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Venoms as Potential New Therapies Targeting the Epidermal Growth Factor Receptor Family

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Abstract

The discovery and development of new breast cancer treatments is vital as current therapies, both targeted and non-targeted have been shown to have toxic side effects and cancers have become resistant to the treatment. The pursuit of new appropriate therapies can be directed towards naturally occurring proteins or peptides found present in nature such as venomous secretions. Understanding where and how breast cancer management started and where it currently stands is key in avoiding failure and covering as many avenues as possible. Although there are a variety of treatment strategies available, breast cancer management is still limited in terms of survival and prognosis. Understanding what those limits are and how they affect treatment response is relevant for future clinical trials. The present review focused on targeted and non-targeted therapies, molecular cancer subtypes and available alternatives for drug development in breast cancer. Among notable alternatives for drug development, venoms are now being utilised in drug discovery research due to their rich variety of small peptides, organic and inorganic compounds. With an estimated 12 million peptides awaiting identification and characterisation, venoms are a collection of diverse and attractive drug development platforms. Currently there have been a number of preliminary studies undertaken using venom and cultured human cancer cells, but more research is needed, not only to inform on the effects, but the need for more research is as relevant as ever, in order to identify as many venom compounds as possible. This paper will report a number of these studies.

Introduction

With a list of six FDA approved drugs, venom-based drug discovery is a relevant field of research for future anti-cancer therapies. Studying and identifying toxins, peptides and other compounds from venomous animals will improve our knowledge of the potential of these naturally occurring peptides against human cancer cells.

Methods

The following work investigates the effects of three types of venom *Acanthoscurria geniculata (A. gen)*, *Heterometrus swammerdami (H. swa)* and *Naja Naja* (*N Naja*) on the human breast cancer cell line MDA-MB-468 using SDS-PAGE and analysing protein expression – Epidermal Growth Factor Receptor. This investigation will also report the results of using *A gen.* venom fractions on MDA-MB-468, which overexpresses EGFR, the first member of the Epidermal Growth Factor Receptor family of tyrosine kinases using HPLC, SDS-PAGE and Western Blot methods. Using the venom from the above species on human phospho-array blots, the effects the types of venom have on phosphorylation levels on a range of clinically important cell surface receptors was revealed.

Results

It was found that crude venom (*A gen., H swa* and *N Naja*) as well as venom fractions (*A gen.*) affected receptor phosphorylation in a time and concentration dependent manner, however more experimental research is required to understand the optimal conditions for the experiments to be carried out appropriately i.e. venom time incubation, antibody treatment.

Acknowledgements

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The past, present and future of breast cancer: Venoms as potential new therapies targeting the epidermal growth factor receptor family

1.1 Introduction

Cancer is one of the leading causes of death and disability worldwide (Ferlay *et al*, 2014). In 2015 it accounted for 8.8 million deaths worldwide with breast cancer being accountable for 571,000 deaths at this time (World Health Organisation (2015).

Cancer occurrence is rising in part due to an increasing and aging population but there are other factors that are involved in the onset of cancer pathologies which include smoking, the increasing segment of overweight population, lack of physical activity, shifting reproductive patterns and poor diet (Torre *et al*, 2015).

There are many types of malignancies, such as lung, breast, stomach, colorectal, head and neck and pancreatic carcinomas. In fact there are over 200 types of cancer are believed to exist (Cancer Research UK (2017). Investigations into the mutations that lead to cancer pathogenesis have revealed that these affect a range of regulatory pathways at cellular level that end in tumour formation (Cairns, Harris & Mak, 2011). Consequently, the efficacy of targeting individual signalling molecules is under scrutiny, as a variety of oncogenic signalling pathways are collaborating in order to adapt tumour cell metabolism to ensure growth and survival (Cairns, Harris & Mak, 2011).

The oldest mention of breast cancer known to date was found in an ancient Egyptian papyrus, written around 3000 BC. The papyrus is believed to be a textbook on surgery, describing breast cancer as having no treatment. However, Hippocrates, who was a Greek physician, also known for establishing the foundation for modern medicine, coined the word

carcinoma or carcinos. The term cancer, which was translated by Roman physician Celsus from Greek to Latin, translates into crab and the resemblance is due to blood vessels forming structures that are similar to crab legs (Hajdu, 2010; Sudhakar, 2009)

Developments in the field of cancer research and treatment was a slow process, mainly because most medical trends established during Galenic, Hippocratic or Dark Ages suggested that treating cancer may lead to further suffering and the consensus was that cancer was incurable. Although surgery has been established since ancient Egyptian times, the process was both painful and raw, leading to more complications and blood loss, which fuelled the idea of an incurable disease. It was another Greek physician, Galen, who coined the term *oncos*, which translates into swelling, also is used today to describe experts and specialists in the field of cancer. During the nineteenth and twentieth century, surgery was led into a new era, partly due to the newly installed field of anaesthesia and working in a sterilised environment. This allowed classic cancer operations to be undertaken, in particular a key breast cancer treatment that is still used today, the mastectomy (American Cancer Society, 2017).

1.2 Molecular mechanisms and breast cancer phenotypes

In modern times, to facilitate diagnostic and therapy pathways, breast cancers have been classified into subtypes using gene expression analysis. These subtypes have been identified and characterised via microarray technology (Tamimi et al, 2012; Sørlie et al, 2001). On the other hand, immunohistochemistry and nucleic acid in situ hybridization have confidently been used for the past 25 and 15 years respectively, and are considered cornerstones in identifying endocrine receptor (a receptor molecule that binds to either oestrogen or progesterone hormones) positive or negative and Her-2 amplified or non-

amplified subtypes, respectively (Pusztai et al, 2006). The more aggressive tumours are associated with the presence of Her-2, making the presence or lack thereof of Her-2 in tumours is a relevant prognostic factor and a relevant clinical target (Nahta and Esteva, 2003).

The main breast cancer subtypes are luminal A, luminal B, Her-2-enriched, basal-like breast cancer and normal like breast cancer which is known to represent endocrine receptor positive breast tumours (Tamimi et al, 2012; Creighton, 2012). Zardavas et al (2015) describes these subtypes based on the various levels of expression of the biomarker triplet – oestrogen Receptor (ER+), Progesterone Receptor and HER2 protein. Luminal A type cancer expresses Oestrogen and Progesterone Receptors (respectively ER and PR), with low proliferation index and decreased pathological grade. Luminal B tumours are ER and PR positive, are characterised by high proliferation rate and diminished response to hormonal treatment. HER2-enriched cancers are characterised by overexpression of HER2 gene and are associated with relapse and poor prognosis (Zardavas et al, 2015; Liu et al, 2015). Triple negative breast cancer (TNBC) also known as basal-like cancer does not express ER, PR or HER2 (Liu et al, 2015) and there is no well-established targeted therapy for this subtype as of yet (Creighton, 2012). Triple negative breast cancer subtype is known for is overexpressing EGFR, the first member of the Epidermal Growth Factor Receptor family. This subtype is associated with poor response to therapy leading to poor prognosis (Zardavas et al, 2015). As such, EGFR is a potential target for future drug development endeavours focused on triple negative breast cancer management. In a study conducted by Liu et al (2015), the difference between basal and HER2 enriched subtypes was investigated via immunohistochemical (Dittadi et al, 1993) and statistical analysis. From a clinicopathological point of view, the investigation revealed the luminal type breast cancers are less aggressive compared to basal and HER2 enriched types. Moreover, basal-like cancers overexpressed more EGFR than all other cancer subtypes

(Liu et al, 2015). The relationship between growth factors and respective oncogenes is evident and, as far as EGFR is concerned, it plays an important role in cell growth, proliferation and differentiation, since its phosphorylation activates signalling pathways involved in these cell processes (Herbst, 2004). In other words, many healthy cells express 4×10^4 to 1×10^5 receptors per cell, while some breast tumours express up to 2×10^6 receptors per cell (Herbst, 2004). Other markers used in this study included Ki-67, which is a nuclear antigen with a role in cell proliferation, and p53 tumour suppressor gene, with a role in cell transformation and characterisation (Liu et al, 2015). The result of the investigation revealed that HER2-enriched and basal-like or triple negative breast cancers are more aggressive, given the higher expression of EGFR and Ki-67 compared to luminal breast cancer. Claudin-low subtype was identified in 2007 and the investigations into hierarchical gene clustering revealed this subtype is close related to basal-like subtype (Eroles et al, 2012; Prat and Perou, 2011).

Up to a study on 78 breast cancer samples (Sørlie et al, 2001), the convention for breast cancer classification was rather simple: a basal epithelial-like group, a HER2 overexpressing group and a normal-like breast cancer subtype group. By systematically investigating the gene expression patters in human breast tumours, Perou et al (2000) found different independent gene clusters reflecting the histological variation of breast cancers, such as endothelial cells stromal cells, adipose-enriched cells, lymphocytes, T lymphocytes and macrophages.

1.3 The Epidermal Growth Factor Receptor family

Epidermal Growth Factor Receptor or EGFR is one of the four members of the Epidermal Growth Factor Receptor family, comprising of EGFR, HER-2, HER-3 and HER-4. The four genes encoding the members of the Epidermal Growth Factor Receptor are c-erbB-2/HER2, c-erbB3/HER3 and c-erbB4/HER4 (Herbst, 2004). Dysregulation of their activity or the pathways

related to these growth factor receptors are present in various cancer pathologies, including breast cancer (Lurje and Lenz, 2009; Harbeck et al, 2013).

Multicellular organisms owe their intricacy and complexity to one particular event in evolution: emergence of signalling pathways between cells. These systems are communication channels between cells or environment, fine-tuned to receive, interpret, follow or create instructions for or from other cells and/or environment. A powerful tool in the molecular biology of signalling pathways are cell surface receptors. Receptor tyrosine kinases are skilful chaperones for pathways involved in cell proliferation, differentiation and other cell processes relevant for the pathobiology of many cancer types (Kovacs et al, 2015).

One of the hallmarks of cancer as described by Hanahan and Weinberg (2011) is sustainable proliferative signalling which is described as a fundamental trait in cancerous cells. Breast cancers are the result of clonal expansion events, where daughter cells inherit mutations that provide them with a selective advantage (Curigliano and Criscitiello, 2014). When investigated via genome wide sequencing, these mutations can reveal their role in the selection process, whether they are driver or passenger (not involved in the selective process) (Curiglino and Criscitiello, 2014). By disrupting the normal growth and cell division regulatory circuits, cancer cells become able to sustain chronic proliferation (Hanahan and Weinberg, 2011; Stamos, Sliwkowski & Eigenbert, 2002). The signals involved in cell proliferation and apoptosis begin with the phosphorylation of cell surface growth factor receptors i.e. the epidermal growth factor receptor (EGFR).

EGFR belongs to the ErbB family of receptor tyrosine kinases (RTK's) and was the first receptor tyrosine kinase to be discovered (Bazley and Gullick, 2005, Herbst, 2004). These cell surface receptors possess tyrosine kinase activity and are found exclusively in metazoans (Stein & Staros, 2000). The completed human genome revealed 58 of the 90 tyrosine kinase

sequences are receptor tyrosine kinases (Kovacs et al, 2015). The 11 ligands identified for mammalian EGFR are the Epidermal growth factor (EGF), transforming growth factor-α (TGF α), heparin-binding EGF (HB-EGF), betacellulin, amphiregulin, epiregulin, epigen and the neuregulins (NRG's) 1-4 (Bazley and Gullick, 2005). Initial investigations brought up by the discovery of the EGFR provided insight in the mechanisms by which receptor tyrosine kinases function and aided scientists in identifying a link between an activated oncogene and cancer (Bazley and Gullick, 2005).

All receptor tyrosine kinases consist of three main domains: an extracellular domain with a ligand binding region, a transmembrane domain and a C-terminal, intracellular domain. The intracellular domain comprises of a juxtamembrane region, a catalytic tyrosine kinase domain and a carboxyl terminal tail.

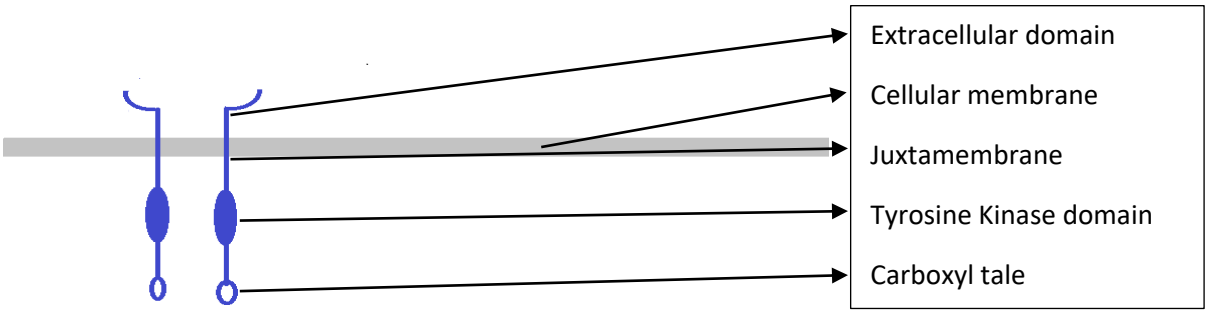


Fig. 1 – Structure of Epidermal Growth Factor Receptor

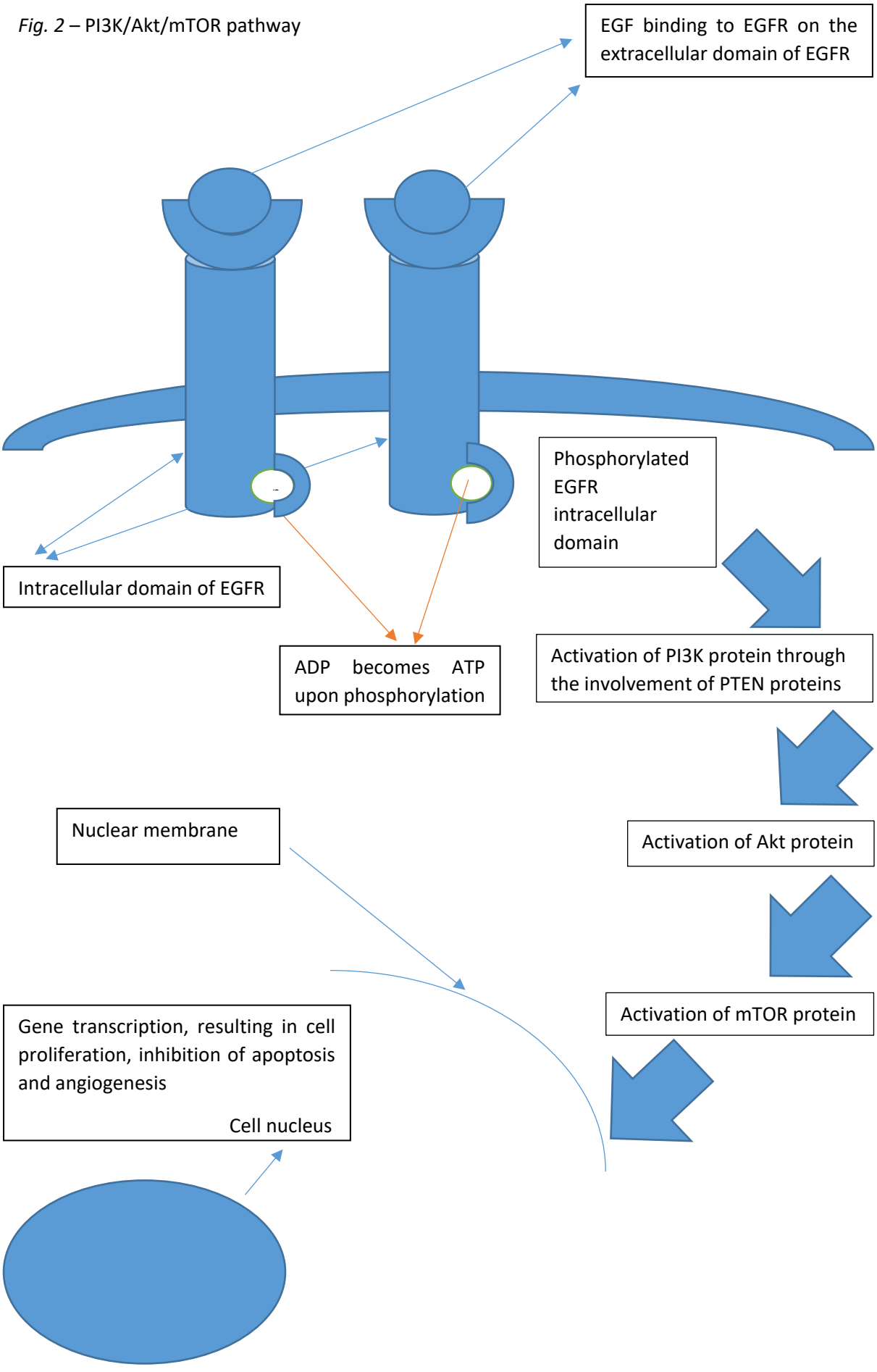
EGFRs are transmembrane proteins, which are activated (phosphorylated) following binding of the EGF and dimerization of the receptor (Normanno et al, 2006). However, there is no known ligand for HER-2 and HER-3 lacks tyrosine kinase activity (Harbeck et al, 2013). Both EGFR and EGF are found to be overexpressed in several cancer malignancies such as breast cancer but also lung, neck and colorectal to name a few. Scientific investigations show that these proteins together are able to induce cell transformations (Normanno et al, 2006). Amplification of the EGFR gene and mutations of the EGFR tyrosine kinase domain are

common in various cancer pathologies and receptor tyrosine kinases have been found to be overexpressed or mutated in some breast cancer subtypes associated with high mortality rates (Normanno et al, 2006, Herbst, 2004, Zhang et al, 2007), such as inflammatory and triple negative breast cancer. In terms of activation, EGFR undergoes a series of structural events meant to integrate various inputs (signals) and yield several outputs (functions) via signalling pathways. The EGFR signalling pathways are involved in a series of important cellular process such as growth, proliferation, differentiation and apoptosis. Abnormal oncogenic phosphorylation of EGFR or any other member of ErbB family of receptors will disturb the normal course of the cellular processes these control. The structure of the cytoplasmic region of EGFR consists of three domains. The juxtamembrane domain is required for feedback by protein kinase C; the noncatalytic carboxy-terminal tail with the required six tyrosine transphosphorylation sites is required for binding to adaptor/effector proteins and finally the central tyrosine kinase domain which is responsible with relaying transphosphorylation of the six carboxyl tyrosine residues (Bazley and Gullick, 2005; Kovacs et al, 2015). This region is the focus for some targeted therapies (Zhang et al, 2007). The EGFR family of receptor tyrosine kinases is known to affect various cellular and physiological processes in breast cancer and deregulated EGFR pathways promote tumorigenesis and metastasis (O'Brien et al, 2014). As such, EGFR overexpression is associated with poor prognosis and differentiation. In addition, the phenomenon is more common in triple negative breast cancer (see previous section) and inflammatory breast cancer, the two most aggressive breast cancers subtypes (Ueno and Zhang, 2011; Eroles et al, 2012). Inflammatory breast cancer is very difficult to manage as treatment is lacking appropriate strategies and clinical targets (Van Laere et al., 2013; Morrow et al., 2017). Although limited by their efficiency and clinical response, there are treatment approaches available for triple negative breast cancer, but the consensus

dictates there are no standard procedures (Oakman et al, 2010). Treatments include anthracyclines, taxanes, platinum based chemotherapeutic agents and bevacizumab in European countries (Oakman et al, 2010; Swain et al, 2013).

Some of the signalling pathways activated by EGFR family are the Ras-Raf-MAPK, JAK/Stat and PI3K/Akt/mTOR (Figure 1) pathways (Eroles et al, 2012; Masuda et al, 2012). Any imbalance in activation or mediation of these pathways affects a range of cellular and physiological processes directly involved in malignant transformation in breast cancer (Masuda et al, 2012, Eroles et al, 2012). For instance, mutations associated with the PI3K/Akt/mTOR pathway are molecular signatures for aggressiveness and chemoresistance in metastatic breast cancer (Guerrero-Zotano et al, 2016). The PI3K/Akt pathway is a mediator for mammalian target of rapamycin (mTOR), which influences cell growth and proliferation via biosynthesis of proteins, lipids and by limiting catabolic processes (Zaytseva et al, 2012).

Fig. 2 – PI3K/Akt/mTOR pathway



EGFR expression in breast malignancies is associated with lack of oestrogen receptors, which is often rendering hormonal therapy as ineffective (Curigliano and Criscitiello, 2014). EGFR is also a driver for tumour proliferation, meaning the presence of EGFR in breast cancer subtypes basal-like, luminal B and normal like is often a poor prognosis hallmark (Eroles et al, 2012).

In an investigation by Shi et al in 2012 showed that nuclear EGFR was found to activate the cyclin D1 gene, which plays a key role in proliferation regulation, via transcription in the cell nucleus. This new pathway is believed to transport EGFR, following binding with EGF, to the cell nucleus with the aid of binding co-factor STAT3 or via the integral trafficking from the ER to the Nuclear Envelope Transport model (Brand et al, 2013). According to the same study, presence of nuclear EGFR and FGFR is associated with proliferation in breast cancers, and can be potential prognostic factors (Hsu et al, 2009).

Adenosine monophosphate AMP-activated Protein Kinase has been observed to inhibit the overall development of malignancies, by disabling growth factors from stimulating oncogenic processes (Jhaveri et al, 2015). The gaps in knowledge and other underlying issues, such as investigation of secondary tumours (Zardavas et al, 2014) push the continuous research in the field of breast cancer.

Another set of recurrent issues in current research focused on oncology related fields are increased costs. Translating research into clinical trials has a low success rate, due to factors such as disease type, inadequate or insufficient cell lines or insufficient number of experiments (Begley and Ellis, 2012). Available data and literature are the basis for most investigations and drug development endeavours, more so regarding novel therapies and molecular targets (Begley and Ellis, 2012). Traditional treatment courses of therapy provide decent response and survival rates. However, the high grade of non-selectivity of these drugs

leads to cytotoxic symptoms and many patients do not tolerate these drugs very well (Esteva, 2004). Understanding the molecular intricacies of breast cancer is a powerful tool in developing novel targeted drugs (Nahta and Esteva, 2003). The purpose of many studies on novel therapies is to target the molecular aspects of transformed cells, leading to better response rates and less toxicity to normal cells (Esteva, 2004).

1.4 Current therapies

Traditionally, all types of cancer malignancies have been managed via the three main therapies: surgery, chemotherapy and radiotherapy. However, more tailored treatments have now been developed for many cancers including breast cancer. These treatments include hormonal therapy, antibody therapy and immunotherapy (Adams, 2017).

In modern medicine, surgery is used in a multidisciplinary setting, to improve prognosis. Surgery is employed in early stage disease, when the tumour is accessible and is not affecting any vital organs or elements thus limiting its effectives in cases were the tumour spreads termed metastasis (Dimitrov et al, 2017). As common practice, a border of healthy tissue is removed during the process, to diminish the chances of recurrence. As far as breast cancer is concerned, there are two types of surgery. The first is lumpectomy, which conserves most of the breast tissue and removes primary tumours, also known as wide local excision. The second is mastectomy, which is removal of all breast tissue (Ohsumi et al, 2007, Dimitrov et al, 2017). Historically, William Stuart Halsted developed radical breast removal as a surgical anti-cancer therapy during the nineteenth century. In 1970, clinical trials and new developments led to the conclusion that local excisions will be just as effective in treating breast cancer, followed by radiation therapy (Dimitrov et al, 2017). John Collins Warren (1778-1856) together with Joseph Colt Bloodgood (1867-1935), two American surgeons,

ushered a new trend in surgical treatment of breast cancer by advocating for pre-surgery biopsy (Lakhtakia & Chinoy, 2014). Following suit, Sir George Lenthal Cheatle (1865-1951) and Dr Max Cutler documented all their findings from performing surgery and biopsies and thus creating the first modern textbook of breast cancer (Lakhtakia & Chinoy, 2014).

Radiotherapy found a niche in the field of cancer treatment following Wilhelm Conrad Roentgen's discovery of a new type of ray, in 1896. The name X-ray was given since 'x' was the algebraic symbol for unknown quantity. In the next three years from discovery, x-rays were used in cancer treatments. As a result, in 1901 Roentgen was awarded the first Nobel Prize in Physics. Soon after the discovery of the x-ray, Pierre and Marie Curie, in France, discovered the radioactive potential of uranium and isolated radium. Consequently, radium was introduced in the radiation therapy of cancer (Hajdu, 2012). However, radiotherapy in its early years was a raw process, consisting of prolonged exposures to radiation, with severe side effects, including the onset of malignancies (Hajdu, 2012). The initial machines were not very accurate and healthy tissue was often damaged in the process. To alleviate side effects caused by prolonged exposure, smaller doses over a short period of time were proposed as an alternative. In 1911 Claudius Regaud proved that smaller doses of radiation are just as effective but with less side effects and thus fractionated radiotherapy became the norm (Connel and Hellman, 2009). In contemporary medicine, radiotherapy is used in palliative treatment, as adjuvant to chemotherapy or surgery and to manage and alleviate metastasis symptoms.

In 1878, Thomas Beatson observed how removing ovaries in mice would stop breast milk production. This observation led to an experiment where he removed ovaries in patients with advanced breast cancer. In turn, he discovered the stimulating effect of the female

ovarian hormone – oestrogen. Beatson's discovery laid the foundation for both modern hormonal therapy and endocrine surgery (Hajdu, 2010; Lakhtakia & Chinoy, 2014).

Hormonal therapy is effective in cases were the tumours overexpress oestrogen receptors. The effects of endocrine therapy are not fully understood for tumours overexpressing progesterone receptors. It is mainly used as adjuvant therapy, following surgery, to prevent recurrence, secondary tumours or metastasis. It can also be used before surgery, to reduce the size of the tumour or when surgery is not an option (Locker et al, 1998).

Unfortunately, all these options present with their own limitations, highlighting the need for novel anti-cancer therapies. These limitations are found in all three traditional lines of treatments. For instance, surgical removal of tumours should be carried out on patients with non-metastatic cancers (Rashid and Takabe, 2014) however, the role of mastectomy in breast cancer is still questioned. It is also believed that removing the primary tumour in a metastatic disease can promote proliferation, but the study was not critically evaluated (Rashid and Takabe, 2014). Due to faster multiplication than normal cells, transformed cells can acquire resistance to chemotherapeutic agents, making chemotherapy a rather limited cancer management strategy (Eroles et al, 2012). Hormonal therapy, on the other hand depends on the presence of hormone receptors (HR) in a tumour and is therefore restricted to less aggressive tumours, not usually associated with negative clinical outcomes (Eroles et al, 2012).

1.5 Targeted therapies

Tumour microenvironment and molecular subtype dictates the therapy strategy, which is the premise for personalised therapy (Curigliano and Criscitiello, 2014). The role the members of the Epidermal Growth Factor Receptor Family plays in the onset and evolution

of solid tumours has been widely recognized (Arteaga and Engleman, 2014). Beatson, who discovered removal of ovaries in premenopausal women with advanced breast cancer leads to tumour regression, was a forerunner of hormone therapy (Locker et al, 1998).

Tamoxifen was the first agent in a class of drugs called selective oestrogen receptor modulators, which was originally developed as an oral contraceptive (Brown, 2009). The mechanism of action of tamoxifen has been described as a partial oestrogen antagonist in humans, blocking the effect of oestrogen in the mammary glands by binding to the oestrogen receptor alpha (ERα) (Ward et al, 2014; Brown, 2009). Adverse effects include increase in occurrence in vein thrombosis or pulmonary embolism (Brown, 2009). Reports describe resistance mechanisms acquired by patients treated in the long term with tamoxifen and disease relapse (Ward et al, 2014). Tamoxifen resistance in mammary tumours is linked with activation of the PKA pathway (Bentin Toaldo et al, 2014).

Aromatase is an enzyme complex present in the placenta and in the granulosa cell of the ovarian follicles (Smith and Dowsett, 2003). The same complex is a catalyst in the conversion of androgens into estrogens (Lephart and Simpson, 1991). Hurvitz et al., 2015 report in breast cancer cell lines to determine drug response of Everolimus but these need validating. The first aromatase inhibitor was aminoglutethimide, initially meant as an anticonvulsant (Smith and Dowsett, 2003). Due to reports of adrenal insufficiency, the drug was withdrawn and redeveloped for use in breast cancer treatment (Smith and Dowsett, 2003). Following the discovery of aminoglutethimide, new series of aromatase inhibitors have been developed, named as first-, second- and third-generation inhibitors, respective to their chronological order of discovery (Smith and Dowsett, 2003, Fabian, 2007). Third-generation aromatase inhibitors are reportedly associated with better performance, fewer side effects, and higher specificity for the aromatase enzyme (Fabian, 2007). In spite of their association

with fewer side effects, the use third-generation aromatase inhibitors is linked to hot flushes, vaginal dryness, fatigue, nausea and loss of bone mineral density (Fabian, 2007). All third-generation aromatase inhibitors are subdivided into steroidal and non-steroidal which bind to aromatase irreversibly and reversibly, respectively (Fabian, 2007). Anastrozole, letrozole and exemestane are all third-generation aromatase inhibitors, which are orally administered on a daily basis (Smith and Dowsett, 2003, Fabian, 2007).

Traditional lines of treatment in cancer management are accompanied by severe cytotoxic events due to disruption of several cellular processes present in both normal and transformed cells, as these treatments cannot distinguish between cancer and normal cells (Arora and Scholar, 2005). Understanding the molecular differences between normal and transformed cells is critical in designing drugs and developing treatments with limited cytotoxic consequences (Arnedos et al, 2015). Cells found in solid tumours exhibit special features compared to normal cells, such as high levels of the EGFR family of cell surface growth factor receptors, a dissimilarity, which can be exploited in drug development endeavours (Hanahan and Weinberg, 2011). By forming homo or hetero dimers, these cell surface receptors undergo internalisation followed by phosphorylation of the intracellular tyrosine kinase domain. This in turn will trigger a cascade of signals, ending in alterations impacting proliferation, apoptosis and metastasis (Zhang et al, 2007; Herbst, 2004). The first rational targeted therapy to exploit the aberrant EGFR presence in transformed cells was monoclonal antibody therapy (mAbs) which targets the extracellular domain of the receptor. The first monoclonal antibody was found to cause reversible downregulation of the cell surface receptors and thus reverse the phenotype of transformed cells grown on soft agar (Zhang et al, 2007). The first study was carried out in 1985, on nude mice (Drebin et al, 1986). Initially, the scientists immunized mice with the cell line B104-1-1, transformed to express the

her2-neu oncogene in order to produce these antibodies (Zhang et al, 2007). However, this antibody had reduced affinity for the human homologue of HER-2 (Zhang et al, 2007). A human homologue was developed later on -4D5- which had inhibitory effects on breast cancer cells through HER-2 expression dependant antibody-dependant cellular toxicity (Zhang et al, 2007). To control the immunological aftermath of using mouse antibodies in human cancers, a recombinant version was developed, engineered on human framework regions (Drebin et al, 1986; Carter et al, 1992, Chowdhury and Ellis, 2014). The humanised version of 4D5 was named trastuzumab, also known as Herceptin and was approved as appropriate for the treatment of metastatic breast cancer (Slamon et al, 2001). The human recombinant version of 4D5 had a greater affinity for HER2, its target, and controlled antibody-dependant cellular toxicity. Trastuzumab targets the extracellular domain of HER2 (Chowdhury and Ellis, 2014) and it is mainly used for metastatic breast cancer patients in combination with chemotherapeutic agents (Zhang et al, 2007). By binding to the extracellular or IV domain of the receptor it prevents proteolytic cleavage of HER2 extracellular domain (Kovacs et al, 2015). Trastuzumab prevents ligand dependant signalling however cannot prevent ligand activated HER-2/HER-3 or HER-2/EGFR, which is believed to be a resistance mechanism (Harbeck et al, 2013)

Cetuximab resulted from efforts to reduce immunogenicity of EGFR-specific monoclonal antibodies by making a chimeric antibody version containing the variable region of EGFR-specific mAbs and human Immunoglobulin G1 constant region (Zhang et al, 2007). Cetuximab has been used as a therapy for triple negative stage IV breast cancer, both on its own and in combination with carboplatin (Carey et al, 2012). The Translational Breast Cancer Research Consortium carried out the study, as there were no previous investigations done on Cetuximab in metastatic breast cancer. Another investigation reports the positive results of

Cetuximab and cisplatin together as treatment for triple negative breast cancer (Baselga et al., 2013).

Pertuzumab (Perjeta, Genentech, Inc.) is the first drug of its kind, a new class of HER2 dimerization inhibitor and it was developed based on the human immunoglobulin G1 sequence. This particular monoclonal antibody targets the second member of the EGFR family, HER2 (Amiri-Kordestani et al, 2014) by preventing dimerization with HER-3 (Dall'Ora, 2015). Trastuzumab and pertuzumab bind to different epitopes of the HER-2 extracellular and are used in combination therapy for HER-2 positive metastatic breast cancer (Swain et al., 2015, Harbeck et al., 2013). Clinical studies revealed pertuzumab is well tolerated with adverse effects such as diarrhoea, rash, asthenia, vomiting, nausea and abdominal pain (Harbeck et al, 2013).

Margetuximab is a chimeric monoclonal antibody targeting the extracellular domain of HER-2 receptor. Margetuximab is a modified molecule with enhanced antibody dependant cellular toxicity (ADCC) (Perez, Rugo and Vahdat, 2013). This antibody has gone through phase I clinical trial, showing positive results and is now entering phase II clinical trial, in which patients with metastatic breast cancer will be enrolled (Perez, Rugo and Vahdat, 2013). The same authors also report new data for everolimus, an agent targeting the mTOR protein. mTOR together with PI3K pathways are associated with endocrine therapy resistance in patients with hormone positive tumours (Perez, Rugo and Vahdat, 2013). The potential of therapies targeting downstream signalling pathways in breast cancer are being tested currently in clinical settings, with promising results, as it is believed these strategies will affect tumorigenesis at multiple levels (Brufsky, 2014). Other agents in clinical development include glembatumumab vedotin and neratinib (Perez, Rugo and Vahdat, 2013).

Another form of targeted therapy includes tyrosine kinase inhibitors, which bind to the ATP intracellular binding site of the cell surface growth factors receptors (Opdam et al, 2012).

Lapatinib is a tyrosine kinase inhibitor, which affects both EGFR and HER-2 (Opdam et al, 2012; Arora and Scholar, 2005). When scientists discovered the role EGFR and HER-2 played in various types of cancer cellular processes, they also realised cancer patients would benefit from treatments inhibiting both of their activity simultaneously (Rusnak and Gilmer, 2011). Finding the appropriate active compound, 4-anilinoquinazoline (Rana and Sridhar, 2012; (National Centre for Biotechnology Information, 2017) depended on a series of improvements in drug discovery such as novel chemistry, broad kinase screening tests and cell testing platforms, which included both EGFR and HER-2 cell lines together with appropriate controls (Rusnak and Gilmer, 2011). Lapatinib has potent inhibitory effects on both EGFR and HER-2 and thus negatively affects the subsequent pathways involving MAPK and AKT pathways. This in turn induces apoptosis in a range of cancers, including breast cancer (Zhang et al, 2007). In addition, the combination between trastuzumab and lapatinib has proven efficient in tumours overexpressing HER-2 and the use of lapatinib in trastuzumab resistant cell lines reduces phosphorylation of the trastuzumab resistant pathways (Rana and Sridhar, 2012). Relapse in patients treated with lapatinib is still a subject of research, but a few mechanisms have been proposed, such as mutations within the HER-2 receptor, mutations and over phosphorylation of the PI3K pathway and over expression of other receptor involved in tumorigenesis (Rana and Sridhar, 2012). Clinically, lapatinib has been approved in combination with capecitabine for HER2 positive metastatic breast cancer (Geyer et al., 2006) and in combination with letrozole for HER2 and hormone receptor positive metastatic breast cancer (Rana and Sridhar, 2012). Headache, rash, cold and gastrointestinal

symptoms were among the side effects exhibited during treatment with lapatinib (Rana and Sridhar, 2012).

1.6 AURORA: the international initiative for the investigation of metastatic breast cancer

Breast cancer treatment and management are not only limited by the effectiveness of the existing medicines, but also by the gaps in knowledge. For instance, many investigations into the molecular diversity of the tumour have been focused on primary tumours (Zardavas et al, 2014). In order to gain more knowledge on the subsequent mutations and changes in tumour and cell environment in secondary tumours, a representative number of patients need to be involved (Curtis et al, 2012). AURORA is a programme started by Breast International Group – (BIG) in order to not only identify mutations and aberrations occurring between first and secondary tumours (Zardavas et al, 2014) but also to innovate clinical trials by characterising metastatic breast tumours and identifying appropriate clinical targets (BIG, 2017). One of the goals would be for next-generation sequencing tools to create a smooth path towards personalised medicine (Russnes et al, 2011).

Variations between breast cancer patients and variations within a single tumour have been labelled tumour inter- and intra-heterogeneity respectively and the causes for these events are not yet fully described as breast cancer tumour progression paths remain undefined (Russnes et al, 2011). Tumour heterogeneity is also associated with drug resistance (Russness et al, 2011; Fisher et al, 2013). Both intra- and inter-tumour heterogeneity are drivers for mutations, leading to metastatic diseases exhibiting new molecular aberrations that in turn will affect the course of treatments (Zardavas et al, 2014). Acquired resistance to a drug is a response to molecular evolution and selective pressures to various drugs used

during the course of treatment (Zardavas et al, 2014). The programme will include 1300 initially and will include clinical setting in Europe (Zardavas et al, 2014). Patients who will be part of the study will provide at least one metastatic tissue sample, tissue from primary tumour, blood, plasma and serum samples. The samples will be subject to DNA and RNA sequencing and a dedicated software will report results to clinicians (Zardavas et al, 2014). Breast International Group will launch clinical trials in order to identify any emerging clinical targets for metastatic breast cancer (Zardavas et al, 2014).

1.6 Alternative molecular biology niches

The cancer phenotype exhibits a range of critical traits that provide the context for development of novel therapies (Sledge & Miller, 2003; Hanahan and Weinberg, 2000). These features or "hallmarks" are described by Hanahan and Weinberg first in 2000, when the authors initially counted six such traits. Since then, the paper became a landmark in the field of oncology (Sledge & Miller, 2003). The authors revised their initial paper and in 2011, publishing a second piece of work that not only presents the original hallmarks in more detail but also introduces four new traits that emerged as a consequence of progress in research. The hallmarks, in no particular order, are as follows: (1) sustaining proliferative signalling, (2) evading growth suppressors, (3) activating invasion and metastasis, (4) enabling replicative immortality, (5) inducing angiogenesis and (6) resisting cell death, with (7) avoiding immune destructions, (8) tumour promoting inflammation, (9) genome instability and (10) deregulating cellular energetics introduced in the paper published in 2011 as emerging traits (Hanahan & Weinberg, 2011).

For instance in the case of breast cancer, scientists are looking at the gene makeup of the tumour to determine which genes are switched on and therefore tailor an appropriate therapy (Deluche, Onesti and Andre, 2015).

Systems Biology is another pathway for studying and distinguishing the different breast cancer pathologies (Song et al., 2017). This relativity new discipline involves the use of mathematical modelling and high throughput technologies with the purpose of identifying the right combination of treatments and predict clinical outcome (Yarden and Pines, 2012).

1.7 Venom components as potential EGFR targets

Since the mammary cancer treatment landscape consists mainly of hydrophobic, chemotherapeutic substances that are often accompanied by side effects, poor clinical outcomes and acquired resistance (Lage, 2003), the need to look for alternative drugs is relevant and vital. Some of the currently used tyrosine kinase inhibitors are also limited in clinical strategy in terms of their multiple targets (Appert-Collin et al, 2015).

Venoms are intricate cocktails of disulphide rich peptides, small molecules and salts (Saez et el, 2010, Sannaningaiah et al, 2014). A large number of species from the class Arachnida employ venom as either a hunting or defence strategy. Since spiders are present in many ecological niches and are successfully adjusted to diverse food availability, their adaptive traits and dietary preferences reflect in venom composition. For instance, some species from suborder Araneomorphae use a venom rich in insect specific toxins. On the other hand, the venom of larger species from suborder Mygalomorphae contains mammalian specific toxins (Matavel et al, 2015) as these species feed on larger organisms, such as birds, amphibians and small fish. As such, venom composition is species specific (Vassilevski et al, 2009). Many of the molecules in these venoms have been selected throughout evolution

based on their effectiveness in killing or paralysing prey and predators (Sunagar et al.,2014; Sunagar and Moran, 2015; Matavel et al, 2015), each species dosing their venom according to factors such as sex, nutrition and climate (Vassilevski et al, 2009). Venoms act on both a local and systemic level, causing toxicity and dermatological issues at the bite site and affecting hosts' nervous, motor and circulatory systems. However, many venoms are currently recognised for their influence on ion channels, making them ideal candidates in the search for new pain killers and analgesics (Trim & Trim, 2013). The reason behind this niche filling strategy is the diverse range of systems, surface receptors and channels these toxins disturb once entered the host bloodstream. It is the reason why these toxins are ideal research subjects for scientific investigations focused on treating neurological disorders, pain or cancer (King, 2013).

Most spider venom compositions comprise of polypeptides and target specific toxins which affect vital systems once injected into a host (Calvete et al, 2009). Each venom contains over one hundred different biologically active components, making each venom a vast, protein diverse media (Vassilevski, 2009). Given the large number of species and the protein richness of each secretion, it is estimated that over 12 million peptides are predicted to be identified from spider venoms (Saez et al, 2010). This variety and the high specificity exhibited by venoms in suborder Mygalomorphae make venomous secretions a novel tool in modern pharmacology (Vassilevski, 2009). Consequently, biopharmaceutical community recognizes the value of arachnid venom in research.

Since venoms have been employed in hunting, natural selection has been a driving engine for toxin efficacy. Consequently, venom toxins exhibit high levels of affinity for molecular targets (King, 2013). The toxins can be classified according to their biological activity as neurotoxic, cardio toxic, hemolytic, digestive, hemorrhagic and algogenic (Kumar,

2014). Hemotoxins affect red blood cells and target the circulatory system and muscle tissue (Kumar, 2014; Tambourgi, D.V., 1994) Neurotoxins affect the central nervous system, inhibiting ion movement across cell membrane (Kumar, 2014). Cardiotoxins affect the heart and cardiac muscle by binding to particular sites and stopping cardiac muscle contractions (Kumar, 2014). The six FDA approved drugs that have been synthesized following research on venom target mainly cardiac pathologies and are derived from snake venoms (King, 2013). Although spiders are considered the most successful synthesisers of venom, the amounts obtained from one extraction were considered scant for any scientific investigations (King, 2013). Recent advances in the field of omics have allowed for this niche to be slowly filled (King, 2013).

Some compounds are commonly found in venoms, such as alkaloids, terpenes, polysaccharides, amino acids and small proteins called peptides. The later are a very important part of venom composition as they exhibit catalytic effect, are thermally stable and proteolysis resistant (Kumar, 2014). Scorpion, spider and snake are three of the main clades with medically relevant venomous secretions.

Snake venoms, according to its systemic effects, can be: hemotoxic, affecting cardiovascular functions and circulatory system; cytotoxic, targeting cellular sites, cellular membranes and affecting signal transduction or neurotoxic, damaging for the host' nervous system (Vyas et al, 2013, Casewell et al., 2014, Costa, 2014). Unlike spider venoms, snake venoms rarely exhibit more than one major system effect, following envenomation. Disintegrins are abundant in snake venom and have been utilised in many studies, particularly for their role in platelet aggregation, inflammatory action and inhibitory effect on tumour cells (Chakrabarty and Chanda, 2015; Thangam et al, 2012). They have been shown to limit the progression of breast cancer when delivered to the tumours using liposomes in a

xenograft model (Swenson et al., 2004). Disintegrins are low molecular weight molecules, with varying structure, strength and affinity for various molecular targets and were initially found through investigations on viperid venom (Vyas et al, 2013). Suto et al (2005) report the identification of two snake-derived Vascular Endothelial Growth Factors, which are a family of four proteins with an important role in the creation of new blood vessels in tumours. Although similar, both VEGF proteins identified from snake venoms exhibited different features, in comparison to other VEGF proteins and to each other (Suto et al, 2005). Osipov et al (2014) report the isolation of neurotrophin found in cobra venom. The concentration of neurotrophin was found to be as high as 0.5% (Osipov et al, 2014). Both mammalian and snake-derived neurotrophin are similar and structure and function, making the costs of neurotrophin plummet, given the easy isolation of the protein from snake venom (Osipov et al, 2014). Another study reports the interactions between *Cerastes Cerastes* viper and VEGF (Ben-Mabrouk et al, 2016). Two disintegrins found in this species venom have been consequently found to have pro-apoptotic effects and down-regulate signalling pathways involved in tumorigenesis (Ben-Mabrouk et al, 2016). Al-Asmari et al (2016) investigated the effects of snake venom on cell line MDA-MB-231 in a time and concentration dependant experimental setting.

Venoms are valuable in the development of novel drugs (Zambelli et al, 2016).

1.8 Conclusion and perspectives

Given the drawbacks of all of the current therapies, such as the difficult management of triple negative and inflammatory breast cancer (Masuda et al, 2012), the lack of appropriate clinical targets in the case of both triple negative and inflammatory breast cancer (Carey et al, 2012) and the increasing number of breast cancer occurrence worldwide (Torre

et al, 2015), the field of drug development needs more alternatives to look into for future drug design efforts. Scientific literature is plentiful in examples of investigations made on venoms research in different settings and for various purposes. Breast cancer overexpressing EGFR is a niche topic, for which more research is needed to overcome the shortcomings of current breast cancer management. Most of the drugs derived from venoms target cardiovascular or chronic pain according to literature thus allowing the mammary cancer management niche yet unfilled. At the same time, there are plenty of ongoing studies on the interactions between human cancers and various types of venom. At the same time, the need to identify, describe and understand the effects of venoms and venomous peptides, more research is needed.

1.9 References

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Investigating the effects of venoms on the Epidermal Growth Factor Receptor and receptor tyrosine kinases as a potential breast cancer therapy

2.1 Background

Evolution has ensured the peptides and proteins present in venomous secretions are fine-tuned and appropriate for the environment the species are present in (Murao et al, 2013). All the proteins and other components present in spider venoms are the result of roughly 400 million years of evolutionary strategies (Sunagar et al, 2015). These traits make venoms an ample collection of high affinity compounds which can potentially be used in drug development research.

Venom derived drugs are present in the current medicinal landscape, with six FDA approved drugs and ten more in clinical trials (King, 2013). Early scientific investigations regarding venomous compounds were focused only on species with potentially fatal secretions (Escoubas et al, 2006), but in 1980's new research showed the variety of pharmacological proteins within the venom of the same species (Adams, 2004). Venoms have been since then studied, mainly as an ion channel and receptor ligands source (Escoubas et al, 2006). Peptide based treatments found a niche in drug development (Craik et al, 2012) following the need to overcome side effects and resistance occurring in cancer treatments based on traditional or targeted therapies (Thangam et al, 2012). Their high specificity and high affinity shown in *in vivo* investigations lead to not only highly potent effects but also to fewer side effects (Craik, 2013). With over 10 million predicted bioactive molecules, spider venoms are a wealthy collection of potential drug discovery candidates (Saez et al, 2010). The consensus is that these biomolecules have had millions of years of evolutionary fine-tuning,

making them the stable and complex weapons currently employed against both prey and predators (Garrison et al, 2016). A fundamental component of spider venom is the disulphide rich peptides and an estimated 0.1% of these peptides have been studied so far (Klint et al, 2012).

Following the long line of general limitations encountered in existing anti-cancer therapies such as side effects and resistance, looking at other potential treatments and naturally occurring small amino-acid chains protein could provide a scaffold for future treatments (Almeida and Stankiewicz, 2016).

Breast cancer is defined as a heterogeneous disease, comprising of multiple molecular entities, with distinctive features (Carey et al, 2012; Koboldt et al, 2012). Some members of the Epidermal Growth Factor Receptor family of tyrosine kinases have been identified as key regulators of cell growth, transformation and other cellular processes in some breast cancer subtypes, including triple negative breast cancer (Jacot et al, 2015). Two of the four members of the EGFR family – EGFR and HER-2- have been considered as relevant and attractive clinical targets in breast cancer drug discovery (Kele et al, 2012). Moreover, the presence of EGFR is considered a marker of triple negative breast cancer subtype (Masuda et al, 2012) which lacks both hormone receptors (Progesterone and oestrogen) and HER-2 (Straehle et al, 2012; Jacot et al, 2015; Oakman et al, 2010). Existing anti-EGFR and HER-2 therapies include monoclonal antibodies such as Trastuzumab, which target the extracellular domain of HER-2, or small molecule tyrosine kinase inhibitors such as Lapatinib which targets the intracellular ATP binding site of both HER-2 and EGFR (Zhang et al, 2007; Rusnak and Gilmer, 2011). As mentioned above, these treatments present their own limitations. For instance, Trastuzumab does not properly infiltrate solid tumours and the cost associated with manufacturing the recombinant protein are known to be high (Zhang et al, 2007). In the case of the small

molecule tyrosine kinase inhibitor Lapatinib, limitations occur in regards to dose and toxicity as reported by an investigation focused on the combination of mTOR inhibitor Everolimus and Lapatinib in metastatic breast cancer (Rana and Sridhar, 2012).

Some studies have utilised snake and scorpion venom to investigate their effect on breast cancer cell lines (Sikkema et al, 2009; Swenson et al, 2004; D'Suze et al, 2010). The diverse range of venoms produced in nature means that there is a wide array of peptides and chemical compounds that can be screened for potential new breast cancer drugs. The need to investigate as many types of venom is as relevant as ever, given a sparse venom peptides library and the number of species and types of venom available. Most of the efforts to produce treatment drugs from venoms are focused on producing treatments for the cardiovascular system (King, 2013) or treatments that target acid sensing ion channels (Zambelli et al, 2016).

2.2 Venom-based drugs and peptides

Venom systems are present in a diverse range of animals. There are three clades with medically important venom compositions: snake, spider and scorpion (Fry et al, 2009). Any active peptides within these venoms target all main biological pathways and tissue types accessible by bloodstream (Fry et al, 2009). Of the three species of interest, snakes are widespread and one of the most often encountered species by humans. Their lethal venom is believed to have acted on primate brain and sensory functions (Casewell et al, 2013). Snake venom is mainly composed of disintegrins, hemotoxic and cytotoxic small peptides and has been shown to degrade tumor tissue (Sarfu-Poku, 2015). On the other hand, arthropods are found in most ecosystems on Earth, a flexible trait present in their venom composition as well (Almeida and Stanckiewicz, 2016). The most common compounds found in spider venoms are

low molecular mass toxins, amino acids, neurotransmitters, disulphide rich peptides, enzymes and small non-disulphide bonded peptides. (Almeida and Stanckiewicz, 2016). Scorpion venom has made possible the research of potassium voltage-gated ions, due to compounds such as polypeptide channel blockers (Kuzmenkov, 2015) and was also found to disrupt pancreatic and blood cancers (Al-Asmari, 2017).

One of the first venom-based drugs was Captopril, marketed under the name Capoten (Smith and Vane, 2003). The discovery came about after an extract of the venom of *Bothrops jararaca* called bradykinin potentiating factor was tested on angiotensin converting enzyme, an important regulator of blood pressure (Smith and Vane, 2003). Capoten received FDA approval in 1980 and became the first venom-based drug, developed to treat hypertension (Smith and Vane, 2003). In 1998, two non-enzymatic compounds eptifibatide and tirofiban were introduced on the market (Harvey, 2014). Both compounds were identified and discovered due to research carried out on venomous snake species *Sistrurus miliarius barbouri* and *Echis carinatus*, respectively (Harvey, 2014). Both compounds are used in treating heart disorders (Harvey, 2014).

Thangam et al (2012) report the identification of a disintegrin protein from the venom of *N Naja*, with pro-apoptotic effects on MCF-7 breast cancer cells line. MCF-7 breast cancer cell line is an oestrogen-dependent cell line, as opposed to MDA-MB-468 which is oestrogen independent (Cos et al, 1998).

2.3 Rationale for use of the venoms in this study

Acanthoscurria geniculata is a theraphosid species, part of the monophyletic group Mygalomorphae. There is literature available detailing the use of *Acanthoscurria gomensiana* as an anti-tumour agent (Soletti et al, 2010). Gomensin (one of the active components of

Acanthoscurria gomensiana venom) has been found to affect the MAPK/ERK signalling pathway. Protein kinase C and PI3K signalling pathway are also shown to have an involvement and consequent tumour cell apoptosis has been detailed (Soletti et al, 2010; Kumar et al, 2010; Heinen & da Veiga, 2011). However, there is little information on the composition of *Acanthoscurria geniculata* venom and the present investigation will contribute to increasing current knowledge on venom effects.

Naja atra (formerly *Naja naja atra*) venom was previously studied due to the apoptosis inducing effects Cardiotoxin III (an active *Naja atra* venom component) exhibited on JAK2/PI3K signalling pathway. (Lin et al, 2010; Das et al, 2011). *Naja naja* venom was chosen as there are investigations detailing the cytotoxic and apoptosis inducing effects of *Naja naja* venom (Das Gupta et al, 2011; Thangam et al, 2012).

Heterometrus Swammerdami venom was chosen due to several accounts of Bengalin, an active component of *Heterometrus bengalensis* venom, being an effective apoptogenic protein that acts via ERK-MAPK pathways (Das Gupta et al, 2007; Das Gupta et al, 2010; Das Gupta et al, 2013).

The aim of this research is twofold: first to analyse and describe the types of venoms used in this research in order to expand knowledge on the subject and second to identify any potential clinically active compounds following treatment of the breast cancer cell line MDA-MB-468 cells with the three types of venom from three different species - Spider, Snake and Scorpion - and to investigate fractions of this venom.

2.4 Methods

For this study human breast cancer cell line MDA-MB-468 were used. These originated from pleural effusion and are known to be oestrogen-independent cells, usually expressing

more than 10^6 EGF receptors per cell (Modjtahedi et al, 1993, Zhang et al, 1991). Basal MDA-MB-468 cell line forms grape-like structures when grown *in vitro* (Holliday et al, 2011).

2.4.1 A gen., H swa and N Naja venom extraction

Venoms for these experiments were supplied by Venomtech Ltd. Venom extractions were carried out at Venomtech Ltd., in a laminar flow hood, under the supervision of trained specialists, according to their proprietary methods. Venoms were collected using Venomtech's optimised procedures to maintain maximum venom quality in safe and controlled conditions. Snakes are 'milked' using the voluntary bite protocol. Invertebrates are milked under anaesthesia with electrical stimulation. Following the extraction, the venom was either freeze dried or frozen until needed at -20°C. Venom concentration was measured using a DS11 spectrophotometer (DENovix, US). Studies with these experiments have not been carried out before therefore the concentrations to use in each step had to be optimised before the experiments were undertaken.

2.4.2 Mammalian cell culture

The human breast cancer cell line MDA-MB-468, was originally obtained from ATCC. Cells were cultured in 25cm^3 culture flasks (Sarstedt, Inc, Newton, USA) and were cultivated in Dulbecco's modified Eagle's medium (Gibco, UK). L-Glutamine (1%), penicillin-streptomycin (1%) and Foetal Calf Serum (10%) (all from Invitrogen, Paisley UK) were added to the media. Cells were incubated at 37 °C and 5% CO_2. To sub-culture the cells when needed, the cells were washed twice with sterile Phosphate Buffered Saline pH 7.4 and then incubated in Trypsin-EDTA (0.05% trypsin, 0.53 mM sodium EDTA, Invitrogen, Paisley) for 10 minutes. The trypsin-EDTA was removed and replaced with 0.2 mL of the same reagent until the cells had

detached from the flask. The cells were re-suspended in a total of 5 mL of media after being re-aliquoted as required by the experiment carried out. For instance, frozen cell stocks would be prepared similarly, but the final trypsin stage would only require 0.1mL of trypsin. The cells were re-suspended in 1 mL Dimethyl Sulfoxide 10% (Sigma, Poole, UK) and Foetal Calf Serum 90%. The mix was then pipetted in a cryotube, left at -20 C overnight and then moved to a -80^{o}C freezer for long term storage. For indefinite storage, the cryotubes were stored in liquid nitrogen.

2.4.3 Bradford assay

For each of the samples obtained, a Bradford assay was carried out to measure protein concentration and thus ensure each sample has the same protein concentration when analysed via gel electrophoresis. The protocol was carried out in 96 well plates, with Bovine Serum Albumin (Sigma, UK) as a control, used at 100µg/ml working dilution. Further serial dilutions were obtained from the working dilutions, which are outlined in table below. The 96 well plates were analysed using a BMG Labtech (UK) plate reader. However, samples obtained after the treatment with fractions of venom were analysed using a DS 11 spectrophotometer to ensure accurate measurements.

2.4.4 Venom treatment and sample preparation

Venom treatments were carried out using the following protocol. MDA-MB-468 cells were trypsinised and plated out in 12 well plates. Cells were grown to at least 70% confluency before any treatment was carried out. Venom serial dilutions from 1:10- 1:100000 were prepared on the day of the experiment, prior to incubation and were prepared in Dulbecco's Modified Eagle Media (Gibco, UK). Cells were incubated with venom for 2h at 37^{o}C. After 2h,

the venom was removed and media containing a final concentration of $1x10^{-7}$M EGF (Sigma, UK) was added to all wells, except for the negative control well. After the 5 min. EGF incubation, all the media was removed and the cells were washed with a final concentration of 2mM EGTA in PBS pH 7.4 (Sigma, UK). Radioimmunoprecipitation assay (RIPA) buffer was prepared and kept on ice, by mixing 1000µl of the buffer, with 10µl each of the protease inhibitor cocktail (100x) and phosphatase inhibitor cocktail (100x) and EDTA (100x) (Thermo Scientific, UK). The amount of total RIPA buffer was adjusted to make up to 150µl of buffer per well. For the venom lysate samples used in the human phospho-array experiment, full flasks of MDA-MB-468 cell line were lysed, using the same reagents, but different amounts i.e. 500µl RIPA buffer, and 5µl of the protease and phosphatase inhibitors and EDTA per flask, After all the wells were added the 150µl of lysis buffer or the flasks were added 500µl lysis buffer, the plates or flasks were kept on ice for approx. 5 minutes and checked for total lysis i.e. until the consistency has visibly changed. The contents of each well were transferred into individual 2mL tubes, appropriately labelled and spun at 4 °C for 10 minutes or at least until a pellet became visible. The supernatant was transferred into a fresh tube and the appropriate amount of 5x sample buffer was added to make up a final concentration of 1x sample buffer. Where the sample required spectrophotometry analysis to determine protein concentration, a small amount of the supernatant was transferred into another tube and stored at -80 °C. Positive and negative controls were used throughout the experiment, with positive control meaning samples were not treated with venom but treated with EGF, and negative control meaning samples were treated with neither venom nor EGF.

Venom samples obtained following fractionation protocol were freeze-dried. The fractions were re-suspended in 12 µl RNAse and DNAse free water (Thermo Scientific) and 2

µl was used to carry out spectrophotometry measurements. The 10 µl left were further diluted to 1 in 100, using Dulbecco's Modified Eagle Media (Gibco, UK).

2.4.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

7% and 9% bis-acrylamide (30%, Bio-Rad, UK add info from D) gels were used for these experiments, made up following the standard protocol and using Tris HCl 1.5M (Fisher Scientific, UK), SDS 10%, distilled H_2O, N,N,N,N'-Tetra-methyl-ethylenediamine (TEMED, Bio-Rad, UK) and APS 10% (Ammonium persulfate, Fisher Scientific, UK). The gels were set for 45min to 1 hr in 0.75mm glass plates.

A Bio-Rad Mini PROTEAN Tetra Cell (Hemel Hempstead, U.K.) was utilised to run 1D SDS-PAGE gels. The glass plates for pouring gels were cleaned with ethanol and assembled as per supplier instructions.

The sample amount was chosen carefully, by using the protein concentration in each sample, identifying the lowest concentration, allocating the lowest concentration the standard amount to analyse through SDS-PAGE i.e. 15µl, dividing the lowest protein concentration by each protein concentration and multiplying by the amount allocated to the lowest protein concentration to be analysed through SDS-Page. For experiments with very limited amounts of sample, i.e. venom fraction analysis, protein concentration was determined using a DS11 spectrophotometer. Alternatively, a 96 well plate Bradford assay was used. After loading, the samples were run at 70V until the molecular weight ladder started to separate. The voltage was set to 170 until the dye front reached the bottom of the gel.

Stacking gels were made at a 3.75% concentration. The stacking gel (3.75% Acrylamide/Bis solution 29:1, 0.1M Tris/HCL pH 6.8, 0.1% SDS, 0.1% APS, 15 µl TEMED) was

poured and allowed to set with either a 0.75mm or 1.5mm thick comb with 5mm width wells inserted.

Gel tanks were filled with 1x running buffer (0.025M Tris, 0.192M glycine, 0.1% SDS, pH8.3) (Bio-Rad, Hemel Hempstead, U.K.). The acrylamide gels analysed for the Coomasie stain step have been incubated in Coomasie blue stain (Bio-Rad, UK) for an hour and destained in 10% acetic acid and 10% methanol overnight.

2.4.6 Immunoblotting

The gels were placed between three 3mm Whatman filter paper (Maidstone, UK) sheets on top and bottom, with a nitrocellulose (Amersham Biosciences, Little Chalfont UK) or PVDF membrane (Immobulin, UK) between the gel and the bottom three filter paper sheets. The PVDF membrane was first activated by immersion in 100% methanol (Fisher, UK). The filter paper and nitrocellulose/PVDF membranes were immersed in semi-dry blotting buffer prior to setting on the three-filter paper stack. Two instruments were used to undertake the protein transfer step: an Invitrogen semi-dry western blotter or a Bio-Rad transblotter. Protein transfer conditions for Invitrogen semi-dry blotter were: 45-60 minutes at 10-15V. The Bio-rad transblotter settings were 30 min at 25V, 1A. The nitrocellulose was stained before being with Ponceau S solution (0.1% w/v in 5% acetic acid) to ensure appropriate protein transfer. Once the protein transfer was confirmed, the membranes were incubated for 1 hour in blocking buffer containing PBS Tween 20 0.1% (Fisher BioReagents, UK) and 5% Marvel milk powder. Following the blocking step, the membranes were washed 5 times for 5 minutes in PBS Tween 20 at 0.1%. The membranes were cut in half, with the top half of one blot incubated overnight in PY20 (1/2000) and the top half of an identical blot with F4 (1/1000) primary antibody, and bottom half incubated in anti-β actin (1/20000) primary

antibody. The latter was used as a control to ensure protein load concentration is equal. Monitoring phosphorylation levels in each sample analysed via SDS-PAGE and Western blot was done using anti-phosphotyrosine monoclonal antibody PY20 (Sigma, UK). In this experiment, the PY20 antibody was used at a concentration of 0.0005mg/mL. Actin monoclonal antibody was used to determine visually if the protein concentrations in each sample were equal. For β-actin antibody, 1µl of the stock (0.2mg/mL) was diluted in 20mL. The use of this isoform of actin as a loading control is due to its ubiquity. It is a housekeeping protein, meaning it is present in all cells under normal patho-physiological conditions. Following the overnight incubation in primary antibody, the blots were washed 5 times for 5 minutes in PBS in 0.1% Tween 20. After the washing step, the blots were incubated for 1h in rabbit anti mouse secondary antibody (1:20000). Enhanced chemiluminescence reagents were used to image the blots after the following protocol: solution A: 10 ml 1M Tris pH 8.5, 90 mM p-coumaric acid (Sigma, UK), 250Mm Luminol (Sigma, UK), dH_20 up to 100mL and solution B: 10 mL 1M Tris pH 8.5, 64 µl 30% H_20_2 (Sigma, UK), dH_20 up to 100 mL. Both solutions are mixed in equal amounts before use on blots in the Chemidoc Touch Imaging System.

Table 1: Primary and secondary antibodies used in this study			
Primary Antibody	**Host and Class**	**Target**	**Dilution used at**
PY20 (Sigma, Poole UK)	Mouse Monoclonal	Phosphotyrosine	1/2000
F4 (Gullick et al., 1986)	Mouse Monoclonal	EGFR	1/1000
CB11 (Abcam, Cambridge UK)	Mouse Monoclonal	HER2	1/1000
21N (Gusterson et al., 1987)	Rabbit Polyclonal	HER2	1/200
RTJ-2 (Hunt et al., 1995)	Mouse Monoclonal	HER3	1/500
HFR-1 (Srinivasan et al., 1999)	Mouse Monoclonal	HER4	1/300
Anti-β-actin antibody (sigma)	Mouse Monoclonal	β-actin	1/20,000
Anti-α-Tubulin antibody (DM1A clone) (Sigma, Poole UK)	Mouse Monoclonal	α-tubulin	1/5,000
Secondary antibody	**Host and class**		**Dilution Used at**
Rabbit antimouse Radish Peroxidase (HRP-Tagged)	Rabbit Monoclonal		1/20,000

2.4.7 Kinome analysis - Phospho-array human receptor tyrosine kinases kit

The proteome profiler antibody array is procedure that enables the measurement of the level of protein phosphorylation in a cell lysate sample. It is different to a normal immunoblot as it allows the simultaneous of several RTK's. The kit has a range of specially formulated buffers and three nitrocellulose membranes coated with 49 specific kinase antibodies. It requires the pre-treatment of the cell lysate with protease inhibitors. The reagents were left to reach room temperature before using. The nitrocellulose membranes were incubated for one hour at room temperature in array buffer 1, which acted as a blocking buffer. Cell lysates were diluted to the appropriate concentration -300mg/µl- using array

buffer 1. The blocking buffer added initially was aspirated and the cell lysates were added to the membranes. As per the instructions, the cell lysates were incubated with the membranes overnight at 2-8 C°. This step requires the membranes to be completely submerged in cell lysate solution. The following day, the membranes were washed in 1x washing buffer three times. The membranes were incubated in 2 mL horse radish peroxidase detection antibody for two hours at room temperature. Membranes were washed again following this step. The exception from the instructions as per handout is the use of the maximum protein concentration 300mg/µl for the overnight incubation step. The next day, for the last two hours of the incubation step, the remainder of the venom lysate sample was added to the blots, to ensure maximum coverage and ensure maximum protein concentration is available. The results were analysed using ImageLab software, Bio-Rad.

2.4.8 Epidermal Growth Factor Receptor Family phosphorylation profile

In order to assess how the venoms affect the expression of the rest of the members of the Epidermal Growth Factor Receptors, an assay was conducted on three cell lines that are known for expressing HER-2, HER-3 and HER-4 using *A gen., H swa and N Naja* venoms. The cell lines selected were HEK293, which is known to overexpress HER-3, SKBR3, which is known to overexpress HER-2 and NIH3T3, which is known to overexpress HER-4. In order to probe for the different receptors, the following antibodies were used: RTJ2 for HER-3 receptor overexpressing cell line, CB11 for HER-2 receptor overexpressing cell line and HFR1 for HER-4 receptor overexpressing cell line. Cell lines were grown and subcultured in 6 well plates as per the same protocol for MDA-MB-468 cell line, incubated in venom for 2 hours and lysed as per the instructions in Venom treatment and sample preparation section above. SDS-PAGE analysis was carried out via the instructions in the sections above, using 9% acrylamide gels.

2.4.9 Venom fractionation

A. geniculata venom was fractionated using a two dimensional method. The first dimension was ion exchange and the second dimension was reverse phase chromatography. Crude venom protein concentration was measured using the DS-11 microspectrophotometer (Dynovix, US) and 20 mg of protein is diluted to 100ul in reverse phase buffer A (0.05% trifluoroacetic acid [TFA]), centrifuged in a bench top microcentrifuge (Scientific Lab Supplies) and injected on to a C18 column with 5um bead size 250mm x 4.6mm column (Vydac). Reverse phase HPLC was then performed with a gradient against 80% acetonitrile in 0.045% TFA with a gradient from 5-100% (See Table 1). Peaks were detected by slope and threshold changes of absorbance at A280nm by the automated fraction collector. HPLC was performed on the Agilent technologies 1100 HPLC system.

Table 2: Reverse phase HPLC gradient against 80% acetonitrile in 0.04% TFA buffer (%B)

Time (Minutes)	**%B**
0	5
10	5
20	20
100	60
120	100
130	100
131	5
140	5

2.4.10 Freeze drying venom

Fractions were frozen at -50^{o}C in the Virtis Advantage Freeze Drier (Biopharma Process Systems, UK) and Vacuum set point of 500 µbar. After 24hrs temperature was gradually warmed at 10 °C intervals until final drying at 0 ^{o}C.

2.4.11 ImageLab analysis

All the western blot images for these experiments were obtained using a Bio-Rad chemidoc imaging system (Bio-Rad, UK) and chemiluminescence detection. Analysis made on Human Phospho-array kit western blot was undertaken also using the ImageLab software. The images were cropped using the Image Tools tab, bands and lanes have been appropriately labelled on images using the Lane and Bands tab. The Lane Profile setting will allow visualization of the bands and any adjusting to be done before reporting results via the Report setting. All the intensity readings were input in an excel file and organised using Insert tab and Chart Tool.

2.5 Results

2.5.1 Evaluation of optimal venom dose to use in Human Phospho-array experiment using Western blotting

In order to establish the effects the *A gen., H swa and N naja* venom had on more than one receptor tyrosine kinase and to be able to get simultaneous data on all of them, a proteome profiler human phosphor-RTK array kit (R&D Systems UK) was used. However first the optimal dose to use in the kinome blot had to be determined. Concentrations were chosen for the kinome blot by looking at the highest concentration of venom used that did not affect the expression of the control actin. Results revealed the threshold venom concentrations to

be as detailed below. All the types of venom were used at the same dilutions from 1 in 10 to 1 in 1000000 and the wells were labelled from A to F, respectively.

Although not obvious from the Coomassie stained acrylamide gels (*Fig. 1a*) the Western Blot analysis revealed the protein levels in the 1:100 treatment were much higher than protein levels in the 1:10 treatment and the actin levels were not affected. The affect at 1:10 is possibly the result of potential cytotoxic effects occurring when using *A gen.* venom on MDA-MB-468 human breast cancer cells at a concentration of 3.772 mg/ml. The dilution was

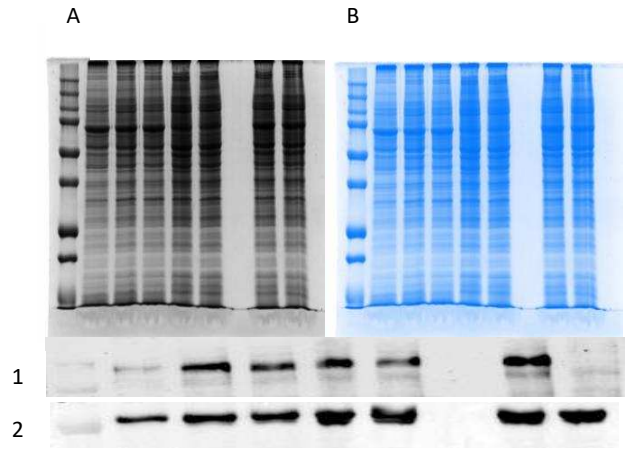


Fig. 1a A &B are a polyacrylamide gel of *A gen.* cell lysates. The six wells loaded contain 5 different dilutions of the venom as follows: 1 – molecular weight marker, 2 – 1/10, 3 – 1/100, 4 – 1/1000, 5 – 1/10000, 6 – 1/1000000. Lane number 7 is empty. After the empty lane, the next well was loaded with a positive control and the last lane contains the negative control.

The first blot below the gel images 1 - is the PY20 antibody treated blot. The last image 2 - is the actin antibody treated blot. These correspond with the polyacrylamide gels above.

chosen following the immunoblotting of the anti-β actin primary antibody treated membrane (bottom of *Fig. 1a*). Therefore, the relevant dilution for *Accanthoscurria geniculata* theraphosidae venom was determined to be 1 in 100, with a concentration of 0.3772mg/ml as the highest concentration showed an increase in EGFR phosphorylation in comparison to the rest of the dilutions.

Samples treated with *Naja Naja,* formerly known as *Naja Naja Atra*, have also been analysed using coomassie stained gels and Western Blots. Results revealed the protein levels in first three samples, meaning dilutions 1 in 10, 1 in 100 and 1 in 1000, were too low to be detected via standard methods (*Fig. 1b*). The working dilution was determined to be 1 in 10000, with a protein concentration of 0.003206 mg/ml. The venom concentrations in the

first three samples was also affecting the levels of actin since the protein levels are also too low to be detected in the first three lanes of the actin antibody treated blot 2 in *Fig. 1a*.

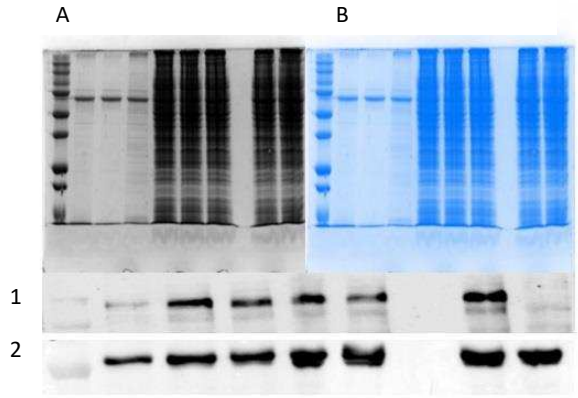
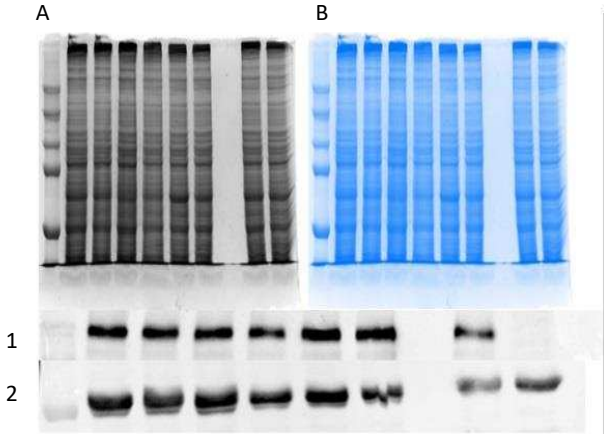


Fig 1b shows the polyacrylamide gel run with Naja naja cell lysate. Six different dilution have been used for this venom as follows: first well – molecular weight marker, second well – 1/10, third well – 1/100, fourth well – 1/1000, fifth well – 1/10000, sixth well – 1/100000, seventh well – 1/1000000. One well was left empty on purpose. The next lane was loaded with positive control and the last lane with negative control.

The two images below are western blots of the acrylamide gel above.

Heterometrus Swammerdami venom treatment results are shown in *Fig. 1c*. In comparison with the positive control, which was a sample of MDA-MB-468 cells treated with EGF and no venom, *H swa* venom treated samples showed no visible difference in the protein levels when analysed through coomasie stained gels, moreover, preliminary analysis using immune-detection methods showed an increase in phosphorylation levels when compared to the positive control. As a result, the working dilution for H swa venom was determined to be 1 in 10, with a protein concentration of 4.012 mg/ml.



Fib 1c shows the acrylamide gel run with H swa cell lysates. Six dilutions, from 1/10 to 1/1000000 have been loaded from left to right. The first well was loaded with molecular weight marker and one well was left intentionally blank between the dilution lanes the control sample lanes. The next lane after the gap was loaded with positive control and the next lane was loaded with negative control.

The two images below are western blots of the polyacrylamide gel above.

2.5.2 Human Phospho-array analysis to determine venom effect on 49 receptor kinases

Fig. 2 below contains the human phosphor-array blots. Only the blots labelled were analysed in this study as the other blots were the work of another student. Each pair of dots on each blot represents a human tyrosine kinase receptor. An intense signal is the result of high levels of phosphorylation of the respective receptor, in response to venom treatment. Equally, less intense signals are represented by receptors with low levels of phosphorylation as a consequence of the blots being treated with the venom.

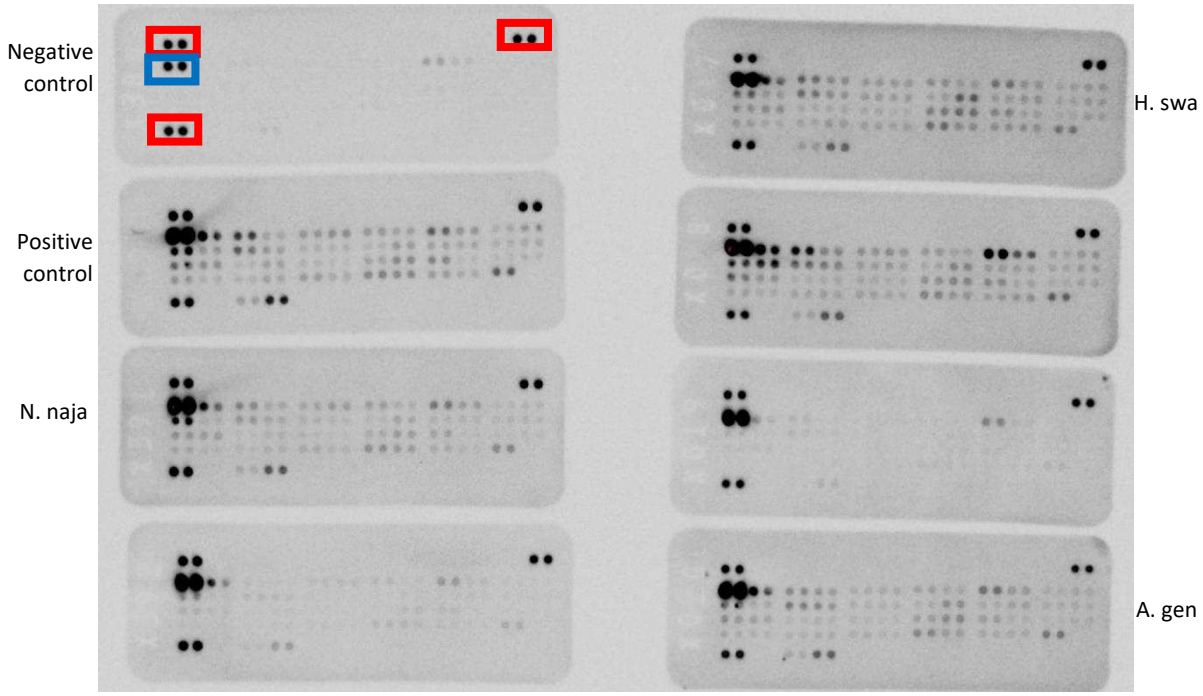


Fig. 2 Human Phospho-array immunoblots. The samples used in the current investigation have been labelled accordingly on the side of the image with the venom used to treat the immunoblots. The spots on each immunoblot are capture and control antibodies to which specific receptor tyrosine kinases bind during the incubation step. Treatment with conjugated horseradish peroxidase HRP was used to detect the signal (pixel density of each spot) by chemiluminescence. Spots boxed in red are reference spots to show the blot is working. Spots boxed in blue are EGFR.

The proteome profiler nitrocellulose membranes were spotted with 49 different specific RTK antibodies in duplicate. The signal (pixel density) data was produced using ImageLab software, analysing each set of duplicate spots.

Following the analysis with ImageLab, the human phosphor-array data was organised in graph form to aid in explaining the interactions between venoms and receptors.

Fig. 3 Phosphorylation levels of FGF receptor family in response to venom

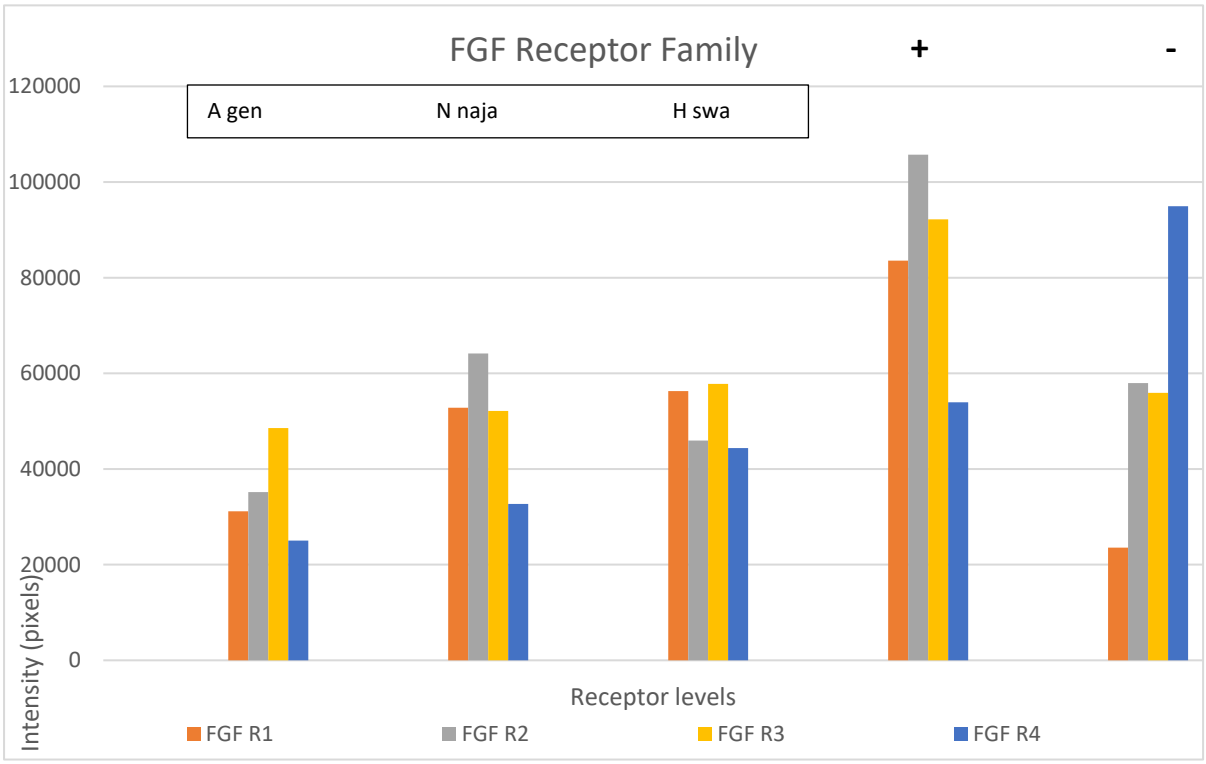


Fig. 3 above represents the information obtained from analysing the effects of the three types of venom analysed on the Fibroblast Growth Factor receptor family. The intensity of each pair of dots on each of the five blots included in this investigation has been organised in the form of graphs. Positive control blots which have been treated with EGF but not venom were also prepared to compare with the venom treated samples. EGF will bind to EGFR and the antibody will detect the phosphorylation levels, which in turn is an indication that the experiment has worked. In this experiment, the levels of phosphorylation in the positive

sample can also be used as comparison in relationship with the levels of phosphorylation in the rest of the samples.

Negative control samples have not been treated with either EGF or venom. This control will allow to identify any non-specific binding of the antibody.

Fig. 3 compares the three venoms and the two control samples (x axis) to signal (y axis) exhibited by each pair of dots on the human phosphor-array blots (*Fig. 3*). By comparing the phosphorylation levels in the three venom treated samples to the positive control, all three venoms have inhibitory effects on FGF receptors at the concentrations used.

Fig. 4 Phosphorylation levels of EGF receptor family in response to venom treatments

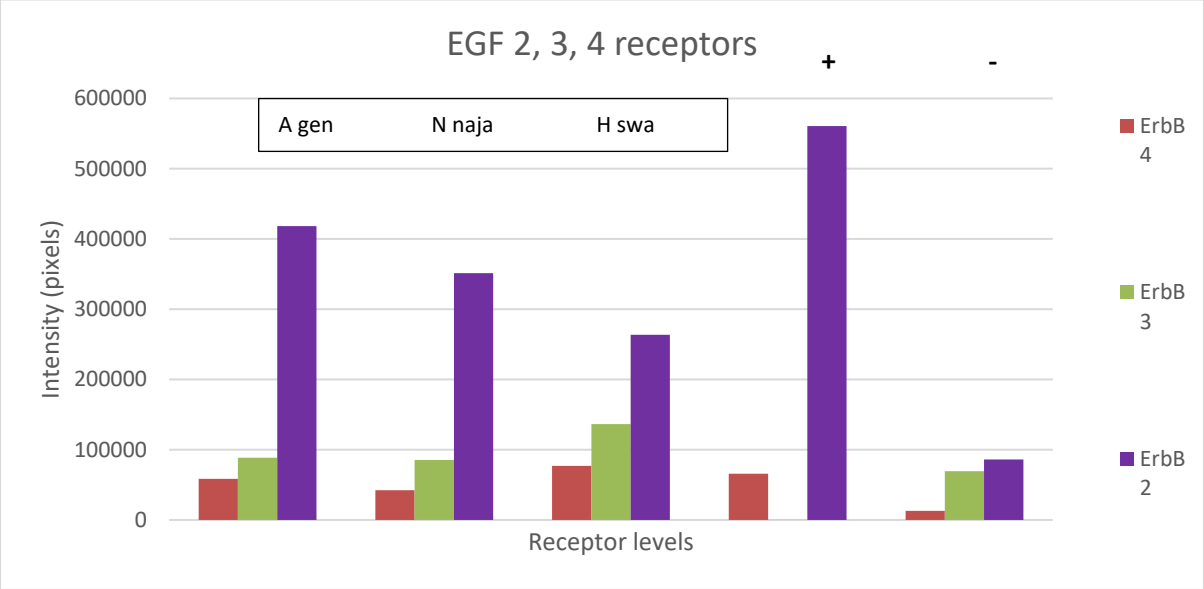


Fig. 4 above represents the levels of phosphorylation in the EGFR family of receptors (excluding EGFR) following treatment with the three types of venom. When compared to the positive control the levels of phosphorylation in ErbB2/HER-2 are decreasing following treatment with all three venoms. In comparison with the positive control, ErbB3/HER-3 receptor shows an increase in phosphorylation levels across all three types of venoms. In the case of ErbB4/HER-4, *N naja* and *A gen.* venoms are inhibiting phosphorylation since the

intensity values of *A gen.* and *N naja* venoms are below the positive control intensity readings, whilst treatment with *H swa* increased the levels of ErbB4.

Fig. 5 Phosphorylation levels of PDGF receptor family in response to venom treatments

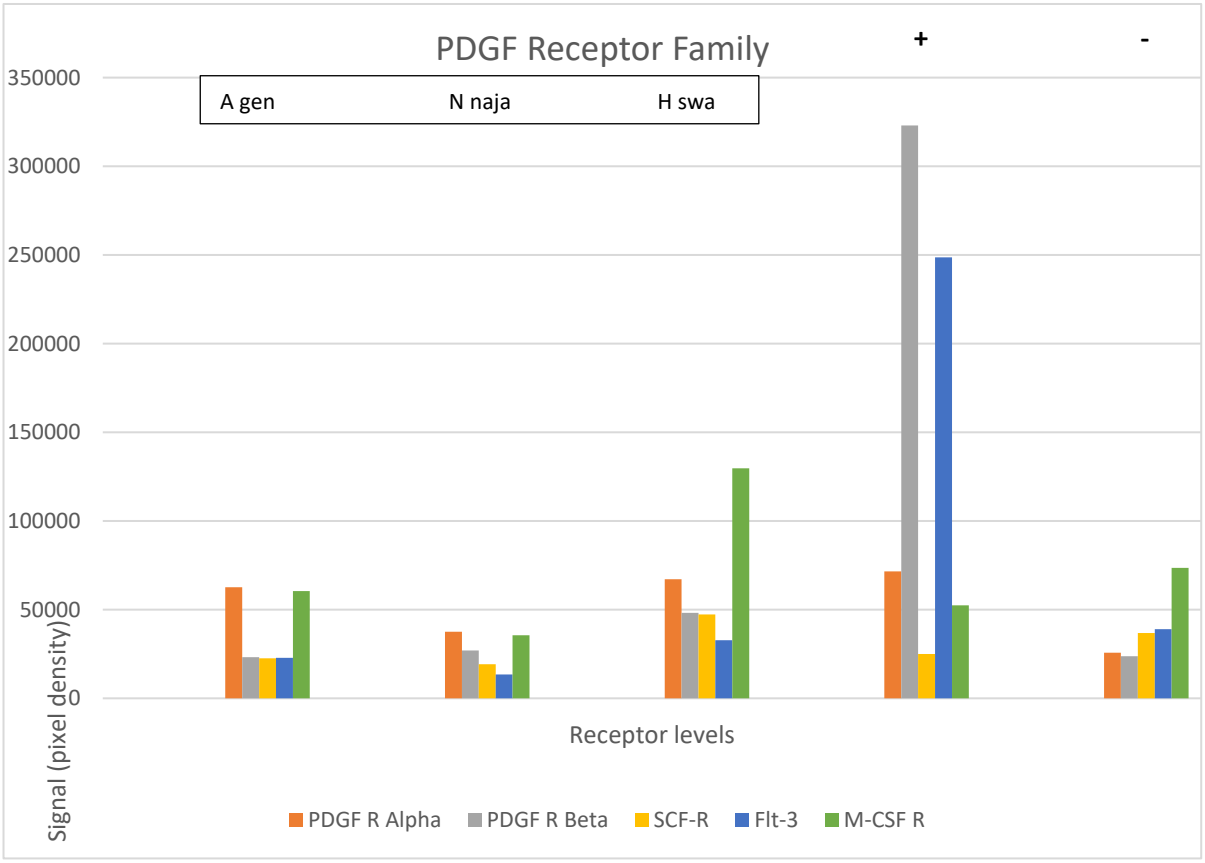
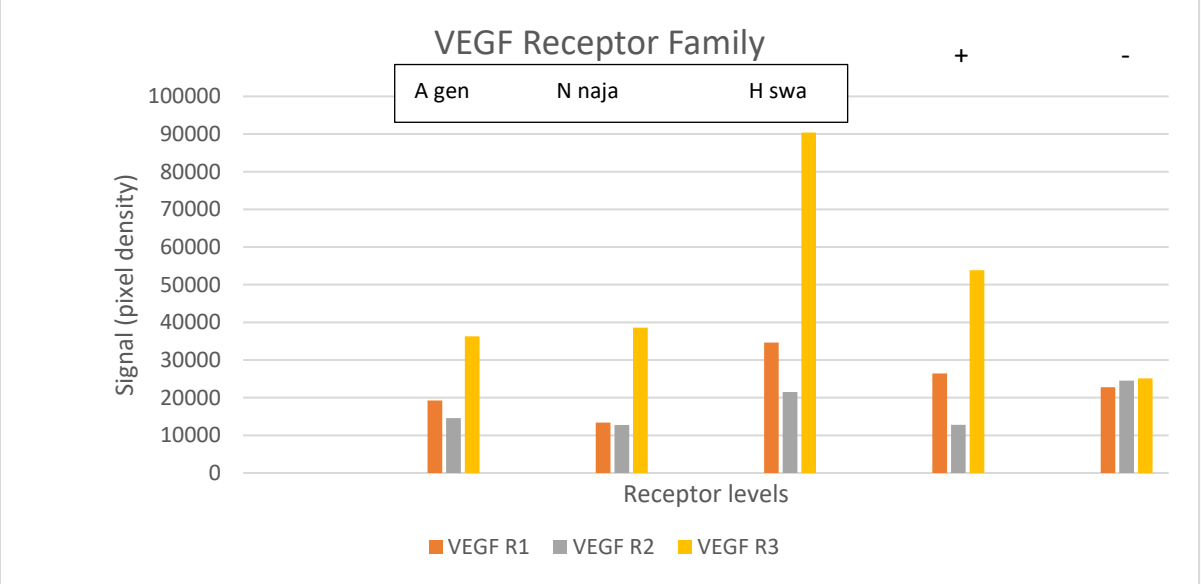


Fig. 5, the phosphorylation levels of Platelet-Derived Growth Factor receptors in response to venom treatment are presented in graph forms. In comparison to the positive control intensity readings, all three types of venoms are inhibiting Flt-3 receptor and PDGF R Beta. The levels of phosphorylation in M-CSF receptor are inhibited by *N naja* venom but not by *A. gen* and *H. Swa*. SCF-R and PDGF R alpha phosphorylation is inhibited by *N.naja* but not *A.gen* and *H.Swa*.

Fig. 6 Phosphorylation levels of VEGF receptor family in response to venom treatments



Vascular Endothelial Growth Factor plays a significant role in angiogenesis, the creation of new blood vessels in solid tumours. Figure 6 above shows how treatment with *H swa* venom increases the phosphorylation levels in all three VEGF receptors. VEGF R2 phosphorylation levels are increased following treatment with *H swa* and *A gen.* venoms.

2.5.3 Epidermal Growth Factor Receptor Family profile

The samples obtained for use on the human RTK profiler immunoblots were run on acrylamide gels together with three more cell lysate samples obtained from cell lines overexpressing the rest of the Epidermal Growth Factor Receptor family. The cell lines used for this experiment were SKBR3, HEK293 and NIH3T3, which overexpress HER-2, HER-3 and HER-4, respectively.

Fig 7 Immunoblots ran with venom lysates and ErbB family receptors.

Fig. 7a

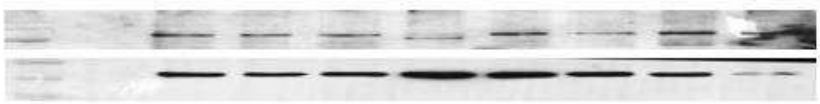


Fig. 7b

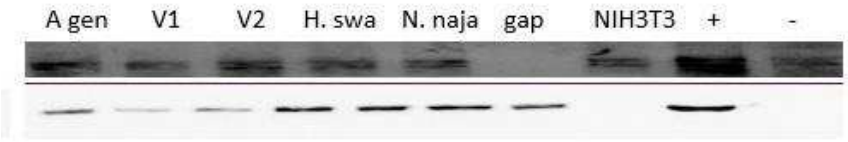


Fig. 7c

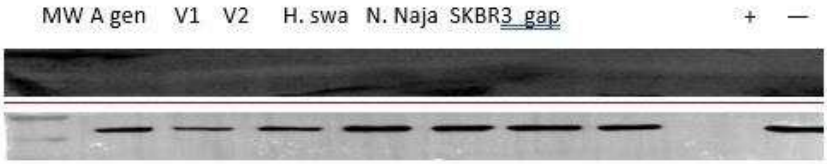
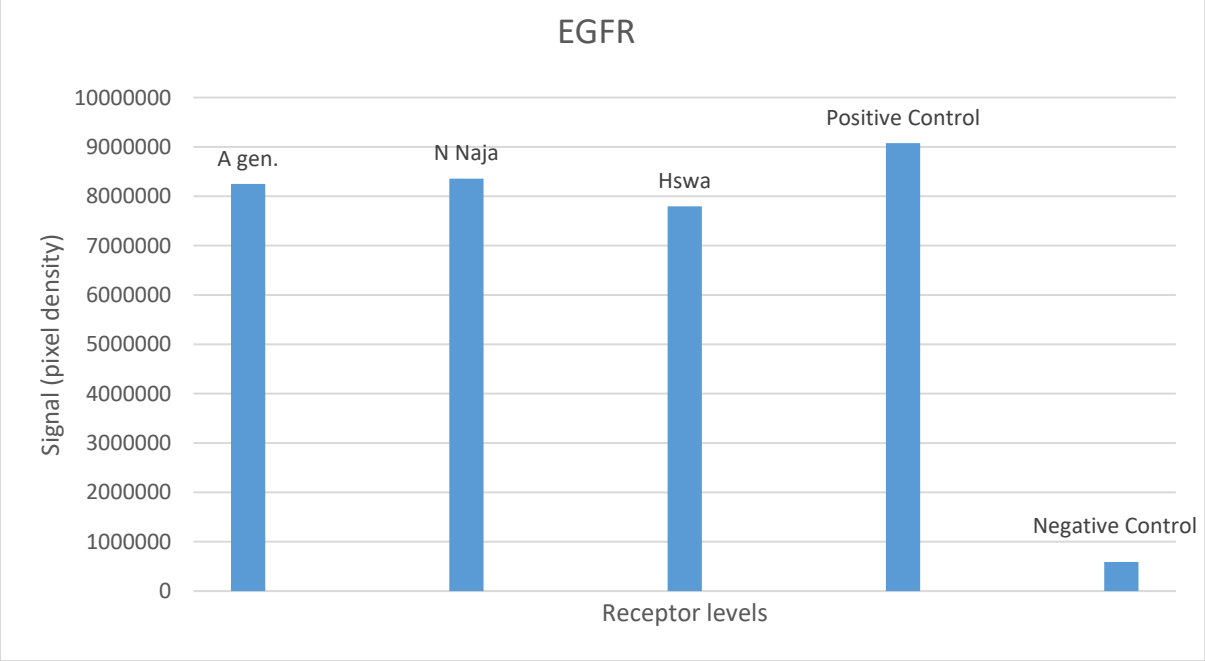


Fig. 7a shows HER3 expression (in the top blot), *Fig. 7b* shows HER4 expression (in the top blot) and *Fig. 7c* shows HER2 expression (in the top blot). The bottom blots are the actin loading control. The resulting blots were not clear enough for an appropriate interpretation of the levels of their respective receptors are. Since the blots probed with actin show that there are proteins in the samples, the underlying issue could be related to antibody optimisation, as all the preliminary work done with PY20 antibody also required optimising. Since the immunoblot images in *Fig. 7a* shows non-specific binding, the blocking step should be longer for these particular antibodies. Also, in order to optimise this experiment, more attempts at running the protocol are needed.

2.5.4 Analysis of changes in expression of EGFR family members shown from human proteome profiler immunoblot

Fig. 8 below shows the results of treating EGFR with the three types of venom used in this investigation. The effect of the three types of venom*: A gen., H swa* and *N naja* on EGFR has been analysed using the results from the human proteome profiler experiment, the data organised in Excel and the intensity of the relevant pair of dots plotted in graph form. The signals (y axis) measured for the three venoms are in the range of 7000000-9000000 (in pixels), with the positive control showing an intensity reading of over 9000000. This is potentially the result of minor inhibition of phosphorylation levels. Arguably, it is not known yet which domain of the receptor does the venom target, either extracellular or intracellular. Should the venoms target the intracellular domain, the venom incubation step should be longer or shorter, depending on the effect and the size of the peptides in each individual venom sample.

Fig. 8 Venom effects on phosphorylation levels of the Epidermal Growth Factor Receptor



2.5.5 Venom Fractionation of A gen.

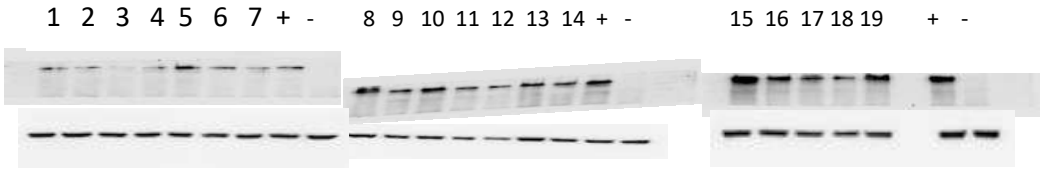


Fig. 9 Venom Fractions Western blots

A gen. venom was fractionated. The resulting fractions, 19 in total, were analysed via SDS-PAGE and western blot methods. The blots have been organised starting with PY20 antibody treated blots first (top images), followed by β actin antibody treated blots below each PY20 (bottom images).

Using High Pressure Liquid chromatography (HPLC), *A gen.* venom was fractionated. This step yielded 19 samples that were incubated with MDA-MB-468 cell lines via the protocol in Methods section. The samples obtained following fractionated venom treatment were analysed via SDS-PAGE and Western Blot methods. The reason for this step was to assess whether there are any peptides in *A gen.* venom that target specifically the EGF receptor. The use of PY20 antibody informed the level of phosphorylation within each samples and β actin will show whether the results seen on PY20 antibody treated blots was the result of the interaction between venom fraction and receptor or just unequal loading. By comparison with positive control lane, some fractions are exhibiting inhibitory effects on EGF receptor. Figure 9 is structured in two parts, with the PY20 antibody on top and β actin antibody on the bottom. The loading map for each blot is the following: Top blot lanes 1-6 were loaded with fraction 1-6, respectively. The last two lanes have been loaded with positive and negative control, respectively. Middle blot lanes 1-8 have been loaded with venom fractions 7-14, respectively. The last two lanes have been loaded with positive and negative control. Bottom blot, lanes 1-5 have been loaded with venom fractions 15-19 and the last two lanes after the gap are the positive and negative control. However, the positive control samples shows up as

a very intense band in the bottom blot and is not present on the middle blot. This is potentially a transfer issue. The step requires optimisation and therefore multiple runs, which are going to be made difficult by the small amounts yielded after venom extractions. A DS11 spectrophotometer was used to measure the protein concentration in each fraction. 2 µl of the re-suspension was used with DS11 spectrophotometer to measure the protein concentration. Each sample was measured 5 times and an average was calculated. The protein concentration was relevant to make sure the results obtained from PY20 antibody treated blots were not the influence of unequal protein concentrations in the samples. Given the nature of fractionating protocol, the fractions are freeze-dried.

Table 3a (top) and 3b (bottom) *A gen.* venom fractions protein concentrations

Table 3 – Fraction concentrations																		
Fraction	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Conc. mg/ml	40.367	185.834	258.981	30.720	3.798	3.540	5.098	13.435	14.535	106.113	5.561	5.991	8.247	6.724	15.578	12.161	11.580	7.396	0.17

2.5.6 Evaluation of optimal time for venom incubation

An assay to determine the optimal time for incubating the cells in venoms was carried out. The results of the experiment were analysed via SDS-PAGE and Western Blotting. The venom used in this step was *A gen.* The time intervals considered for this experiment were 20 min, 40 mins, 1hr, 1hr 20 mins, 1hr 40 mins, 2hrs, 2 hrs 30 mins, 3 hrs, 4 hrs 30 mins, 6 hrs, 24 hrs and 48 hrs. Positive control sample was treated with EGFR but not with venom, and the negative control sample was not treated with either EGFR or venom. Positive and negative control samples were collected at the 48 hr timepoint. PY20 antibody was used to determine the levels of receptor phosphorylation following treatment with *A gen.* venom. Venom dilution used for this experiment was 1 in 100, which was a concentration of 0.3772 mg/ml.

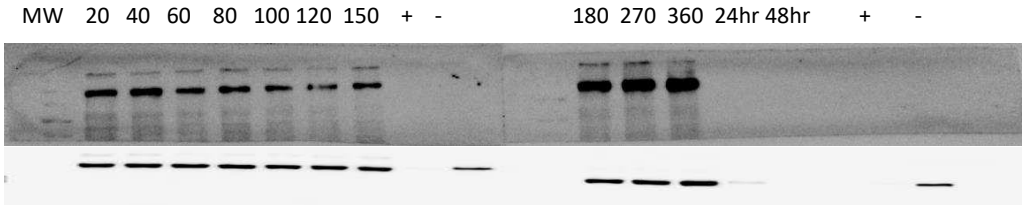
The cells were cultured in 12 well plates and allowed to grow until the bottom of each well was completely covered with cells. The media used for this step did not contain any Foetal Calf Serum, as the proteins in FCS can skew the results by masking the proteins in the samples collected. Each well was incubated in 250 µl of media, no FCS added.

As shown in *Fig. 10*, the lanes on the western blot images were labelled from 1-14. Lanes 8-9 and 15-16 were positive and negative control, respectively. In the same order as represented in *Fig. 1*, the samples represent the following time intervals: 20 minutes-lane 1, 40 minutes-lane 2, 1 hour-lane 3, 1hr 20 mins-lane 4, 1hr 40 mins-lane 5, 2hrs- lane 6, 2 hrs 30 mins-lane 7, 3 hrs-lane 10, 4 hrs 30 mins-lane 11, 6 hrs-lane 12, 24 hrs-lane 13 and 48 hrs-lane 14, respectively. The sample used as positive control has been treated with EGF but not with venom to assess the normal phosphorylation levels under the condition imposed by the experimental settings. Therefore, positive control lane should have represented the level of EGFR phosphorylation in MDA-MB-468 human cancer cell line. However, both negative and positive samples have been collected after 48 hours and the possible reason the protein levels are too low to be detected via immune-detection is the prolonged exposure to low volume of media and the lack of the proteins and nutrients provided normally by FCS. Another reason for the results observed would be destruction of cells following extended exposure to venom.

To determine the appropriate time incubation interval, more research is required. In *Fig. 10* below, the sample collected after two hours was loaded in the 6th lane. After visually analysing the blots in Fig 10, the 2-hour and 2 hour and 30 mins samples showed a clearer band with very little protein degradation or receptor breakdown. The ghost bands at the top of the blot represent potential non-specific binding of the PY20 antibody. The aim of this experiment was to inform on the best time interval for the breast cancer cells to be treated with the venom to interact with the Epidermal Growth Factor Receptor and display the effect

on the phosphorylation levels at different time points. The decision was made difficult by the lack of bands in positive and negative control lanes, which could be a result of long incubation intervals. The results seen in samples left for 24 hours and 48 hours in lanes 13-14 are consistent with positive control lanes 8 and 15, where actin is present in very small amounts or not present at all. This can be due to either transfer issues or a consequence of the cells being incubated for long intervals, with cells eliminating small molecular weight proteins into the media. However, an avenue worth exploring for any future time dependent experiments is to have positive and negative controls for every time interval analysed.

Fig. 10 Venom Incubation Assay Western Blot treated with PY20 antibody (top) and actin (bottom). Time in minutes unless otherwise stated.



2.6 Discussion

The present study investigated the effects of three different venoms from three different clades: Spider, Scorpion and Snakes on breast cancer cell line MDA-MB-468. After optimisation of the concentrations to be used for the kinome blot the kinome assay was undertaken. All the venoms studied had an inhibitory effect on EGFR phosphorylation as shown by the kinome blot study. Compared to the positive control, which represented the sample treated with EGF but no venom, scorpion venom *H.swa* had the highest overall inhibitory effect. The least inhibitory effect were recorded on the membranes treated with *N.naja* venom. The overall effect of the *H. swa* venom is due to the presence of disulphide and non-disulfide bridged proteins in its composition, which have a wide range of activities such as targeting ion channels, antimicrobial, haemolytic and anticancer effects (Almaaytah

and Albalas, 2014). Looking at the effects of ErBb2, *H. swa* had the most obvious inhibitory effect compared to positive control readings, followed by *N. naja* venom and *A gen* with the least inhibitory effects, compared to the scorpion and snake venom. This could be due to the presence of proteolytic linear peptides in spider venoms, which would disrupt the cell membrane completely and interfere with the cell surface proteins such as EGFR (Kuhn-Nentwig et al, 2011). *Naja* species venom has been shown to affect breast cancer cells by the activity of a protein called Cytotoxin (Munawar et al, 2017). ErbB2 has the highest presence in the positive control compared to the other two members of the EGFR family and as such any inhibitory activity of the venoms is of scientific interest. An inhibitor of HER2 in HER2 positive breast cancer could potentially lead to a new breast cancer drug. ErbB 3 is present in very low amounts in positive control and thus seems to be upregulated by all venoms, more so by *H. swa.* ErbB 4 is inhibited by *N. naja* venom and upregulated by *H. swa* venom. A very important factor to consider here is that despite various investigations reporting the interactions between venomous compounds and cancers, there is not a great deal of work done with the MDA-MB-468 breast cancer cell line. Previous investigations focused on MCF-7, A-431 or He-La cell lines mainly.

Fibroblast Growth Factor receptor 3, FGFR3 is present in highest numbers according to the positive control readings. *A. gen* venom had the highest inhibitory effect on this protein out of the three clades. Fibroblast Growth Factor Receptor FGFR and Vascular Endothelial Growth Factor Receptor VEGF are involved in neoangiogenesis – the process by which tumours create their own blood vessels (Presta et al, 2005; Turner and Grosse, 2010). Targeting any or both these cell surface proteins means more avenues to explore in anti-cancer treatments. Snake venoms are rich in disintegrins, a diverse range of proteins which

disrupt the activity of integrins which in turn regulate key stages in cancer initiation and progression (Kele, 2015).

Given that Mygalomorphae species' venom is rich in mammalian-specific compounds, this investigation was relevant by revealing the effect *Accanthoscurria geniculata* venom had on various tyrosine kinase receptors including on the members of the Epidermal Growth Receptor Family, which have been a relevant clinical target since their discovery in 1960 (Lo, 2010). Spider venoms are also known to contain non-peptide compounds such as inorganic salts (Heinen and da Veiga, 2011) and as such more research is needed to identify how these various compounds in either crude venom or venom fractions interact with various biological targets and what is their nature i.e. peptides or non-peptides. Also, the costs associated with designing new drugs can be reduced by looking at naturally occurring molecules (Almeida and Stanckowickz, 2016). According to Almeida and Stanckowickz (2016), there are no patent applications that involve venom from *Accanthoscurria* genus. However, venom from *Accanthoscurria Gomesiana* has been used in experimental settings to assess its effects on human cancer cell lines, including human breast cancer cell line SKBR3. Since 1986, 76 patent applications were filled following spider venom research results (Almeida and Stanckowickz, 2016). *Accanthoscurria geniculata* genome has been recently described to be approx. 6 Gb which in turn can prove helpful in investigating venom evolution and toxin selection (Pineda et al, 2014). Notably, theraphosidae venom investigations have been facilitated by advances in biochemistry tools (Craik et al 2013; Pineda et al, 2014).

Snake venoms are known to be rich in various compounds such as disintegrins, which are believed to disrupt key process in tumors (Kele et al, 2015). Integrins present in tumour cells play a role in proliferation, invasion and survival (Kele et al, 2015). Disintegrins are capable of binding specifically to integrins and thus interfere with vital tumorigenesis

processes (Kele et al, 2015). Thangam et al (2012) describe a disintegrin identified in *Naja Naja* venom with cytotoxic and apoptotic effects on human cancer cell lines in vitro. However, this study used MCF-7, HepG2 and A549 cell lines, which show different EGFR densities compared to cell line MDA-Mb-468. Investigations on snake venom have been facilitated by the amounts venom extractions would yield (King, 2013; Pineda et al, 2014). According to Mirtschin et al (2006), some *Naja* species wet venom ranges between 1.161 and 0.741 grams on average. Tarantula milkings usually results in amounts measuring between 2-10 µl. Thakur et al (2016) studied the effects of ruviprase, a protein identified in *Daboia Russelli* venom on various human cancer cell lines including MCF-7 breast cancer cell line, which showed the apoptosis inducing effects of the protein in a time dependent manner.

The human proteome profiler assay revealed the effects the venoms had on the level of phosphorylation of various receptors. Of particular interest is EGFR. The phosphorylation level graphs in the results section can be used to determine new targets for future breast cancer therapies. For instance, PDGFRβ has been linked to shorter survival rate in breast cancers (Paulsson et al, 2016). PDGFRα has been associated with tumour progression in a study carried out on MCF-7 and HS578T breast cancer cell lines (Carvahlo et al, 2005). In a study carried out on MDA-MB-231 and MDA-MB-157 triple negative breast cancer cell lines, PDGFRβ was shown to promote angiogenesis (D'Ippolito et al, 2016). Issa et al (2013) carried out an investigation on 4T1 and 67NR breast cancer cell lines to determine the efficacy of combination targeted therapy which revealed targeting both FGF and EGFR can have inhibitory effects on basal-like breast cancers (also known as triple negative breast cancer). Neovascularisation in an important process in tumorigenesis following which new, small tumours can grow, given the access to nutrients and blood supply (Sitohy et al, 2012). However, most clinical trials carried out on targeted VEGFR therapies had little success (Sitohy

et al, 2012). In a study carried out Bevacizumab in triple negative breast cancer, the scientists reported various adverse events, which led to discontinuation of the clinical trial for most of the patients involved in the study (Cameron et al, 2013). Bevacizumab is a monoclonal antibody that targets the VEGFR (Cameron et al, 2013). Fig 5 shows the levels of PDFR Receptor family. Positive control measurements revealed elevated levels of PDGF R Beta and FR 3. PDGFR Beta is inhibited by the activity of the *N. naja* venom to a higher degree compared to *A. gen* and *H. swa.* However, M CSF R is upregulated by the activity of *H. swa* and *A. gen* venom and inhibited only by the activity of *N. naja* venom.

A. gen venom was fractionated as there is no available data about this procedure done on the venom of this species. The resulting fractions, 19 in total were tested on MDA-MB-468 cell line. Fractions 1-7 had an obvious inhibitory effect when compared to positive control lane. Fractions 8-19 had various effects, with fractions 11, 12 and 18 inhibiting the phosphorylation of EGFR and fraction 15 and 19 upregulating phosphorylation of EGFR. According to Murao et al (2013), in *Acanthoscurria paulensis* venom, the first fractions contain acylpolyamines and other low molecular mass compunds, which may be responsible for the effects of *A. gen* venom fractions, as see in figure 9.

Available data has however several limitations: the human phosphor-array experiment and Western blot of spider venom fractions were carried only once, due to logistics and finance associated restrictions. In ideal conditions, a set of three repeats would have allowed a statistical interpretation. The results do however report findings on previously uninvestigated venoms in different experimental conditions. Given the limitations of both targeted and non-targeted cancer therapies and the variety of venoms in nature, researching, identifying and describing venomous compounds is an effort worthwhile in medicine. At the moment, technical advances and the new focus on naturally occurring peptides are paving

the way for venom peptide based drugs (King, 2013), convergence scientists should take more advantage of.

2.7 References

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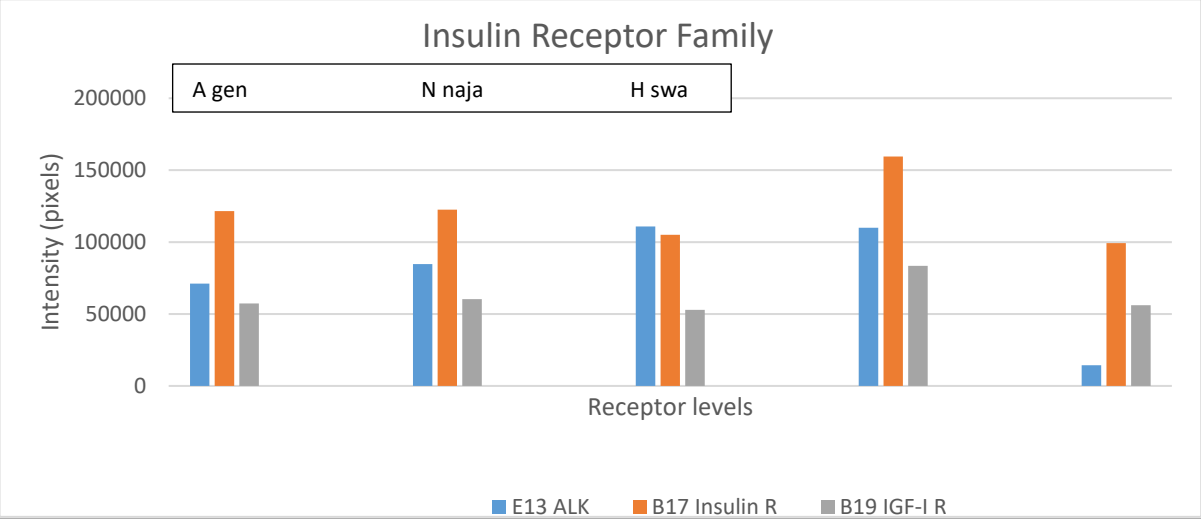
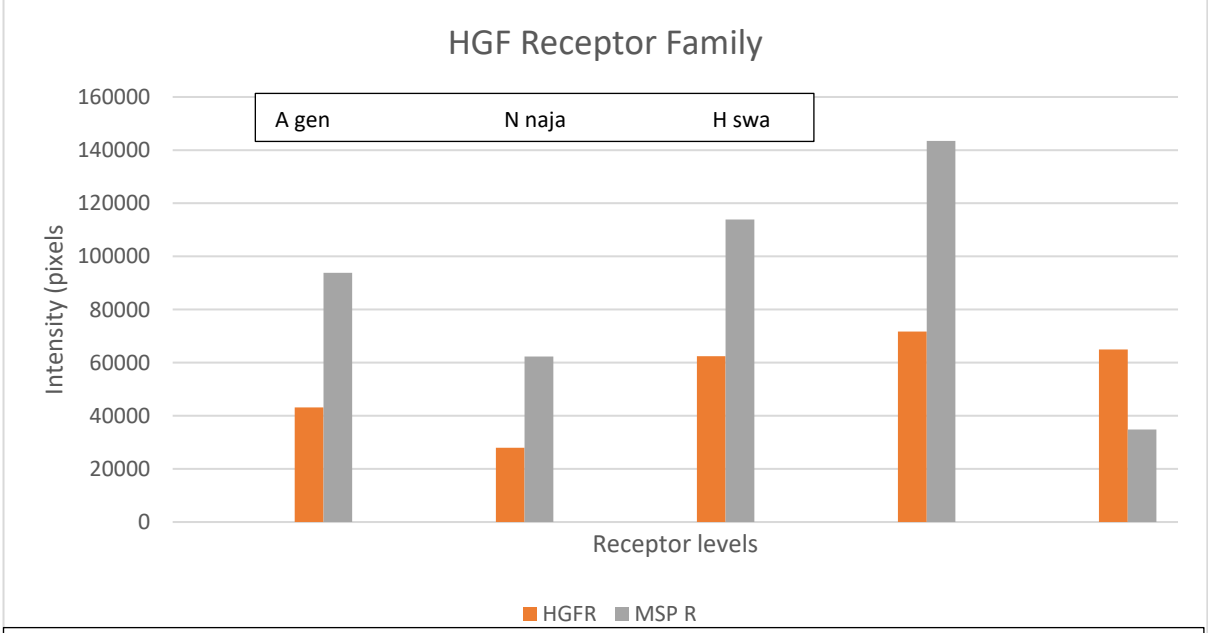
2.8 Appendix

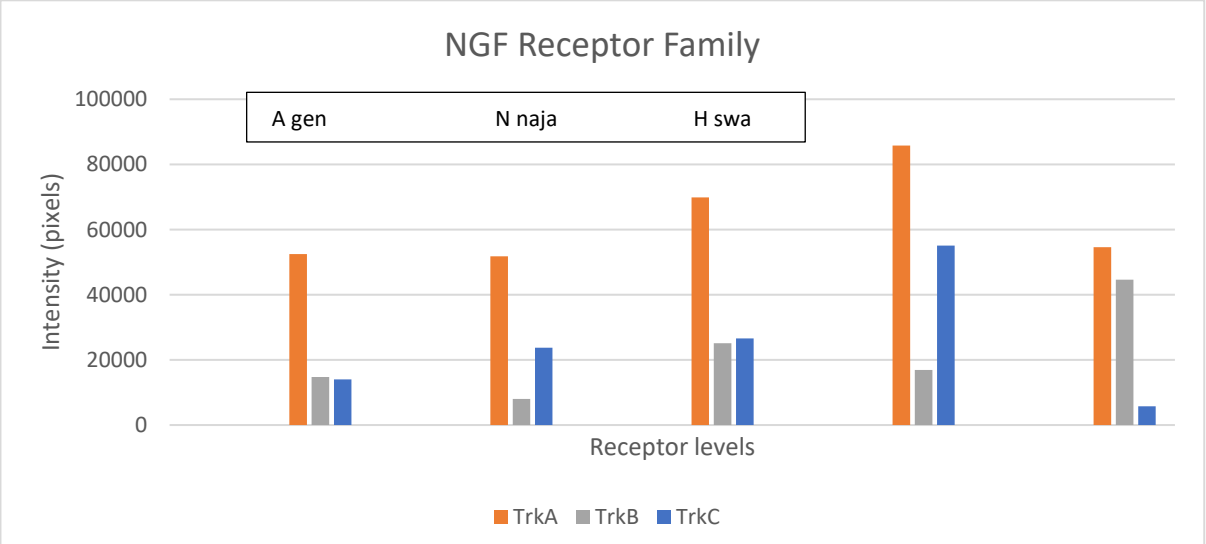
Graphs representing the phosphorylation levels in the remaining receptor tyrosine kinases (Results Section 1- Evaluation of optimal venom dose to use in Human Phospho-array experiment using Western blotting)



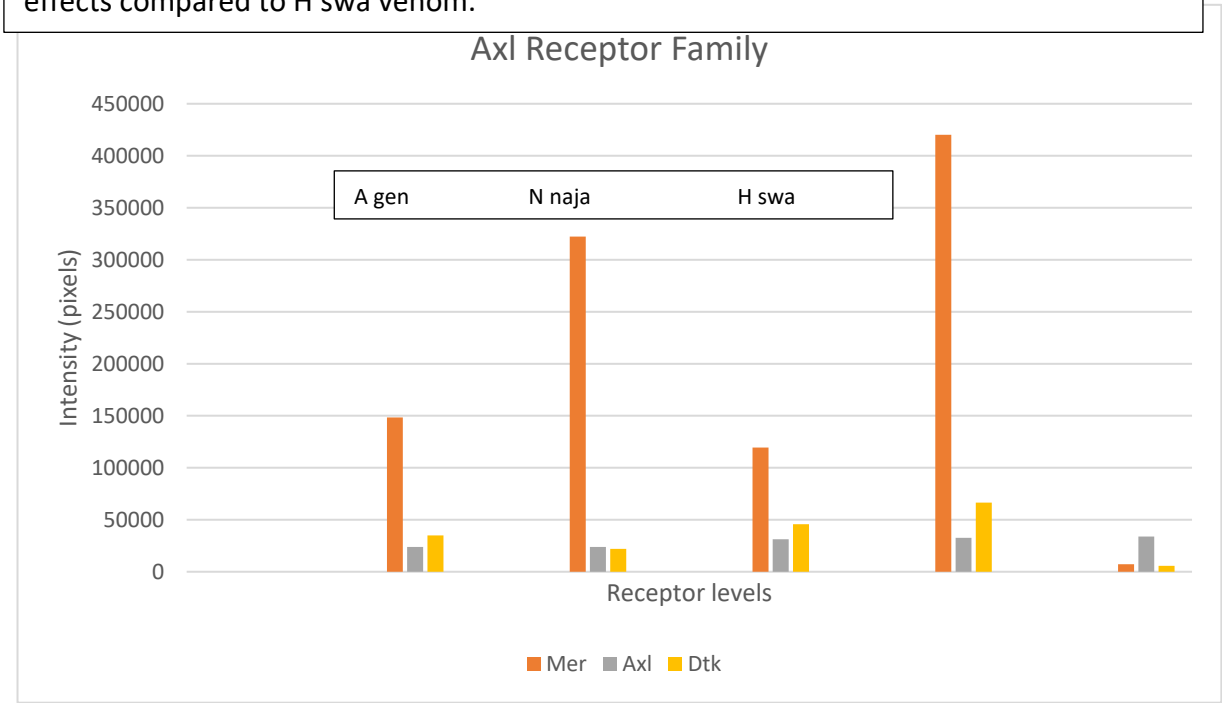
Appendix 1 above shows the effects of the three venoms on Insulin Receptor Family. According to the individual receptor levels in positive control, E13ALK receptor is present in highest amounts in cell line MDA-MB-468. A gen had the highest inhibitory activity on E13 ALK receptor, compared to the snake and scorpion venoms.



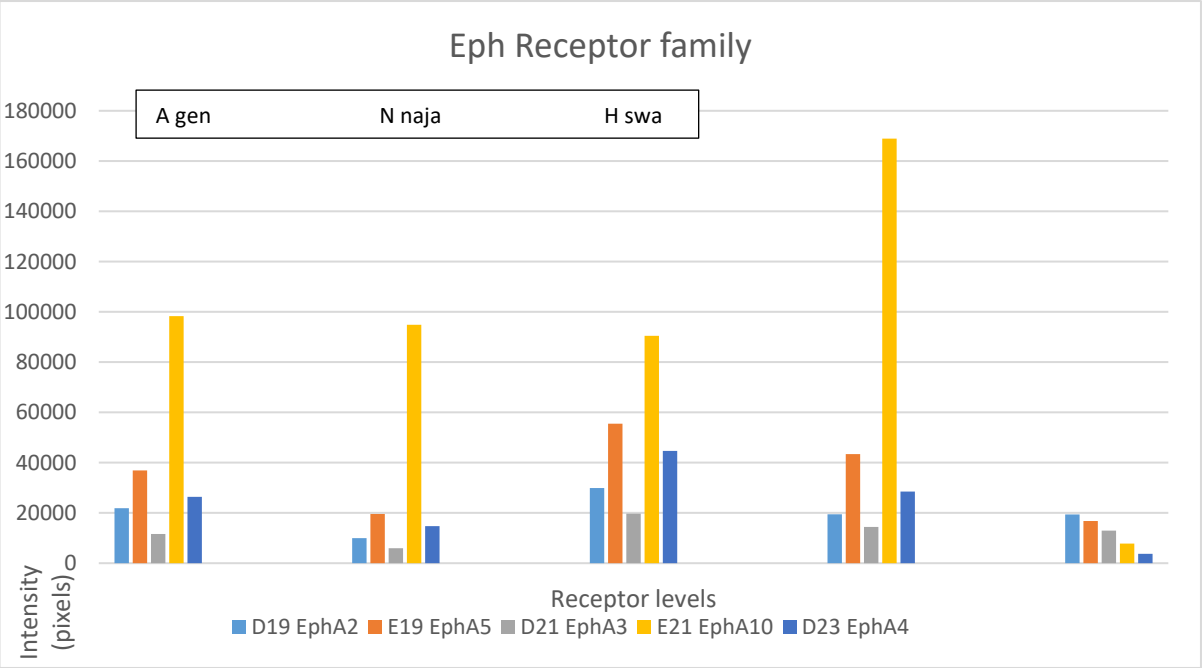
Appendix 2 above shows the effects of the three venoms on Hepatocyte Growth Factor Receptor Family. According to the positive control sample, MSP R is present in highest amounts in cell line MDA-MB-468. N naja venom had the highest inhibitory effect on MSP R, with a signal of 60000 pixel density.



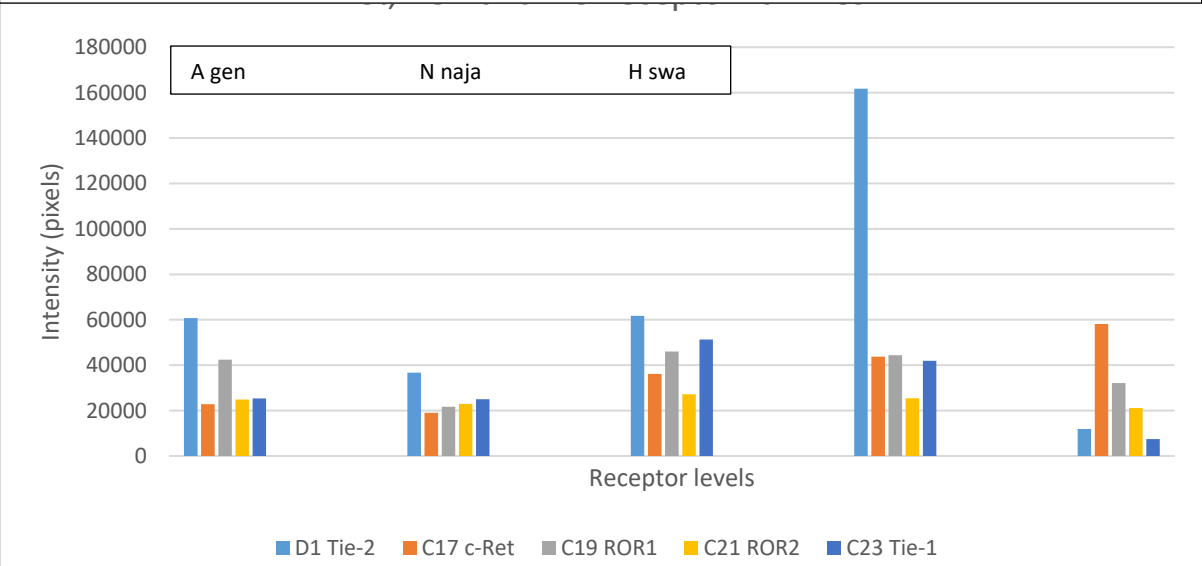
Appendix 3 shows the effects of the three venoms on neutrophin receptors. Present in highest amounts in cell line MDA-MB-468is TrkA receptor, according to the levels measured in positive control sample. Both A gen and N naja had equally high inhibitory effects compared to H swa venom.



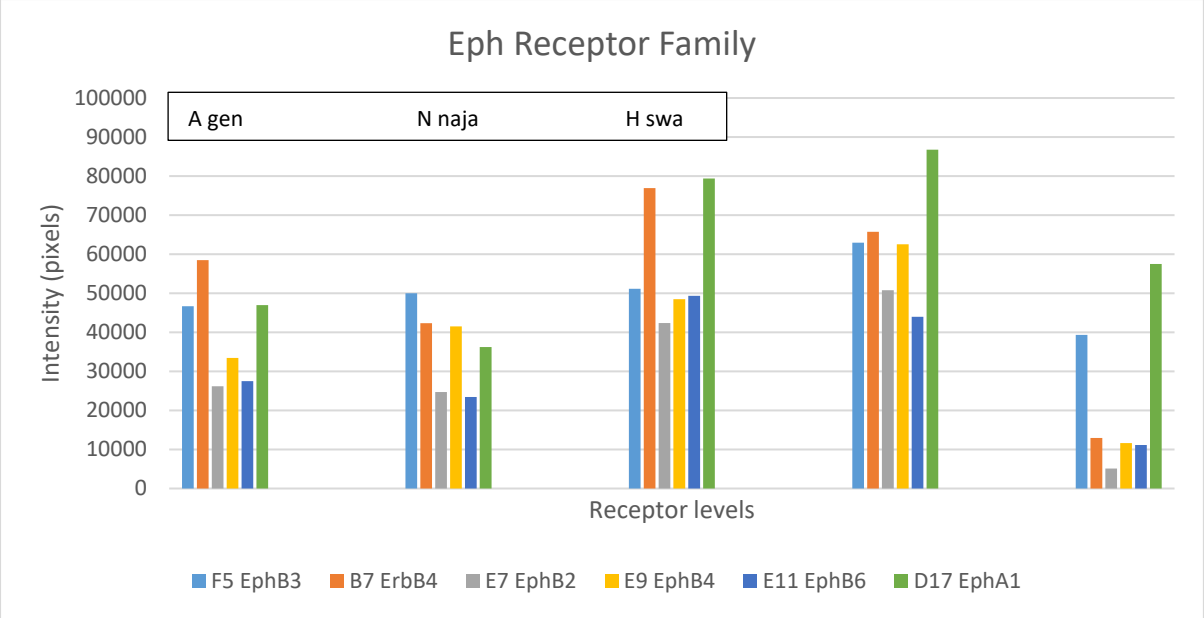
Appendix 4 shows the effects of the three venoms on AXL, which is a cell surface protein. According the readings in positive control sample, Mer Receptor in present in highest amounts in cell line MDA-MB-468. H swa venom had the highest inhibitory activity on the receptor out of the three venoms.



Appendix 5 and Appendix 7 show the effect on Ephrin receptors. According to positive controls samples in the two graphs, E21 EphA10 and D17 EphA1 are present in highest amounts in cell line MDA-MB-468. H swa had the highest inhibitory effect on E21 Epha10. D17 EphA1 were inhibited equally by H swa and A gen, but N naja had the highest



Appendix 6 shows the effects of the three venoms on three receptor families. D1 Tie-2 angiopoietin receptor is present in highest amounts in cell line MDA-MB-468. A gen and H swa had equally inhibitory effects on D1 Tie-2 receptor, with N naja venom showing the most inhibitory activity.



Appendix 7 Ephrin receptors