified from Semenkov et al., 1982.

3.3 Doxycycline

There are many subclasses of the tetracycline family have different pathways and functionality as therapeutics. The most common clinically used semisynthetic tetracycline is doxycycline (DOX), which is a well-tolerated and safe compound. DOX is a semisynthetic, chemically modified, Food and Drug Administration (FDA) approved tetracycline analog, which is rapidly absorbed and has a prolonged half-life (Saivin and Houin 1988). DOX is used in the treatment of various conditions; anthrax, chlamydial infections, community-acquired pneumonia, Lyme disease, cholera, syphilis, Yersinia pestis (plague), periodontal infections and many more.

DOX is made of a four-ring core with other groups attached to the core. The upper half of the structure contains a dimethylamino group at the C4 carbon, which is the portion of the molecule responsible for the antimicrobial activity of the drug (Griffin et al. 2010). The lower half of the structure has actions that influence oxygen-rich mechanisms in both prokaryotic and eukaryotic targets. This region of the molecule is essential to the drug's effectiveness and interference with the oxygen-rich mechanisms and can therefore reduce or eliminate function (Lorne M Golub et al. 1991). The lower region of the structure is reported to be involved in metal chelation, between the drug and proteins (Takahashi, Altschmied, and Hillenj- 1986). The mechanism of action is reported to be chelation of structural and catalytic zinc ions within the MMP enzyme (Lorne M Golub et al. 1991). The strength of the interaction is dependent on the drug-metal relationship, zinc possessing a higher affinity than calcium (Nelson 1998).

3.4 Doxycycline Inhibiting Matrix Metalloproteinases

DOX has many functions that are useful in the treatment of various pathologies. Tetracyclines, specifically doxycycline are well known for their involvement in inhibiting MMPs which are a family of zinc dependent proteases that contribute to tissue remodeling, inflammation, angiogenesis and tumor invasion (Nagase and Woessner 1999). The interaction between tetracyclines and MMPs is not clearly understood throughout the literature. However, tetracyclines are thought to have an impact on direct inhibition of MMPs as well as inhibiting MMP synthesis and expression.

The mechanism of direct inhibition is understood to involve metal chelation between the drug and enzyme. Through the addition of calcium and zinc- MMP inhibition can be reversed (Yu et al. 1991). However, more calcium ions are required to restore activity as they bind with a lower affinity (Lorne M. Golub et al. 1999). The mechanism of action is dependent on the chelation and interaction with structural metals rather than the chelation of the active site of zinc (Smith, Hoh, and Easty 1999). The effectiveness of the tetracycline interaction with MMP is dependent upon the class of tetracycline, MMP subfamily and pH.

There is much speculation on the mechanism of action of tetracyclines on MMPs, however, definitive conclusions have not been drawn. Tetracyclines are thought to interact with the enzymes, through metal chelation, inhibiting MMP activity, inhibiting MMP synthesis and inhibiting the synthesis of pre-cursor cytokines. The tetracyclines are

reported to bind the zinc ion in the active site of the protein, however this process is irreversible, as MMP activity cannot be restored with exogenous zinc (Smith, Hoh, and Easty 1999). Chelation is a type of bonding between a ligand and a single central atom. Chelators work by binding (chelating) to metals, thus preventing the effects that these metal ions possess on the body. Despite its various mechanisms of action, the use of tetracyclines as antibiotics has declined in the clinical setting due to the possibility of antibacterial resistance.
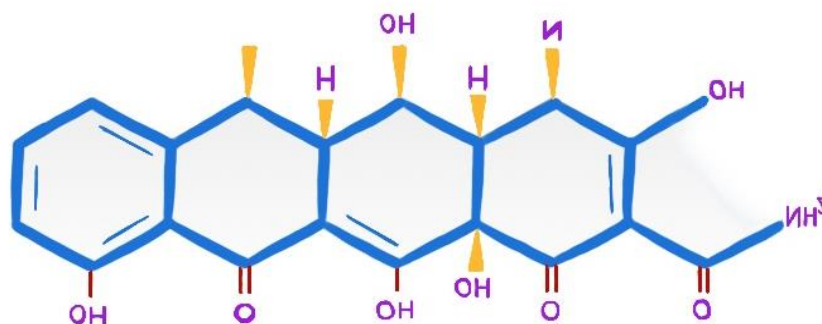
Fig. 8- Doxycycline: The chemical structure of doxycycline. The upper half of the structure contains a dimethylamino group at the C4 carbon, which is the portion of the molecule responsible for the antimicrobial activity of the drug. The lower region of the structure is reported to be involved in metal chelation, between the drug and proteins. Modified from Griffin et al. 2010.

3.5 Chemically Modified Tetracycline (CMT)

De-dimethylamino tetracyclines also referred to as chemically modified tetracyclines (CMT) do not possess the dimethylamino group and therefore lack antimicrobial activity (Golub et al. 1987). This may be a drug or test material of interest, as this will combat the antimicrobial resistance barrier seen with tetracyclines.

CMTs retain the interaction with MMPs warranting their consideration as a therapeutic for disease processes that require inhibition of these MMPs (Golub et al. 1987). CMTs are involved in inhibiting over expression of MMPs, pro-inflammatory cytokines and other mediators of protease activity (Agnihotri and Gaur 2012). CMT is a useful therapeutic as this pharmaceutical is not associated with the development of antibacterial resistance and gastrointestinal toxicity; which is a common disadvantage with parent tetracyclines. Furthermore, the route of administration also possesses various advantages and disadvantages associated with tetracycline antibiotics.
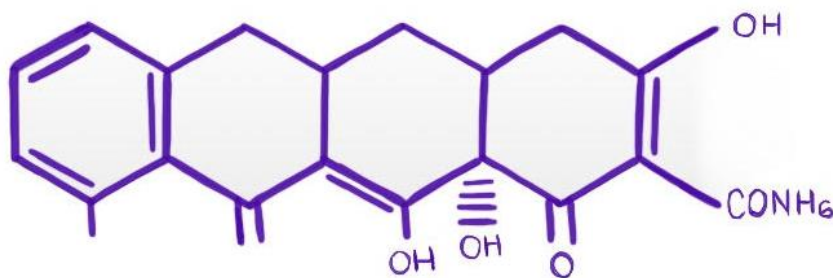
Fig. 9 – Chemically Modified Tetracycline: CMT-3 (6-deoxy-6-demythl-1-4-dedimethyl amino tetracycline) will be the subfamily used as this is the available product for experimentation, which lacks antimicrobial activity unlike the parent tetracycline. Modified from Golub et al. 1987

4.0 ROUTES OF ADMINISTRATION

4.1 Oral

Different routes of administration of drugs exhibit diverse bioavailability of the chemicals being administered (Yu Wang et al. 2015). In oral administration, the drug is absorbed in the gastrointestinal (GI) tract, and then enters the systemic circulation. This is an advantageous method as it is non-invasive, and a safe route that is convenient and cheap. Oral DOX is reported to have high bioavailability of over 80% (Saivin and Houin 1988). In comparison to intravenous administration, the rate of absorption is reported to be between 73-77% (Agwuh and MacGowan 2006). DOX rapidly absorbs in the duodenum with a half-life of 0.85 ± 0.41 h (Welling et al. 1977). This drug is made up of metal ion complexes that are unstable and degrade within acid pH, DOX is absorbed by the duodenum at a greater rate than any other tetracycline analog. The removal of DOX from the body has various mechanisms, with the concentrations of DOX being highest in the excretory organs of the GI tract, kidney and liver (Saivin and Houin 1988). Pharmaceutical molecules must pass through the gut wall and liver in order to enter the systemic circulation. This first pass effect is a disadvantage of the oral route, as the pharmaceutical molecule is partially metabolized during this process, thereby reducing the concentration of the drug before it reaches the systemic circulation (Kwan, 1997). The pharmaceutical molecule is broken down by enzymes of the GI lumen, gut wall, bacterial enzymes and hepatic enzymes. The hepatic metabolism greatly reduces the bioavailability

of the molecule. Furthermore, the ingestion of oral medication, specifically antibiotics can negatively impact microbiomes and leading to long lasting effects (Candon et al. 2015). Drug interactions are another factor that must be considered with oral route of administration. A drug interaction occurs when a substance affects the activity of a drug; increasing or decreasing the drug's effects, and potentially producing side effects (Bushra, Aslam, and Khan 2011). These interactions can be involved with food, herbs or other drugs that are being consumed orally. These disadvantages are potentially even greater amongst a vulnerable population such as individuals with diabetes. Oral DOX possesses a high bioavailability that is reported to be not significantly different to intravenous route of administration (Wormser et al. 2003).
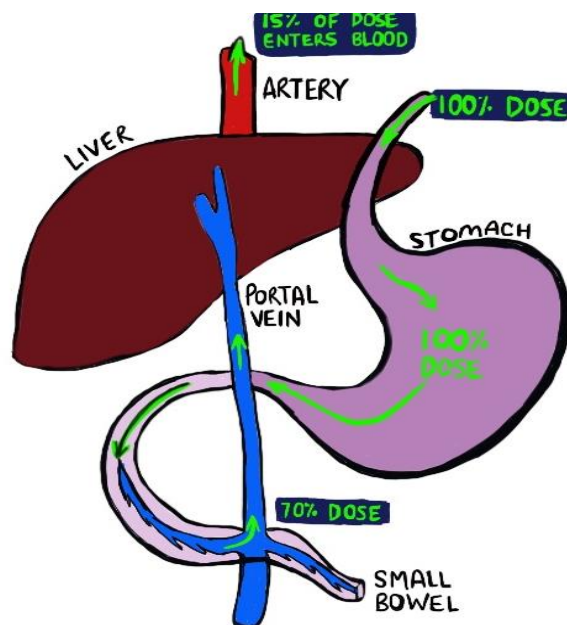
Fig. 10 - First Pass Metabolism: The drug is ingested orally, any product absorbed from the gastrointestinal tract is first delivered to the liver by the portal vein. The 'first pass effect' shows the metabolism of the drug by the gut and liver, leading to relatively low levels of drug reaching the circulation. Figure modified from Kwan, 1997.

4.2 Intravenous

Intravenous is a method of administration in which the drug is directly given by needle into the venous circulation, hence avoiding passage through the GI tract, the liver and the first pass effect. This method of administration ensures 100% bioavailability of the drug and occurs in a quickly with infusion time of 30-60 seconds (Fischer et al. 2003). This also allows for the desired blood concentration to be achieved. However, there are various disadvantages with intravenous treatment such as: technical assistance is required; danger of infection; increased cost; less convenient, invasive and painful; and repeated injections are not always feasible due to the possibility of irritation of the vein and cellulitis (Youmin Wang et al. 1999). The main disadvantages of oral and intravenous administration include – they are not targeted specific and circulate the entire body. Specifically, in treatment of DFUs topical administration would be ideal, as this method is more targeted and bypass the gastrointestinal tract.

4.3 Topical

Topical route of administration is the application of the drug directly to the surface of the skin -this mode of delivery is both non-invasive and convenient. Transdermal delivery occurs in a target specific manner ensuing local therapeutic effects while not opposed impacting the whole body and having less impact on the area of interest (Lipsky and Hoey 2009). The transdermal delivery route can also be used to deliver a steady level of drug into the systemic circulation ensuring stable blood levels effects and bypassing the first pass metabolism (Hardenia, Jayronia, and Jain 2014). Some topical products do not offer efficient penetration and the drug must be potent for a significant impact and result. Also, topical administration may be difficult to monitor the dosage and frequent re-applications may be required (Lipsky and Hoey 2009). New technologies offer various method of penetration for the drug that enable deeper transportation and more efficacious impact of the drug.

Topical DOX can be applied directly to the wound bed of the DFU. Absorption of the drug into deeper levels of the skin or mucous membranes is minute, which lowers the risk of side effects that are associated with the pharmaceutical. Topical doxycycline is employed to treat many disease pathologies such as stomatitis and corneal neovascularization (Jovanovic and Nikolic 2014; Sree Vijayabala et al. 2013). This method of administration was shown to be effective in these pathologies by alleviating symptoms and improving the condition. Topical doxycycline would also be advantageous because the targeted specific route of deliver would not produce the same adverse effect

of photosensitivity seen with oral and systemic circulating doxycycline. There is less of a chance of systemic antimicrobial resistance with topical administration, a major concern with continuous antibiotic treatment. Overall, topical administration has high patient satisfaction resulting in increased adherence and improved outcomes (Lipsky and Hoey 2009). Topical application has many advantages but there can be difficulty with the penetration of the drug through the layers of the skin and therefore its delivery to the site at which the action is intended.

5.0 TOPICAL FORMULATIONS AND TRANSDERMAL SYSTEMS

5.1 Skin Barrier and Permeability

The stratum corneum prevents the penetration of hydrophilic molecules efficaciously in comparison to lipophilic molecules (Joke A Bouwstra and Ponec 2006). Therefore, topical medication delivery can be a challenge as the stratum corneum, outermost layer of the skin is a formidable barrier only allowing small lipophilic molecules at low amounts passage. On the other side, highly lipophilic molecules penetrate the skin easily but embed themselves in the cells of the skin known as depot- not allowing for target specific delivery of the pharmaceutical. Topically applied drugs fill the lipid domains consisting of long chain ceramides, free fatty acids and cholesterol (J.A Bouwstra and Honeywell-Nguyen 2002). Furthermore, the outmost layer possesses a low pH, enzymes, and a transcutaneous concentration gradient that decreases ability for external agents to penetrate through the skin (Geusens et al. 2011). There are various factors that influence the absorption of products into the layers of the skin which include size, shape, superficial charges, lipophilicity, presence of penetration enhancers, type of formulation and physical state of the stratum corneum (Verma et al. 2003). Old topical products only offer small amounts of drug into the stratum corneum and even less penetrates the dermis and subcutaneous tissue. The majority of the drug applied topically is lost through exfoliation, sweat, absorption to clothes, degradation and washing. New topical

formulations utilize encapsulation systems to oppose forces that resist penetration and

enhance delivery of agents through the skin.
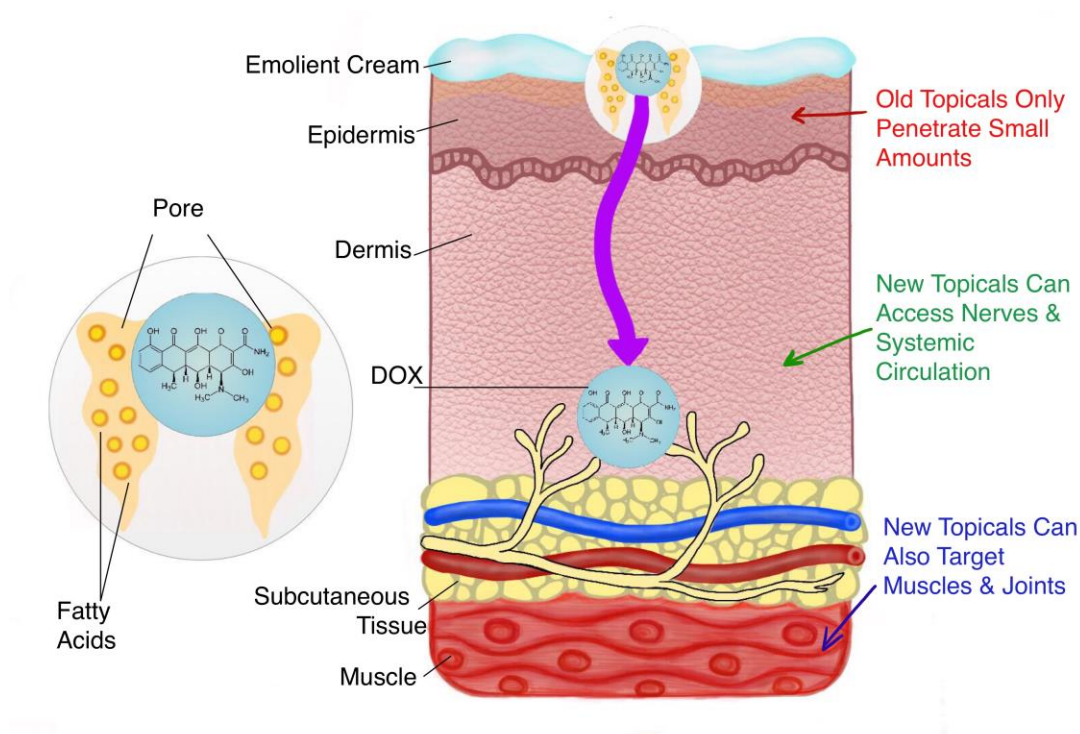
Fig. 11 – Old Topical Vs. New Topical Penetration: The old topical formulations are not able to penetrate deep into the dermis and only small amounts of drug are delivered. Whereas, new topical formulations are engineered to disrupt the stratum coreum for enhanced penetration. New topicals also have the capability to penetrate so deep they are even able to target and treat muscles alongside joints. Figure modified from Delivra Inc. 2015.

5.2 Lipid-based delivery Systems and Penetration Enhancers

Vesicular delivery systems delivering drugs through the skin have been explored previously however, the exact mechanisms are not fully understood. The four mechanisms that have been explored include: intact vesicle heavily loaded with drug; lipid vesicles acting as penetration enhancers through skin lipid fluidizing composition; direct carrier-skin drug exchange through the "collision complex transfer" occurring between the stratum corneum surface and the drug intercalated in the lipid bilayer; and lipid vesicle-mediated enhanced transdermal drug delivery through appendageal pathways such as the hair follicles and sweat ducts (Dubey et al. 2010; Elsayed et al. 2006; Hua 2015; El Maghraby, Barry, and Williams 2008). These mechanisms are influenced and consider the composition as well as the particle size of lipid vesicles. The size of the vesicle has a significant influence on penetration into the skin as larger vesicles are unable to deliver their contents into deeper layers of the skin (Verma et al. 2003)**.** Liposomes are nano-lipid vesicles that formed by one or many lipid bilayers that encapsulate an aqueous phase. These molecules are similar to the epidermis composition allowing for enhanced transportation through the tough mechanical barrier. Furthermore, topically administered liposomes accumulate in the upper layers of the stratum corneum creating a reservoir and ensue a more localized mechanism of action (Geusens et al. 2011; Honeywell-Nguyen and Bouwstra 2005; Kirjavainen et al. 1996). The composition of the vesicle is influential of the penetration capabilities, therefore new classes of lipid

vesicles with flexibility have been created, specifically - Transfersomes R, niosomes and ethosomes.

Transfersomes® are highly elastic or deformable vesicles composed of phospholipids and an edge activate, which destabilizes the lipid bilayers of the vesicle (Cevc and Blume 2001). These vesicles increase their deformity by lowering interfacial tension allowing these vesicles to squeeze through intracellular portions of the stratum corneum despite the transdermal water gradient.

Niosomes are laminar structures, non-ionic surfactant vesicles in combination with cholesterol. Niosomes are structurally similar to liposomes in possessing a lipid bilayer, however, niosomes properties make them more stable (Vora, Khopade, and Jain 1998). Niosomes' surfactant properties are involved in the structural modification of the stratum corneum, allowing this tough layer to be more loose and permeable (Geusens et al. 2011).

Liquid crystal emulsion is a novel emulsion which uses the ordered arrangement of surfactant and oil molecules formed at the oil-water interface (Zhang and Liu 2013). Liquid crystals combine the properties of both liquid and solid states; they have spatial orientation but remain in fluid. The formulation composition and process using non-ionic surfactant emulsifiers is crucial in the formation of liquid crystals. This technology offers stability of the multi-laminar structures, moisturizing and controlled release of the agent (Vilasau et al. 2011) .

Ethosomes is mainly composed of phospholipids, alongside a high concentration of ethanol (20–50%) and water. The presence of high alcohol concentration allows for the

formation of soft, flexible, malleable and highly fluid vesicles (Touitou et al. 2000). The pliable features allow these vesicles to easily and effectively penetrate deeper in the layers of the skin.

Liposomes are advantageous drug transporters that are biodegradable, non-toxic, and able to encapsulate water-soluble and lipophilic substances (Hua 2015). The various vesicles mentioned use lipid- based nano- delivery. These novel penetration enhancers increase vesicle elasticity and deformability useful in modifying the stratum corneum for efficient and effective delivery (Elsayed et al. 2006) Furthermore, these vesicles have shown enhanced therapeutic potency in comparison to the conventional liposome technologies making these structures attractive for topical drug delivery (Geusens et al. 2011).
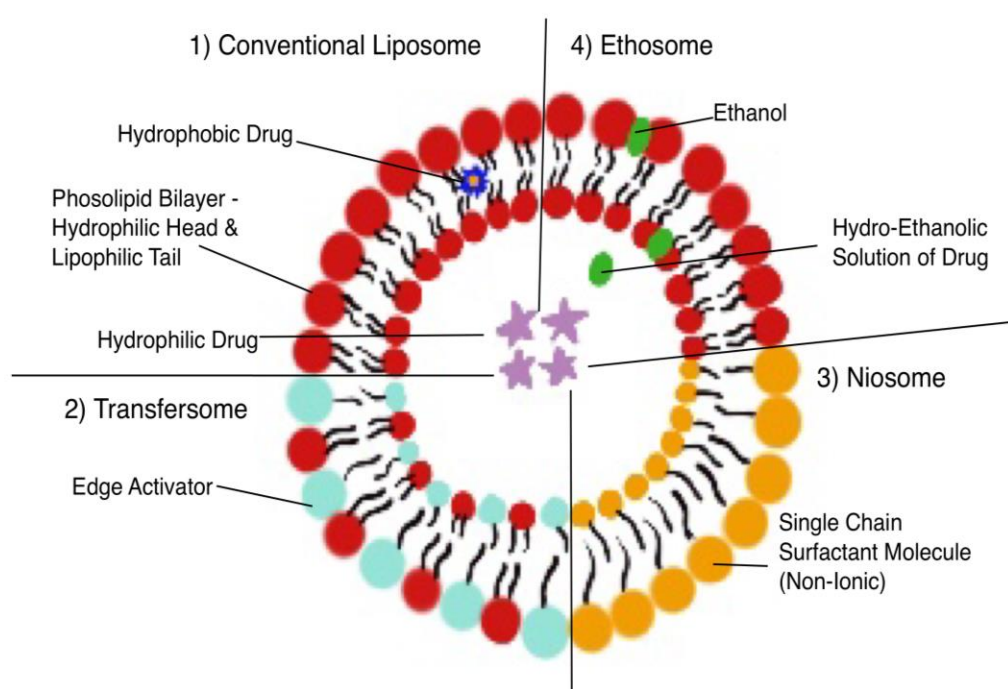
Fig. 12 – Liposomal Structure and Technologies: 1) The conventional liposome does not have enhanced penetrations and the hydrophobic drug gets trapped in the lipophilic tail of the phospholipid bilayer. There are various novel technologies developed to modify vesicles regarding particle size and composition to enhance transportation of agents. 2) Transfersome® possess an edge activator which destabilizes the lipid bilayer of the vesicle allowing for easy transportation. 3) Niosome surfactant composition allows the stratum corneum to be more loose and permeable allowing the agent to pass through. 4) Ethosomes are composed of 20-50% ethanol, creating flexible, malleable and highly fluid vesicles that aid enhanced delivery of agents. Figure modified from Geusens et al. 2011; Honeywell-Nguyen and Bouwstra 2005; Kirjavainen et al. 1996.

5.3 Iontophoresis

Iontophoresis is the process of transdermal drug delivery through a voltage gradient on the skin, in which an externally applied potential difference can increase permeability of the skin (Scifers, 2013). The pharmaceutical molecules are transported across the stratum corneum by electrophoresis and electro-osmosis. Ions prefer the routes of the least electrical resistance; therefore, transporting in areas sweat glands and hair follicles assist in the penetration and delivery of the agent (Grimnes 1984). However, with passive transdermal penetration, hydrophilic molecules usually localize in the hair follicles, and the agents require drug enhancement to assist in delivery. Through electrical stimulation, low voltage and low current density is applied, repelling ions into and through the stratum corneum. The mechanism occurs through electrostatic repulsion where ions repel against one another driving the agent through the layers of the skin. The sweat ducts are a primary path of delivery and the electrical current runs from the anode to the cathode (Grimnes 1984). The active electrode drives the cationic drug into and through the skin, which also extracts and attract the anion from the tissue underneath the skin into the anode. The return electrode (cathode) and anionic buffer ions are driven into the skin and cations from the tissues are attracted and extracted into the cathode. Iontophoresis is a painless, sterile, non-invasive technique that uses electrical current provides an additional force that drives ions through the skin and increases permeability for enhanced drug penetration (Scifers 2013). However, ionophoresis is not practical when applying medication away from your home base.
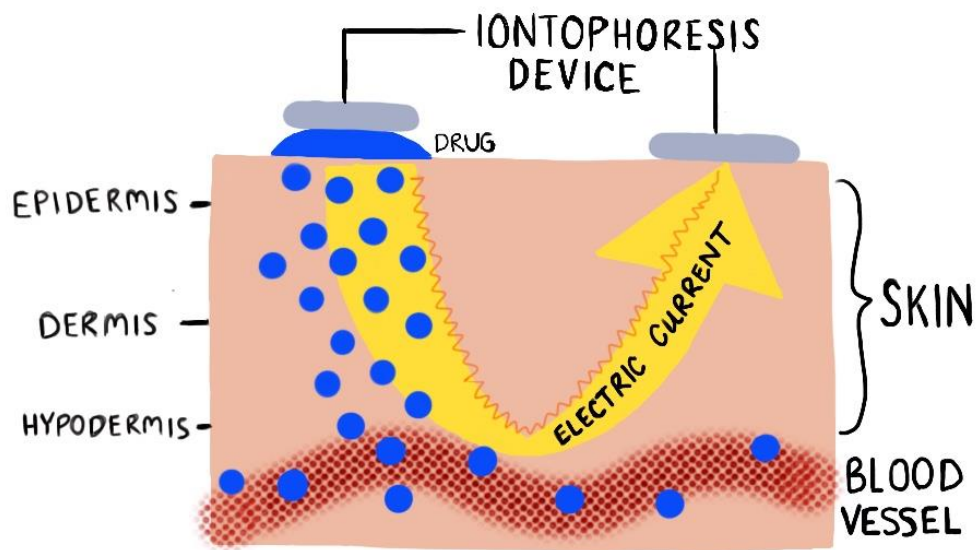
Fig. 13 – Iontophoresis Device: A method to enhance drug delivery into deeper layers of the skin. The electric current passes through the electrode, causing disruption in the skin allowing the drug to drive down into the skin, then the cations from the skin follow the current to the second electrode placed on the surface of the skin. Figure modified from Scifers, 2013.

6.0 DELIVRA^{TM} TOPCAL TECHNOLOGY

Delivra^{TM} is a liquid crystal-like emollient cream manufactured under GMP conditions using naturally occurring plant-based extracts to yield liquid crystals and a polymeric water-oil emulsion. Due to the abundance of antioxidant molecules, the Delivra^{TM} base maintains stability during product distribution networks and upon use - Delivra^{TM} disperses into structurally defined multilaminar structures that act to deliver a bioactive molecule of choice through the skin in a sustained release manner. This system has demonstrated: 1) potent pharmacokinetics; 2) efficacious outcomes in acute and chronic preclinical disease animal models; 3) extended half-life of the pharmaceutical molecules and; 4) is amenable to the formulation of highly hydrophobic, hydrophilic, and otherwise poorly stable compounds. The mechanistic properties of transdermal formulations are highly variable, ranging from gels that merely act as excipients and rely upon a bioactive's inherent lipophilic character to adsorb in and through the skin layers, to complex liposomal carriers that are integrated within stabilizing copolymers. Delivra^{TM} represents the latter, a multilaminar carrier system that incorporates various penetration enhancers that alter the outer epidermis to allow Delivra^{TM}, and its bioactive payload to enter and deliver bioactives within the underlying skin, tissues, or the circulatory system. Furthermore, ongoing research hypothesizes that Delivra^{TM}'s liposomal-like system relies upon both intercellular and intracellular pathways of bioactive penetrance through the skin layers to yielding an efficacious outcome.

Delivra^{TM} has commercialized a novel transdermal delivery system Delivra^{TM}, which can introduce various pharmaceuticals and natural molecules through the skin. DOX-Delivra^{TM} has been compounded through a specialty pharmacy group, NKS Healthcare Pharmacy (Mississauga, Ontario) to formulate DOX topically. There are constraints to the use of topical DOX- Delivra^{TM} for protease inhibition are its instability beyond 60 days and the need to deliver it into the wound tissue, not just to the wound surface. However, DOX- Delivra^{TM} ensures stability up to 6 months as the formulation can generate a long-term stable drug product with clinical efficacy. As DOX, the molecule is quite unstable in solution - the Delivra^{TM} chemists have been able to cage the DOX molecule with cyclodextrin and metal ion Magnesium. It is the objective of the company to develop a delivery system platform that can penetrate deep into the wound bed to allow for maximal protease inhibition. Another main objective of the company was to develop a simplified delivery system that will allow for stable product for a minimum of 1 year. Mass Spectrometry Liquid Chromatography will be used to assess the formulation to ensure a stable product.

7.0   MASS SPECTROMETRY

Mass Spectrometry Liquid Chromatography (LC-MS) is an analytical chemistry technique that uses the physical separation properties of high pressure liquid chromatography (HPLC) with the mass analysis properties of mass spectrometry (MS) (Henion and Maylin 1980). The HPLC separates mixtures with multiple components, while the MS provides structural identity of the separated components - providing high molecular specificity and detection sensitivity. The LCMS separates compounds chromatographically before they are introduced to the ion source and MS. LCMS is useful for understanding pharmacological products; specifically, in this study, DOX and degradation products will be investigated. Stability testing provides evidence of the quality of a drug product over a duration of time.

The instrumentation conditions are crucial when using an LCMS - as this device is highly sensitive and various parameters need to be taken into consideration. The conditions are set up to ensure optimal peaks are produced which are narrow and tall peaks that translate to higher sensitivity and resolution due to a higher concentrated analyte band (Henion and Maylin 1980). The standards are a reference tool to validate the method, the standards contain known amounts of analyte to determine parameters. The standard series is employed to ensure the experiment is working properly. The internal standard is the known amount of compound or analyte. The signal of the analyte is compared with the signal from the standard to quantify the analyte. When using LCMS, the matrix effect is a common problem impacting the results and needs to be accounted

for. There are matrix elements of the sample other than the analyte, which can have an impact on the way the analysis is conducted and the quality of the results. The matrix effect can cause suppression or enhancement of the signal, thus producing poor analytical accuracy (Ahumada, Zamudio, and España 2012). The inclusion of a spiked control accounts for the matrix effect and determines if this effect is present. A doped control determines if the extraction process occurred properly and a blank control ensures that nothing in the base of the cream possesses the analyte being tested. In this study, doxycycline was the analyte being tested to determine if the formulation degraded and examining the metabolites present.

DOX-Delivra[TM] claims their product is stable due to the formulation process which cages DOX with cyclodextrin. The LC-MS will be used to confirm stability and assess the cream over a longer duration. The formulation is currently stable when stored at 4 °C and has been anecdotally used at The Mayer Institute.

8.0 ANECDOTAL EVIDENCE

The Mayer Institute located in Hamilton, Ontario, Canada specializes in the care of diabetic foot ulcers. DOX-Delivra[TM] was applied to the diabetic foot ulcers of approximately 30 patients at The Mayer Institute. The wounds were assessed according to the University of Texas Diabetic Wound Classification System. The wounds used for the observational anecdotal study was focused on chronic wounds that had failed to heal with previous treatment and standard of care. The treatment began with debridement, removing the damaged tissue from the site of the wound and re-setting the wound bed. DOX-Delivra[TM] was then administered to the wound bed and the appropriate dressing was then applied to complete the treatment. The cream was applied once daily for duration of 12 weeks with regular follow-up to assess the progression of the wound. The treatment provided anecdotal evidence of DOX-Delivra[TM]'s efficacy in treating chronic diabetic foot ulcers and the potential to improve the standard of care. I was involved and assisted in the treatment and follow-up of these wounds treated at The Mayer Institute. There was an observed improvement in 71% of the population tested and the remaining patients comprising 29% experienced no change in their wound status. Despite the positive results, this is anecdotal evidence that did not have strict guidelines regarding exclusion and inclusion criteria. This treatment option requires further investigation, to prove quantitatively DOX-Delivra[TM]'s efficacy in the treatment of DFUs.

Fig. 14 – Wound Before the Treatment: 79 years old female with a 13-year history of diabetes. The diagnosis is a chronic non-healing ulcer: Grade A1+ ulcer located on the left, medial lower leg measuring 10 cm2 treated on May 21st, 2015 when ulcer appeared (onset: May 21, 2015). Adapted from The Mayer Institute, 2015.

Fig. 15 – Wound After the Treatment: The patient was treated with debridement (standard of care) and the application of DOX-Delivra^{TM}. The treatment was effective, and the ulcer was healed as of Jun 22, 2015. Adapted from The Mayer Institute, 2015.
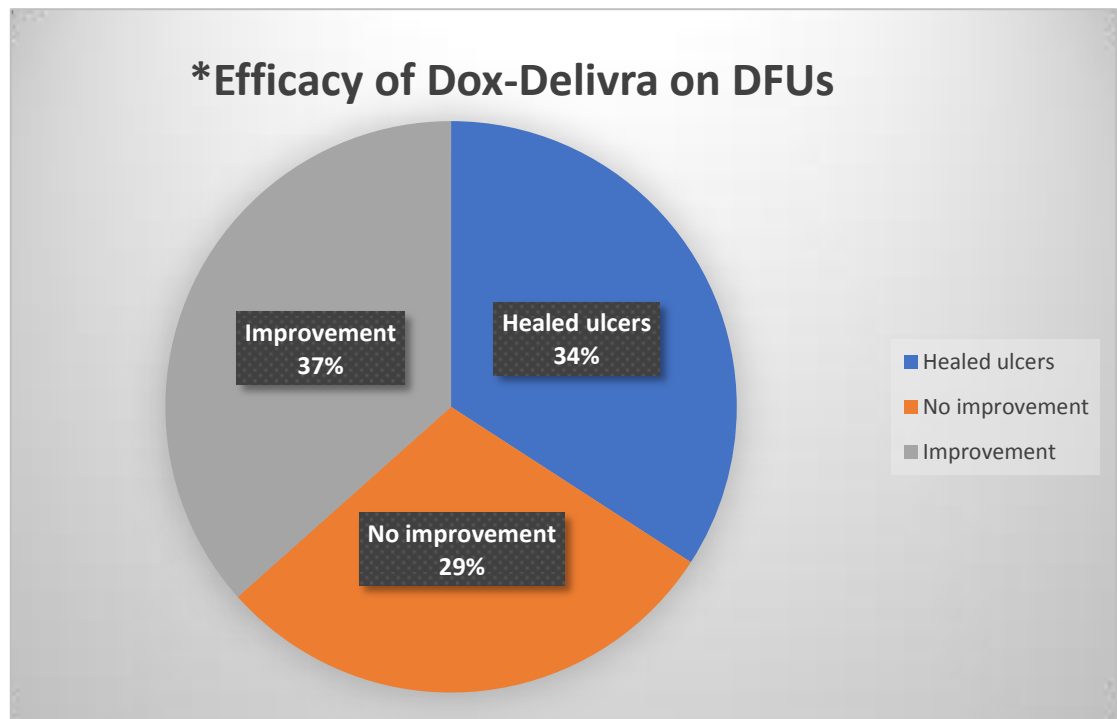
Fig. 16 - Efficacy of DOX-Delivra^{TM} Treatment on DFUs: The graph is anecdotal evidence of DOX-Delivra^{TM}'s effectiveness in healing chronic diabetic wounds. There was a sample size of n=30 at The Mayer Institute in Hamilton, Ontario, Canada. There was a total 71% effectiveness in improvement or complete wound closure observed within the sampled population. There was a total of 29% of the patients that experienced no improvement in the wound, however, with the standard of care in conjunction with the treatment of DOX-Delivra^{TM}, no patients sampled experienced worsening of symptoms or ulcers. Adapted by The Mayer Institute, 2017.

9.0 RATIONALE OF THE PRESENT STUDY

The prevalence and persistence of chronic non-healing wounds, particularly DFU'S, is linked to the prevalence of diabetes and the diseases age-of-onset, respectively. DFU's that persist eventually require amputation, yielding a decrease in quality of life and an associated high cost to the health care system. With few exceptions the therapeutic innovations in DFU treatments have revolved around variants in dressings (e.g. antiseptic and biodegradable) and their incorporation of "grandfathered" antibacterials (e.g. silver salts and synthetic dyes). While these tools are accepted components of DFU wound management their ability to affect a demonstrable change in wound healing is debatable. In general, novel DFU treatments have lagged for the past two decades and innovative non-invasive technologies are required to reduce this complication of diabetes and abrogate or delay the need for amputation. For innovation to occur an understanding of wound healings underlying mechanisms are required and how this system is perturbed in DFUs.

Chronic wounds are trapped in the inflammatory phase of wound healing with an increased presence of neutrophils and macrophages in turn increasing the production of enzymes, such as MMP-9. This construes a self-fulfilling loop, with excess MMP9 degrading ECM and yielding increased inflammatory cytokines that subsequently recruit additional neutrophils and macrophages. As such, the wound is unable to progress to angiogenesis as the ECM is being continuously degraded and not remodeled - essential for wound closure. MMP-9 Inhibition would assist the wound to allow for accelerated

collagen deposition, and adequate fibroblast proliferation that are required for normal wound healing. Doxycycline, a commonly used antibiotic that is also reported to possess enzyme inhibiting properties, would be a useful therapeutic for DFUs. Doxycycline is predominately administered through oral application, however, there are many disadvantages associated with this route of administration including: first pass pharmacokinetics, photosensitivity and increased chances of antimicrobial resistance. More drug is required to actively treat the condition, whereas topical delivery offers a method - delivering the pharmaceutical molecule in a targeted specific manner at a potent level. There are two major disadvantages of topically delivering doxycycline: 1) instability and; 2) penetration into deep layers of the skin. DOX-Delivra^{TM} has created a novel technology delivery of doxycycline that circumvents these disadvantages associated with topical application. The formulation process is novel and offers enhanced penetration and stability of doxycycline making Delivra^{TM} a potential tool for treating chronic wounds. Specifically, the inclusion of cyclodextrin surrounds and protects the agent retarding oxidation. Further, cyclodextrin surrounds and protects the drug, doxycycline, to provide increased stability of this tetracycline-like antibiotic in solution. The formulation process creates a liquid crystal- like emulsion with liposomal structures that aid the moisturizing and preparation of the skin and flexibility for enhanced penetration. The DOX-Delivra^{TM} compounded product has been used anecdotally at The Mayer Institute; observational evidence of this DOX-Delivra^{TM} topical suggests it may promote wound healing in DFUs. These preliminary results support further investigation of the product in relation to MMP inhibition and determine is this is the mechanism of

action that is decreasing MMP expression and increasing wound closure. MMP-9 activity will be assessed, as this is the major MMP associated with inflammation and chronic wounds. The mechanism of this interaction is also of interest to understand how the inhibition is occurring. This mechanism has been hypothesized to occur through metal chelation, which is responsible for inactivating the enzyme. This claim has not been quantitatively proved and one of the objectives of this study is to test this mechanism of action. Stable topical doxycycline that has the capability to penetrate deep into the skin - contains many advantages in the treatment of DFUs, as this is a highly unmet need in today's healthcare.

10.0 HYPOTHESES AND OBJECTIVES

The long-term objective of this work is to understand the topical delivery of doxycycline in the DOX-Delivra formulation, in terms of its stability and function and its impact on the healing of DFUs. That long term objective is beyond the scope of this project which will focus on understanding the stability and the mechanism of action of the DOX-Delivra formulation itself.

Hypothesis

The transdermal doxycycline formulation, DOX-Delivra^{TM}, is stable, retains both the anti-MMP9 and anti-microbial activity with prolonged storage, and has sufficient anti-MMP9 activity to inhibit the levels of MMP9 in DFUs.

Specific Objectives:

1) To investigate the stability of the transdermal doxycycline formulation, DOX-Delivra^{TM}, with prolonged storage.

2) To assess the anti-MMP9 activity of doxycycline and to compare that with methacycline, and ensure this activity is within the clinical concentration.

3) To assess the antimicrobial activity of doxycycline and to compare that with methacycline.

METHODS AND MATERIALS

11.0 STABILITY OF DOXYCYCLINE DELIVRA

The stability of the transdermal doxycycline formulation, (DOX-Delivra[TM],) was assessed using a 2% w/w preparation that was stored for 2 separate time periods of 15 days and 30 days. The DOX-Delivra preparations were stored in airtight, light protected containers at -20, 4 and 25°C. In the first experiment, samples were tested at days 2, 5, 8, 12, and 15. In the second experiment, the samples were tested only at 30 days.

The samples were assessed by Mass Spectrometry Liquid Chromatography to detect both doxycycline and its degradation product epi-doxycyline. In addition to the DOX-Delivra stability samples, assessments were performed on a doxycycline standard, a Delivra base sample, and fresh preparation of 2% w/w DOX-Delivra.

11.1 Materials

Doxycycline hyclate, usp (Medisca 0434-04), Doxycycline-d_3 hyclate (Toronto Research Chemicals D561503), methanol (Omnisolv MX0488-1), acetonitrile (Caledon 1404-7-40), HPLC water (Caledon 8801-7-40), Arlasolv DMI (Uniquma), Delivra[TM] base (Lot 13750-2), sodium hydroxide (Sigma S8045), hydrochloric acid (bioshop, HCl333.500), Trifluroroacetic acid (Sigma, 299537), MgCl$_2$ (Sigma, M8266), β-hydroxypropyl-cyclodextrin (Sigma 332593),

11.2 Instrumentation

High performance liquid chromatography- tandem mass spectrometry was carried out on 5500 QTRAP Mass spectrometer (ABSciex, Concord, Canada) with a TurboV source, equipped with Agilent 1260 (Agilent Technologies, Santa Clara CA, USA) HPLC.
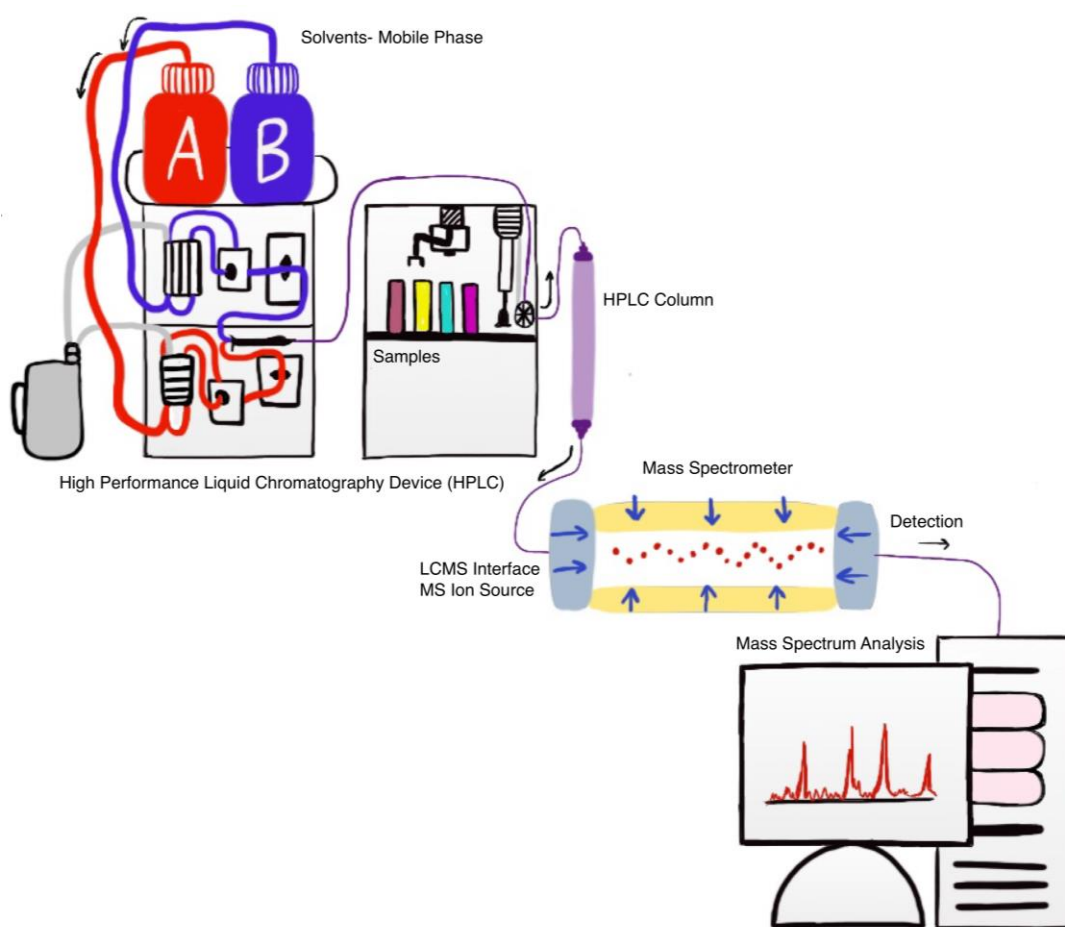
Fig. 17 - High Performance Liquid Chromatography- Mass Spectrometry: The pure mobile phase passes through the flow cell, the slower the samples move through the HPLC column the wider the band. Before the sample reach the detector, the compounds will be separated chromatographically before introduced to the ion source and mass spectrometer. The bands will then be read as peaks via the mass spectrum analysis. Figure modified from ABSciex, Concord, Canada.

11.3 Formulation

A topical formulation of doxycycline hyclate was prepared by first dissolving the drug (100 mg) in Arlasolv (1 mL), then adjusting the pH of the solution to between 5.5-6.5 with a 2 M aqueous solution of NaOH. Delivra^{TM} base (4 g) was then added to the solution, and the mixture was homogenized using vortexing, sonication manual stirring. The formulation was stored in an airtight, light-protected container at -20, 4, and 25 ^{o}C for 30 days until analysis.

A topical formulation of doxycycline hyclate was prepared by first dissolving the drug (100 mg) and β-hydroxypropyl-cyclodextrin (β-HPCD) (268 mgs) in distilled water (1 mL), then adjusting the pH of the solution to between 5.5-6.5 with a 2 M aqueous solution of NaOH. Delivra^{TM} base (4 g) was then added to the solution, and the mixture was homogenized using vortexing, sonication manual stirring. The formulation was stored in an airtight, light-protected container at -20 ^{o}C, 4 ^{o}C , and 25 ^{o}C for 30 + days until analysis.

A topical formulation of doxycycline hyclate was prepared by first mixing the drug (100 mg) and $MgCl_2$ (18.5 mg) in Arlasolv (1 mL), then adjusting the pH of the solution to between 5.5-6.5 with a 2 M aqueous solution of NaOH. Delivra^{TM} base (4 g) was then added to the solution, and the mixture was homogenized using vortexing, sonication manual stirring. The formulation was stored in an airtight, light-protected container at -20, 4, and 25 ^{o}C for 30 + days until analysis.

11.4 Stability Testing Methods

The test article to be analyzed was weighed into scintillation vials in triplicate and then enough extraction solvent (50:50 methanol: water adjusted to pH 2 with 1 M HCl) as added to make a 1 mg/mL solution of the test. The samples were then sonicated for 30 minutes at room temperature and centrifuged at 11,000 rpm for 10 minutes. 10 µL of this solution was then added to 990 µL of a 126 ng/mL solution of d_3-doxycycline hyclate in methanol/water (50:50). The resulting solution was mixed thoroughly and submitted for analysis.

Stock solutions of doxycycline and d_3-doxycycline were prepared by dissolving accurately weighted standards in 60:28:12 water: acetonitrile: methanol to generate 1 mg/mL solutions, and all other working solutions were prepared from these. A solution of 1 µg/mL solution doxycycline hyclate + 125 ng/mL d_3-doxycycline in 50:50 methanol: water ($MeOH:H_2O$) was serial diluted with a 125 ng/mL d_3-doxycycline solution to give a standard series of 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 ng/mL of doxycycline hyclate and a constant concentration of 125 ng/mL d_3-doxycycline hyclate. Ratio of peak area of doxycycline to peak area of d_3-doxycyline was plotted against doxycycline concentrations and used to produce a standard curve in the form of $y = A + Bx$ using weighted least squares linear regression. Each batch of unknown samples was accompanied by a standard series, and a set of quality control samples. Blank samples were prepared from Delivra[TM] base cream, processed and evaluated for specificity. Delivra[TM] base was spiked with doxycycline at a concentration of 2 % w/w for evaluation

of the precision, accuracy and recovery of the method. A blank extract of Delivra^{TM} base was spiked with doxycycline at a concentration of 2 % w/w to assess matrix effects.

11.5 HPLC- MS/MS conditions

Isocratic chromatographic separation was performed on a C18 column (Eclipse XDB C18 column (4.6 x 150 mm, 5 μm, Agilent USKH095544)) with guard using a mobile phase of acetonitrile (0.1% trifluoroacetic acid): water (0.1% trifluoroacetic acid) (30:70) at a flow rate of 0.75 mL/min for 10 min. The first two minutes was sent to the waste and doxycycline elutes between 6.5-7.5 mins. There was no post time. The column temperature was 30 °C and injection volume was 5 μL. A 5500 QTRAP from AB Sciex Instruments equipped with an electrospray ionization (ESI) probe was used in the positive ion mode with multiple reaction monitoring (MRM) for the quantitative analysis. Nitrogen was used as the collision gas and the curtain gas. The curtain gas was 10.00 psi, the collision gas was medium, the ion spray voltage was 5000 volts, the temperature was 500 °C, and gas sources 1 and 2 were 45 and 50 psi respectively. The declustering potential was 40 volts, the exit potential was 10.00 volts, the focusing lens 1 was -10.50 volts and the cell exit potential was 4.00 volts. Quantification was performed using the transitions m/z 444.8 -> 428.2 (CE = 28 V, 100 msec) for doxycycline and 447.8 -> 431.2 (CE = 28 V, 100 msec) for d_3-doxycycline with low resolution. Analytical data was acquired, and quantification processing was performed by using Analyst software.

12.0 ENZYME INHIBITION

The MMP-9 inhibition of doxycycline was measured in order to investigate whether the doses that are being used clinically had the ability to inhibit MMP-9 activity. The activity of doxycycline inhibiting MMP-9 was assessed in an MMP-9 inhibitory screening fluorometric assay. Initially, the assay was performed to exhibit the MMP-9 activity, the tetracycline test materials were introduced into the assay, producing an inhibitory effect on MMP-9 activity. The experiment was conducted using dose-dependent varying concentrations to assess the potency of the inhibition. The IC50 value was determined and was within the clinical concentration being employed.

12.1 Materials

MMP-9 Inhibitor Screening Assay Kit- Fluorometric (abcam; ab139449), Doxycycline hyclate, usp (Medisca 0434-04), Methacycline hydrochloride (Trc can M258_5)

12.2 Instrumentation

MMP-9 Activity Assay Kit (Abcam: ab139449) uses a fluorescence resonance energy transfer (FRET) peptide as a generic MMP activity indicator via florescent micro plate reader. The kit is designed to determine the activity of an MMP enzyme and to screen

MMP inhibitors. In the intact FRET peptide, the fluorescence of one part is quenched by another. After cleavage into two separate fragments by MMPs, the fluorescence is recovered. The kit possesses excellent fluorescence quantum yield and longer wavelength, which is highly sensitive. The signal can be easily read by a fluorescence microplate reader at Ex/Em = 490/525 nm.

12.3 MMP9 Activity Assay Methods

MMP-9 activity was measured by using a commercially available MMP-9 Inhibitor Screening Assay Kit (abcam; ab139449) and the procedure was completed as described within products manuals. Briefly, using provided 96-well microplate 70 μL of MMP-9 enzyme (0.013 U/μL final concentration 0.009 U/μL) in assay buffer along with 20 μL aliquot of assay buffer that included various concentrations of test articles (see below) or positive controls. The microplate reactions were mixed and pre-warmed to 37°C, 10 μL of substrate (40 μM, final concentration 4 μM) was added, and real-time kinetics were collected over 10 minutes at 1-minute intervals using excitation and emission wavelengths of 328 nm and 420 nm, respectively. Relative fluorescence units per minute (RFU/min) were fitted to a linear equation (y=mx+b) and graphical representations of reaction velocity (m) and drug concentration along with IC50 analysis were completed using the software GraphPad Prism.

The experiments involved doxycycline hyclate and methacycline hydrochloride at a 1.0 mM solution of the compound was prepared in deionized water. Using this stock a

1:1 serial dilution was constructed with a range of 1,000 – 15.6 µM in assay buffer. For each serial dilution concentration 20 µL was added in duplicate to enzyme reaction wells. The entire experiment was conducted using two interday repeats to yield a final IC50 value. The plate reader was warmed up to 37°C and SpectraMaxM2; Softmax pro 6.3 was used. The settings included the read type being kinetic at 328 (Excitation)/ 420 (Emission), duration 10 minutes, 1-minute interval (kinetics), Temperature 37°C, shaking 5 seconds before reading. Then 10 µL of MMP9 substrate working solution was added to each well promptly to ensure accurate results.

13.0   ANTIMICROBIAL

The antimicrobial activity of doxycycline was measured to determine whether the dose that is being used clinically possesses antimicrobial activity. The tetracyclines that were assessed were doxycycline, methacycline and CMT-3. The chemically modified tetracycline is of particular interest as this drug has been modified to remove the antimicrobial properties associated with tetracyclines and therefore alleviate the concern of antimicrobial resistance. These molecules were assessed in an E.coli culture assay, in which different dilutions of the test materials were plotted into Agar plates with the bacterial culture solution. The colony forming units was the measure used to assess whether the test materials inhibited possessed antimicrobial activity. The IC50 values were determined from the dose-dependent response to demonstrate the potency of the of each test material.

13.1 Materials

E. Coli Stock BL21 (DE3) pLysS (Promega L119B), LB Agar Medium (Bio Basic; SD7003 S519), LB Broth -Lennox (Bio Basic; SD7005), Doxycycline hyclate, usp (Medisca 0434-04), Methacycline hydrochloride (Trc can M258_5), 4-demethylaminosancycline- CMT-3 (Echelon Biosciences B-0802)

13.2 Instrumentation

The antimicrobial assay was employed with the inclusion of a bunsen burner apparatus and autoclave instrumentation to ensure a sterile environment of experimentation. The broth was prepare using the Nautilus rotating incubator and the plates were stored in the incubator for bacterial growth and to determine inhibition. The colonies were assessed using OpenCFU 3.9.0 colony counting software. The IC50 values and other statistical parameters were derived through GraphPad Prism V5.01 software.

13.3 Antimicrobial Activity Assay Methods

In vitro bacterial activities were examined for tetracycline analogs. This was a standard antimicrobial inhibition assay BL21 E.Coli, to evaluate dose-dependent changes in colony forming units (CFUs) The preparation of agar plates, LB broth and bacterial

broth was performed. The test material, tetracycline analogs, were prepared, producing a 1mg/mL solution that was then serially diluted to prepare work sample solutions, achieving 8 decreasing concentrations of the test material (12.50-0.10ug/mL). A vehicle control was also prepared and examined. The bacterial broth was diluted to achieve a working dilution and the dilution factor was factor was determined in a previous assay (Assay No. DNWH-BGK-170410, WI No. 20170329-01). The plating solutions were prepared by combining the work culture and work sample solutions, by mixing 500uL of sample work solution to 500uL of the diluted broth. The final bacteria dilution in each sample will be 2.18E-7, the optimal cell concentration determined in Assay No. DNWH-BGK-170410. The LB agar plates in 37°C incubator warmed for 10 minutes. The experiment was performed in duplicates and each test material and vehicle control plating solution was ass to an LB plate and spread evenly using a sterile cell spreader. The plates were incubated upside-down overnight at 37°C (18-20 hours). The OpenCFU software was used to count the colonies on each plate. The colony count was compared to the test material concentration to assess each test material's antimicrobial activity. The Graphpad Prism V5.01 software was used for data analysis. The "log(inhibitor) vs. response – Variable slope (four parameter) non-linear fit" algorithm was used to determine the IC50 value of both test materials assessed.

RESULTS

14.0 STABILITY OF DOXYCYCLINE DELIVRA

14.1 Doxycycline Quantification Method Validation

The formulation of doxycycline was assessed over 15-day and 30-day duration. The LC-MS was able to detect doxycycline and d3- doxycycline and ensure the parameters and instrumentation were optimized. A calibration curve is also generated in this process to the determine the concentration of doxycycline from the observed spike. The blank sample of Delivra base did not show any peaks consistent with doxycycline, and the spikes with doxycycline and d3-doxycycline were similar in the Delivra base to those that were in solution only. There was no evidence of interfering peaks from the blank samples.
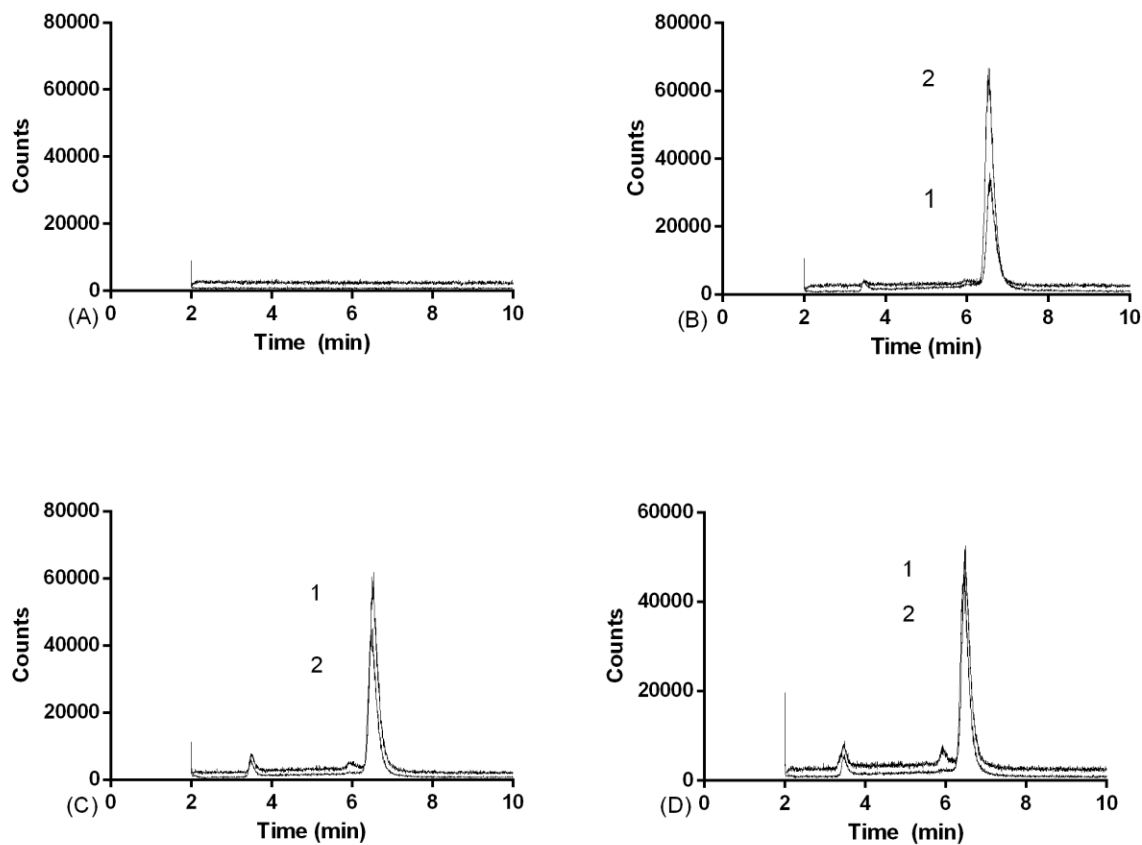
Fig. 18: LC-MS/MS profiles of doxycycline and d₃-doxycycline. Chromatograms of (A) Blank sample (B) Doxycycline (62.5 ng/mL) and d₃-doxycycline (125 ng/mL) standard solution (C) Blank Delivra^TM base spiked with doxycycline (200 ng/mL) and d₃-doxycycline (125 ng/mL), and (DOX) Doxycycline extracted from formulation (stored at 4°C for 30 days) and d₃-doxycycline (125 ng/mL).

14.2 Doxycycline in Formulation Over 15-Day Duration

In stability studies, the preparation was regarded as stable if the stored preparation was shown to have no less than 85% of the doxycycline concentration that was present in the fresh preparation. Figure 19 shows the concentrations of doxycycline in the Delivra preparation at each of the timepoints of 0,2,5,8,12 and 15 days. The measured concentration at day 0 was 2.54%. From this graph it is clear that with storage at 4C and at room temperature, the levels of doxycycline show slight degradation. However, at 37C, the levels show more significant degradation from day 8 and longer of storage.
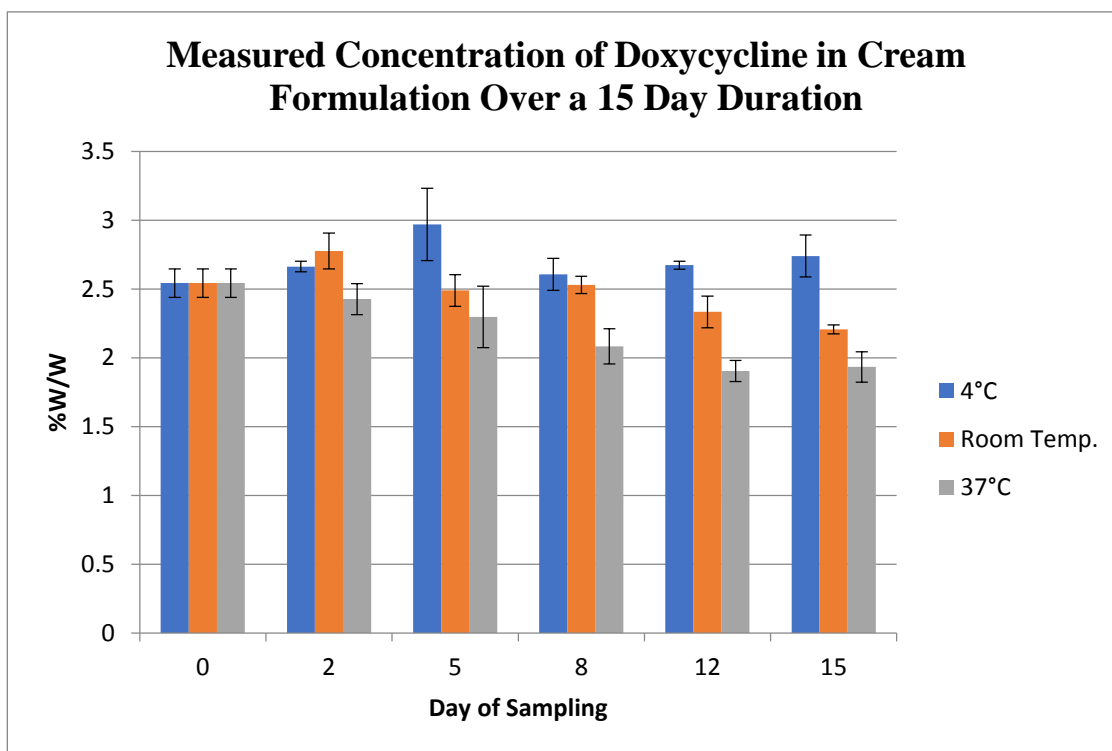

Fig. 19: Concentration of doxycycline hyclate amongst formulations stored under 3 conditions (4°C, Room Temperature, 37°C) were assessed regarding their stability.

14.3 Epi-Doxycycline in Formulation Over 15-Day Duration

As the doxycycline degrades, the degradation product of epi-doxycycline is produced. Figure 20 shows that the concentration of this degradation product gradually increases in all samples and is most noticeable in the samples stored at 37C. This supports the observations made with the doxycycline concentrations over the different durations of storage. The extraction efficiency of doxycycline was out of linear range within this experiment. Therefore, modifications to the isocratic parameters were required and a follow-up experiment was employed to determine if the formulation was stable for 30-days.
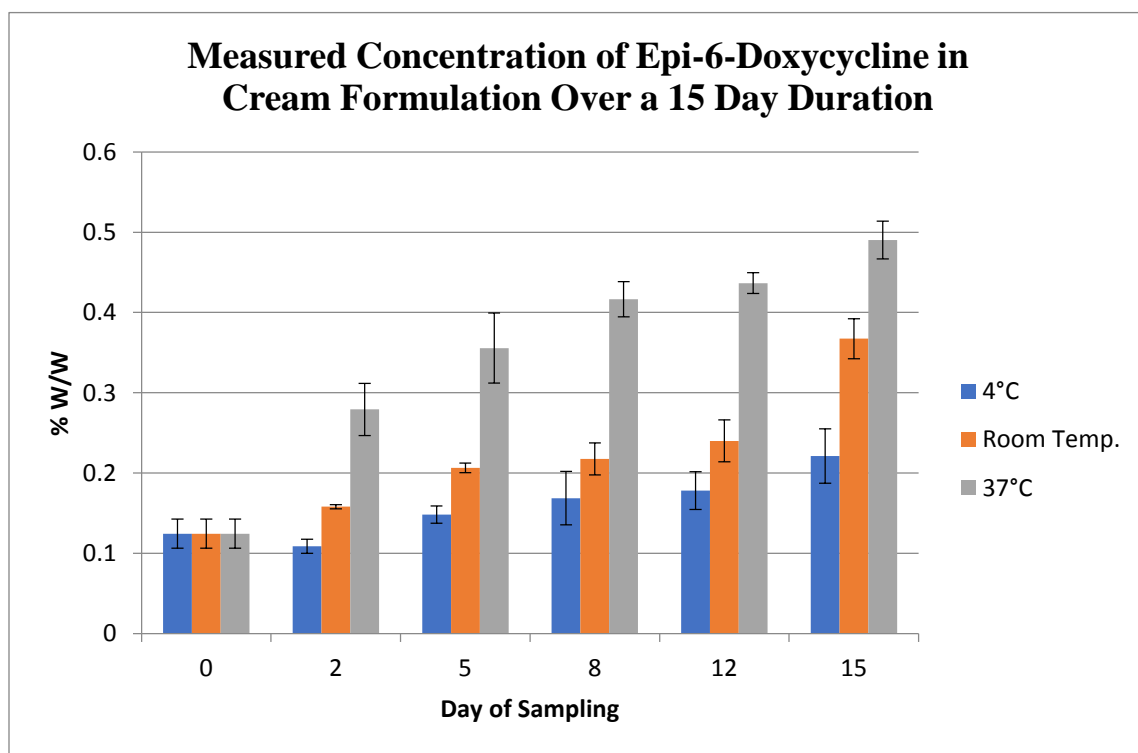

Fig. 20: Concentration of 6-Epi-Doxycycline amongst three formulations prepared at varying temperatures, to assess the stability of the Delivra[TM] formulation.

14.4 Doxycycline in Formulation Over 30-day Duration

A longer period of storage of 30 days was tested with storage at 4C and -20C (Figure 21). When tested at 30 days, the samples demonstrated levels that were within the 85% threshold, thereby indicating stability in storage under these conditions. The stability of doxycycline in formulation proved stable over the duration of 30-days. The difference between the concentrations measured was used to understand the % decomposition. The standard error of the mean between technical replicates gives an indication of the homogeneity of the formulations. The experiment assessing the formulation over 15 days under went degradation due to the LC-MS and required optimization before further testing was executed. The degradation % regarding the extraction efficiency was within acceptable range for the remainder experiments.
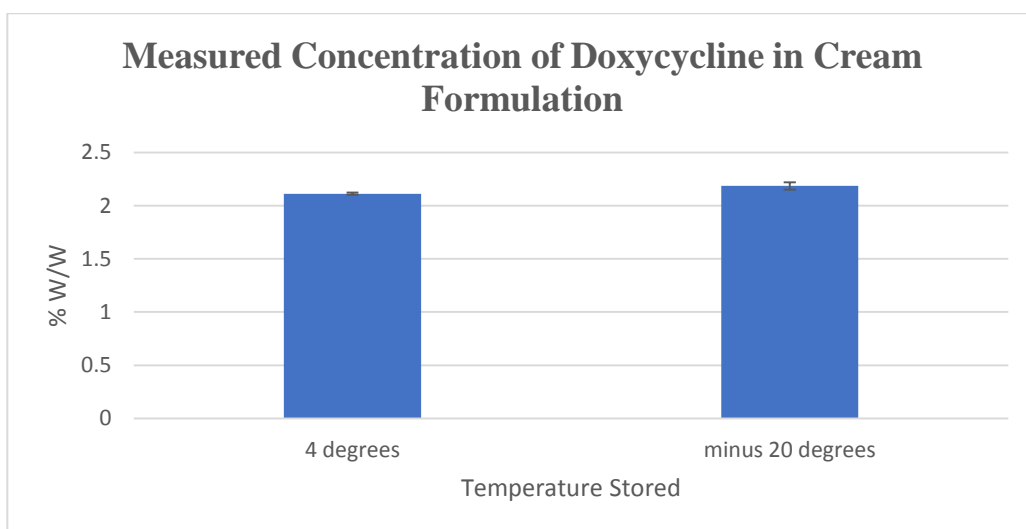
Fig. 21: Stability and concentration of doxycycline hyclate formulation measured over 30-days with storage at – 20°C, and 4°C.

15.0 MMP9 INHIBITION

15.1 Calibration Curve

Tetracyclines- doxycycline and methacycline inhibition of MMP9 was assessed using and enzyme inhibition assay. A calibration curve was generated (Figure 22) to determine the linearity of fluorescence in the assay. This is a method used to quantify the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration.

Calibrator Curve Using Free Peptide Fluorophore

$y = 137.72x + 93.694$
$R^2 = 0.9832$
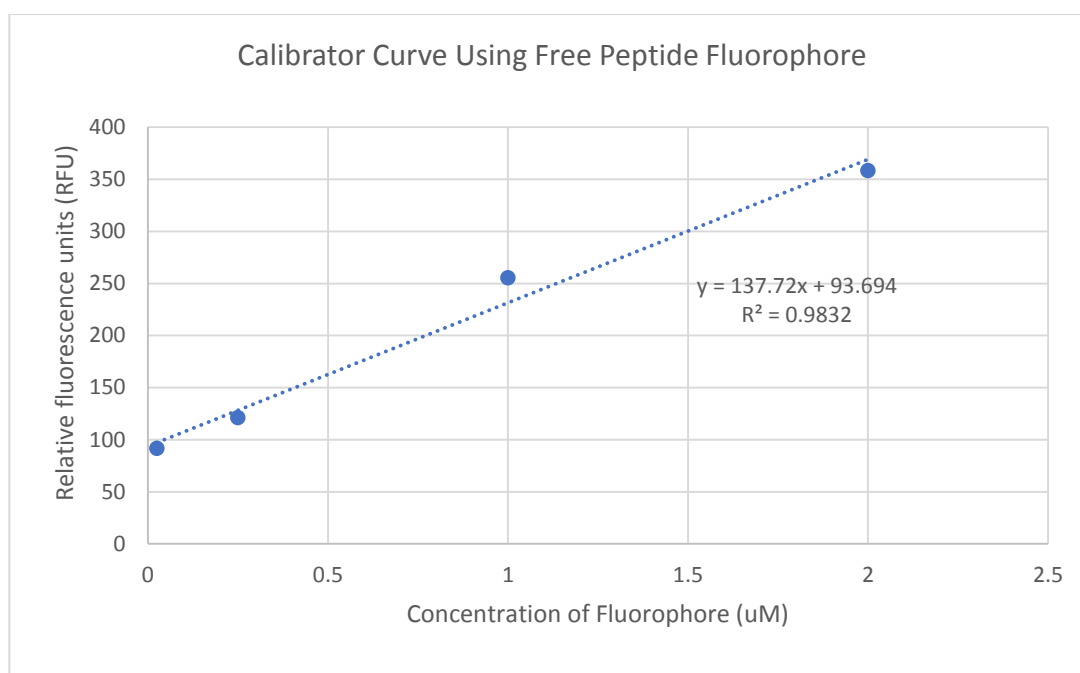
Figure 22. Calibrator curve of free peptide fluorophore. Varying concentrations of free fluorophore was measured to determine the linearity of fluorescence within the assay.

15.2 Activity and Inhibition of MMP-9 Enzyme

MMP-9 enzyme activity was initially determined to ensure the enzyme was active as this enzyme is secreted in its latent form. The kit also possessed an inhibitor which was the positive control of the experiment to ensure the enzyme was being inactivated within the system. These controls were employed (Figure 23) to compare the activity of tetracyclines and determine if the action was inhibition or activation of MMP9.
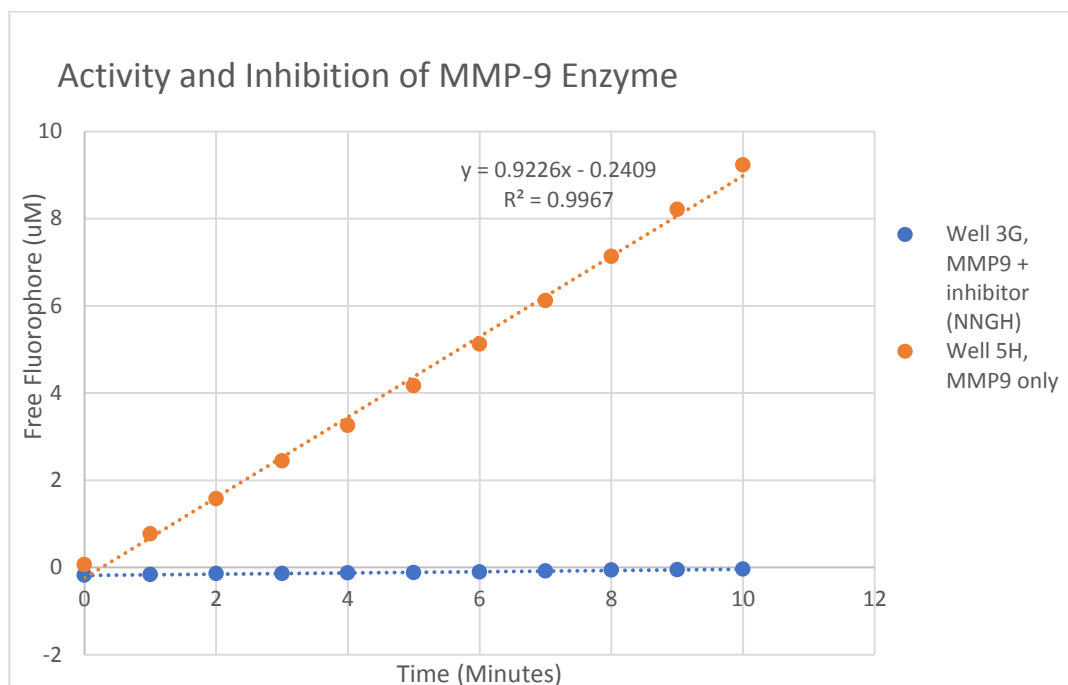
Fig. 23: The activity and inhibition of the enzyme was measured to ensure the system and kit was functioning optimally.

15.3 Activity and Inhibition by Tetracyclines

Doxycycline and methacycline both possess enzyme inhibition properties. The IC50 is the concentration of an inhibitor where the response is reduced by half; this is a quantitative measure to determine how much of an inhibitor is required to inhibit a given biological process. Log(inhibitor) vs. Response variable slope was used to determine the IC50 of the inhibitor. The IC50 values of two inhibitors were compared on their inhibition properties on MMP9. The IC50 of doxycycline hyclate was 48.27 uM and methacycline hydrochloride was 71.99 uM (Figure 24). Tetracyclines successfully inhibited MMP9 enzyme activity and possessed antimicrobial properties, yielding IC50 values that supported previously reported findings.

Figure 24- Log(inhibitor) vs. Response variable slope, comparing the enzyme inhibition activity of the two test materials. The error bars are representative of the SEM. The IC50 of doxycycline hyclate was 48.27 uM and methacycline hydrochloride was 71.99 uM.

16.0 ANTIMICROBIAL ACTIVITY

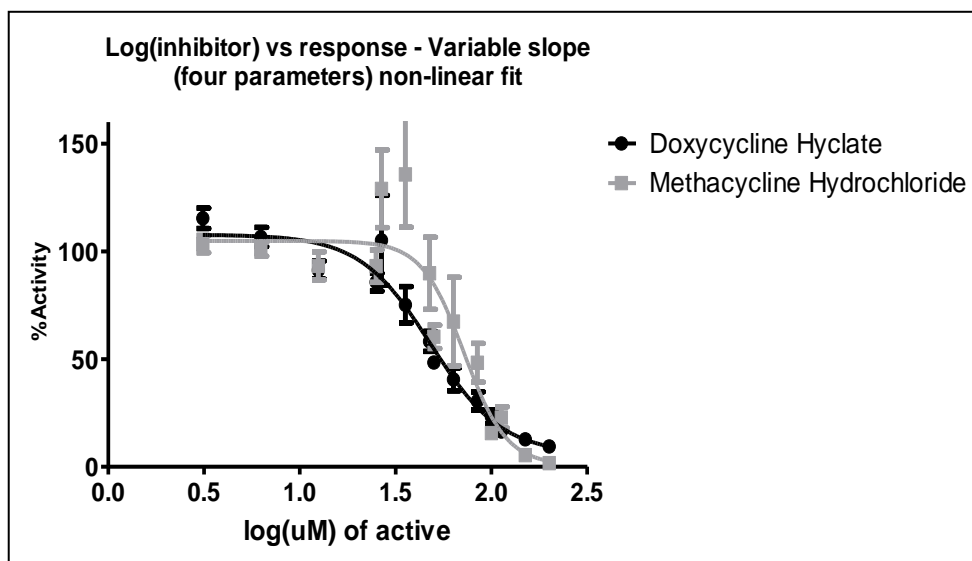Antimicrobial activity was assessed for tertacyclines- doxycycline, methacycline and CMT-3. The inclusion of CMT-3 within this experiment was to investigate this tetracycline as it has been chemically modified to exclude the antimicrobial properties of the molecule that is associated with antimicrobial resistance when repetitively prescribed and used as a therapeutic. The dose-dependent changes were evaluated and with the increasing concentration of tetracyclines less colony forming units were present. Log(inhibitor) vs. Response variable slope was used to determine and compare IC50 values of the various tetracyclines. The IC50 values were doxycycline hyclate 4.32 μM, methacycline hydrochloride 2.53 μM and CMT-3 1.62 μM. Doxycycline and methacycline supported previously reported IC50 values, solidifying the antimicrobial properties of the drug. However, CMT-3 was also determined to possess antimicrobial activity, which was not the suspected result. Therefore, further investigation is required for CMT-3 to understand this chemically modified tetracycline and its role as a potential therapeutic.

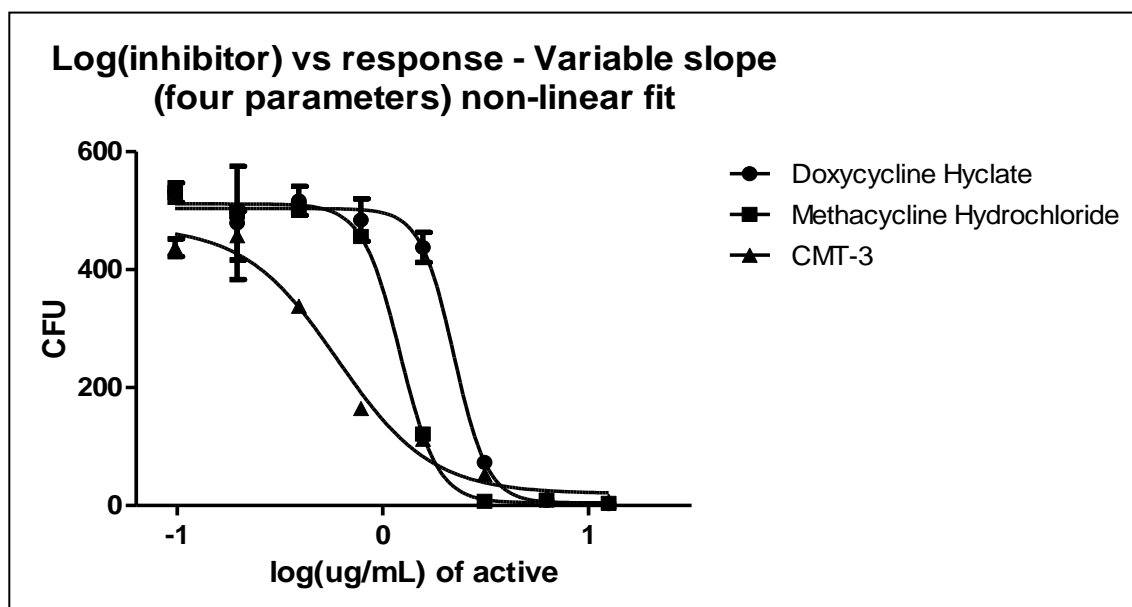**16.1 Antimicrobial Inhibition by Tetracyclines**

Fig. 25: IC50 Values- doxycycline hyclate 4.32 μM, methacycline hydrochloride 2.53 μM and CMT-3 1.62 μM.

DISCUSSION

17.0 General Overview of The Present Study

Diabetic foot ulcers are a chronic problem within the diabetic population and their effective treatment is a relative unmet need for the healthcare system. Wound healing is an orderly multifaceted process that involves MMP activation and inhibition through out the various stages. Patients with diabetes experience impaired wound healing, as their MMP levels are disrupted, trapping the wounds in the inflammatory stage. The standard of care and novel affordable treatments have stalled, and new treatment options are warranted. Tetracycline analogs are a group of broad-spectrum antibiotics, whose primary mechanism of action is to inhibit protein synthesis through the binding of the bacterial ribosome. In addition, tetracyclines act as an anti-inflammatory molecule by inhibiting MMPs, a family of zinc-dependent proteases that contribute to tissue remodeling, inflammation, and angiogenesis – these enzymes are over-expressed in certain pathophysiologies such as DFUs. Doxycycline maybe a suitable treatment option for healing chronic wounds such as DFUs, primarily due to its robust MMP inhibition. Topical DOX is an advantageous route of administration potentially minimizing the risks associated with systemic antimicrobial resistance. However, DOX is known for its instability in aqueous formats and in typical topical formulations. DOX-Delivra^{TM} offers a stable topical formulation with the ability to penetrate deep into the skin and deliver the

molecule efficaciously. The stability of DOX-Delivra^{TM} was examined to determine whether the product remains intact over a duration of time.

In the present study, DOX was evaluated regarding its inhibition of MMP-9 activity and its anti-microbial activity. The present study confirmed that reported results of doxycycline's MMP-9 inhibition may support this molecule as a viable tool for treating chronic wounds. The stability of DOX-Delivra^{TM} was assessed through a simple quantification method using liquid chromatography and mass spectrometry (LCMS). This method of analysis was used to assess whether the DOX-Delivra^{TM} formulation was stable and could therefore be useful as a potential treatment option for wounds.

17.1 Stability of Doxycycline Formulation

The concentration and stability of doxycycline hyclate in Delivra^{TM} formulation was assessed over three different temperatures (4°C, Room Temperature, 37°C). The experiment showed a gradual degradation of doxycycline throughout the duration of 15 days and an increase of its metabolite, 6-epi-doxycycline, over the 15-day duration. This metabolite was an epimer, with the same weight but with varied stereochemistry. Repeating this study would help to understand if some form of experimental error may have contributed to this result. The extraction efficiency surpassed the acceptable range and therefore, a follow-up experiment was required as this was a limitation within this experiment. The instrumentation required modifications and optimization of the protocol before the 30-day experiment was executed.

DOX- Delivra stability was also assessed over 30-day duration to at -20°C and 4°C temperatures. The average concentrations of doxycycline hyclate measured for the formulations stored at each temperature were within 10 % of the starting concentration of 2% w/w doxycycline hyclate. The differences in the measured concentrations of doxycycline hyclate in the formulations that were stored at -20°C and 4°C were not statistically significant (P = 0.09). The standard errors between replicates were relatively small, suggesting that the formulation maintained an appropriate level of homogeneity. Under these conditions and temperatures, the formulation demonstrated stability as doxycycline did not undergo extensive and complete degradation. The accelerated temperature of 37°C demonstrated a more extensive degradation of the formulation than the other two storage temperatures assessed. The samples were also stored in light-shielding containers which assisted in the stability of the formulation.

The DOX-Delivra^{TM} formulation was also assessed under three different temperature variations (4°C, Room Temperature, 37°C) over a 70-day duration. The DOX-Delivra formulations were prepared using the solvent Arlasolv, in addition with β-HPCD in water that resulted in concentrations of DOX within 15 % of target concentration of 2 % w/w after 70 days of storage at 4 °C. The incorporation of magnesium chloride in the formulation resulted in an increase in degradation of DOX at room temperature. All the samples had a measured DOX concentration within 15 % of the intended 2 % w/w over the course of the analysis (1-3 months) when stored at 4 °C in light-shielding containers. The storage conditions had a direct impact on the formulations' stability. These results provide further evidence of the stability of the

formulation as this analysis was conducted over 1-3 months. These experiments indicated doxycycline's stability in this formulation.

17.2 MMP-9 Inhibition

Having established the stability of DOX-Delivra, this study evaluated the bioactivity of doxycycline. The IC50 of doxycycline hyclate was 48.27 uM and methacycline hydrochloride was 71.99 uM. The results are consistent to those of Bannikov et. al., who evaluated neutrophil-purified MMP9 with several low molecular inhibitors and found an IC50 of 48 μM for doxycycline (Bannikov et al. 2009). These results support doxycycline as an exogenous inhibitor and a potential agent for DFU treatment. Exogenous inhibitors are required to inactivate MMP9 and to facilitate wound healing.

Experiments were conducted to determine the inhibition of MMP-9 activity by doxycycline hyclate in solution vs. in the DOX-Delivra formulation. Doxycycline hyclate in solution, DOX-Delivra^{TM} formulation, and vehicle control (Delvira^{TM} base) produced IC50 values 48.27 uM, 62.92 uM and > 250 μM, respectively. The IC50 value of doxycycline in solution is consistent with the literature, however there is limited information on the MMP9 bioactivity of doxycycline within formulations. Doxycycline is a slightly less potent MMP9 inhibitor in formulation compared to doxycycline in solution. The IC50 values produced in the experiment was well within the clinical concentration; therefore the formulation will possess potent inhibitory effects on

enzymatic activity. The formulation would have to be tested directly to make further claims on its inhibitory properties. Formulation optimization may be warranted to produce a more potent inhibitor. Further investigation into the effect of topical doxycycline on MMP9 levels in wound fluid would give an indication of its potential clinical impact on MMP9 inhibition in chronic wounds. Ultimately, future clinical assessments will be needed to determine if the wound fluid expression of MMP9 is reduced by the application of DOX-Delivra to chronic wounds.

17.3 Antimicrobial Activity

Doxycycline hyclate and methacycline hydrochloride possessed antimicrobial activity as reported in previous literature (Modheji et al. 2016) As the concentration of the test product increased fewer colonies were formed, demonstrating the dose-response relationship with the inhibition of bacterial colony formation. The IC50 value of doxycycline hyclate was determined to be 4.22uM and the IC50 value of methacycline hydrochloride was determined to be 2.53uM. The IC50 values produced in the experiment was well within the clinical concentration; therefore the formulation will possess potent inhibitory effects on bacterial activity. The formulation would have to be tested directly to make further claims on its inhibitory properties. CMT-3 antimicrobial activity was also assessed. The expectation for this chemically modified tetracycline was that the drug would not inhibit colony formation as this tetracycline has been modified to exclude the antimicrobial function of the molecule. This previous observation was not

supported by the results, which found that CMT-3 had an IC50 of 1.62 uM. Therefore, further investigation is required as this result did not support the reported literature, which suggested that this chemical modification would combat the problem of antimicrobial resistance.

CONCLUSIONS

The measured concentrations of doxycycline in the cream formulations were within 10 % of the starting concentration after storage for over 30 days at 4°C and were not significantly different from those with the cream stored at -20°C. The results suggest that the concentration of doxycycline was stable over this time frame under these specific storage conditions. The standard error of the mean between technical replicates gives an indication of the homogeneity of the formulations. The instrumentation required further optimization after the 15-day duration experiments and the parameters were modified to increase the precision and sensitivity for the 30-day assessment. The follow-up experiment assessed the formulation over 30-days and this proved stable. When DOX-Delivra was assessed over a 70-day duration, this also proved to be stable. Therefore, given the stability of the DOX-Delivra formulation, it may be able to be used to treat DFUs.

All of the drugs and chelators displayed inhibition activity of the enzyme, MMP-9 within the micromolar range. The test materials: doxycycline, methacycline and EDTA all yielded similar results for inhibitory effectiveness. The mechanism of action responsible for this enzymatic inhibition must be investigated in further experimentation.

All of the tetracyclines assessed for antimicrobial activity demonstrated inhibitory mechanism of action on the colony forming units of the assay. This was the anticipated result, however the chemically modified tetracycline also possessed antimicrobial activity, which was not expected as this tetracycline is meant to only have enzyme

inhibiting function. Therefore, further investigation is warranted to understand this specific drug.

Transdermal DOX- Delivra^{TM} formulation offers a stable drug product for a regular prescription duration suitable to aid in chronic wounds, specifically, DFUs. These studies have confirmed that doxycycline both antimicrobial activity and is able to inhibit the activity of matrix metalloproteinase-9. DOX is adequately potent in order to sufficiently inhibit matrix-metalloproteinase-9 activity. The inhibition by the DOX-Delivra^{TM} is slightly lower than the inhibition from DOX alone but is still at a level that is considered adequate for MMP-9 inhibition.

Clinical trials are needed to demonstrate both the efficacy of DOX-Delivra to inhibit MMP-9 the in chronic wound environment, and in improving ulcer healing. If its efficacy is ultimately proven in wound healing, DOX-Delivra has the advantages for clinical use bit being affordable, easy to apply, and is delivered directly to the wounds, thereby reducing anti-microbial resistance,

FUTURE DIRECTIONS

The current mechanism of action of doxycycline in inhibiting MMP is currently considered to be by chelation of the catalytic zinc atom from the enzyme active site (García et al. 2005). A preliminary experiment was performed to investigate the mechanism of action between doxycycline and MMP9. Zinc was added to the assay to further understand the chelating mechanism of action of doxycycline and comparing that to methacycline and EDTA. With the addition of zinc to EDTA the inhibitory effect on MMP-9 was reversed, and as the zinc concentration increased, the MMP-9 inhibition was further reduced. However, for doxycycline and methacycline the addition of zinc in all concentrations did not reverse MMP inhibition. The questions is whether the mechanism of action between the tetracyclines and MMP9 is due to divalent ion chelation.

Further investigation is warranted to understand the mechanism of action of doxycycline on inhibiting MMP-9 activity, and the role of zinc chelation. Further evaluation could also need explore whether the divalent cation chelation is interacting with the calcium ion rather than with the zinc ion.

The understanding of how doxycycline is inhibiting the enzyme MMP9 is important when developing therapeutics as well as treating conditions with elevated levels of the enzyme. This would allow further understanding of how the drug is interacts with MMPs and this knowledge may have implications for its application in the clinical setting.

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