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Abstract

The search for novel and relevant cancer therapeutics is continuous and ongoing. Cancer adaptations, resulting in therapeutic treatment failures, fuel this continuous necessity for new drugs to novel targets. Recently, researchers have started to investigate the effect of venoms and venom components on different types of cancer, investigating their mechanisms of action. Receptor tyrosine kinases (RTKs) comprise a family of highly conserved and functionally important druggable targets for cancer therapy. This research exploits the novelty of complex venom mixtures to affect phosphorylation of the epidermal growth factor receptor (EGFR) and related RTK family members, dually identifying new activities and unexplored avenues for future cancer and venom research. Six whole venoms from diverse species taxa, were evaluated for their ability to illicit changes in the phosphorylated expression of a panel of 49 commonly expressed RTKs. The triple negative breast cancer cell line MDA-MB-468 was treated with optimised venom doses, pre-determined by SDS PAGE and Western blot analysis. The phosphorylated expression levels of 49 RTKs in response to the venoms were assessed with the use of Human Phospho-RTK Arrays and analysed using ImageLab 5.2.1 analysis software (BioRad). Inhibition of EGFR phosphorylation occurred with treatment of venom from *Acanthoscurria geniculata* (Theraphosidae), *Heterometrus swammerdami* (Scorpionidae), *Crotalus durissus vegrandis* (Crotalidae) and *Naja naja* (Elapidae). Western green mamba *Dendroaspis viridis* venom increased EGFR phosphorylation. Eph, HGFR and HER were the most affected receptor families by venoms. Whilst the importance of these changes in terms of effect on MDA-MB-468 cells' long-term viability and functionality are still unclear, the findings present exciting opportunities for further investigation as potential drug targets in cancer and as tools to understand better how these pathways interact.

Keywords

Breast Cancer, Venom, targeted therapy, Receptor Tyrosine Kinase, MDA-MB-468, Triple negative

1. Introduction

Venom systems present in a diverse range of animals, contain venom components known to target a range of biological pathways and tissue types accessible by the bloodstream (Fry *et al.*, 2009; Estevao-Costa *et al.*, 2018). For example, snake venom produces neurotoxic, haemotoxic and cytotoxic effects in humans and prey animals (Casewell *et al.*, 2014). To date, six FDA approved drugs have been derived from venoms (not including follow ups), with these currently providing treatment options for diabetes, chronic pain, hypertension and coagulation (Robinson *et al.*, 2017). Beyond this, many more drugs derived from venoms are in development or in clinical trials (Mohamed Abd El-Aziz *et al*., 2019) and it is clear that there is potential for many more such discoveries. Currently very little is known about venom effects on receptor tyrosine kinase phosphorylation.

Venom peptides are highly selective and potent (Pennington, Czerwinski and Norton, 2017), thus offering a potential advantage over small molecule inhibitors leading to potentially fewer side effects (Craik *et al.*, 2013). In recent years cancer researchers have turned to venoms to look for drug leads following the need to overcome side effects and resistance in standard and targeted therapies (Thangam *et al.*, 2012; Ma, Mahadevappa and Kwok, 2017). Disintegrins are an example of abundant proteins in snake venom which acts as a potent inhibitors of platelet aggregation and cell adhesion (Arruda Macedo, Fox and de Souza Castro, 2015). This has also been shown to have an effect on cancer cells (Chakrabarty and Chanda, 2015). Haemotoxic and cytotoxic venom components have also been shown to degrade tumour tissue (Tasoulis and Isbister, 2017) and to

have the potential to show clinically useful anti-metastatic and antiangiogenic properties (Kerkkamp *et al.*, 2018). Here for example, a range of scorpion venoms have been shown to affect Erk1/2 and STAT3 pathways and to cause DNA damage in colorectal and breast cancer lines in culture (Al-Asmari, Riyasdeen and Islam, 2018). Evidence for venoms affecting phosphorylation of receptor tyrosine kinases are limited to *Naja atra* cardiotoxin III blocking EGFR (Tsai *et al.,* 2016), insulin signalling within *Heloderma* and *Conus* venoms (Ahorukomeye *et al*., 2019), neurotrophins (Katzir *et al*., 2003).

Here we have investigated the effect of a diverse range of venoms from spiders, snakes and scorpions on the activity of receptor tyrosine kinases from the Epidermal Growth Factor Receptor (EGFR) family and related families. The EGFR family of tyrosine kinase receptors are key regulators of cell growth, transformation and other cellular processes in some breast cancer subtypes (Jacot *et al.*, 2015). The EGFR family consists of EGFR (HER1,ErbB1), HER2(ErbB2), HER3(ErbB3) and HER4(ErbB4), with EGFR and HER2 considered to be relevant and attractive clinical targets in breast cancer drug discovery (Hsu and Hung, 2016). Existing anti-EGFR and HER2 therapies include monoclonal antibodies such as Trastuzumab, which targets the extracellular domain of HER2, or small molecule tyrosine kinase inhibitors such as Lapatinib which inhibits receptor phosphorylation by targeting the intracellular ATP binding site of both HER2 and EGFR (Zhang *et al.*, 2007; Rusnak and Gilmer, 2011). These treatments are however limited as Trastuzumab does not properly infiltrate solid tumours (Zhang *et al.*, 2007) and Lapatinib has toxicity issues (Rana and Sridhar, 2012). Resistance has also been reported with Lapatinib and other small molecule inhibitors (Escriva-de-Romaní *et al.*, 2018). Our assays have utilised the triple negative breast cancer cell line MDA-MB-468, an oestrogen-independent cell line originating from a pleural effusion that usually expresses more than 10^6 EGF receptors per cell (Zhang, Fidler and Price, 1991; Modjtahedi, Styles and Dean, 1993).

These analyses indicate that whole venoms have an effect on phosphorylation of EGFR family members and related receptor tyrosine kinase which have the potential to block cancer related pathways and thus could contain useful tools to understand these pathways better.

2. Materials and Methods

2.1 Venom extraction

Venoms from the Brazilian whiteknee tarantula (*Acanthoscurria geniculata*), the Uracoan rattlesnake (*Crotalus durissus vegrandis*), the Western green mamba (*Dendroaspis viridis*), the giant forest scorpion (*Heterometrus swammerdami*), the Indian cobra (*Naja naja*) and the African Black Tail Scorpion (*Parabuthus liosoma*) were supplied by Venomtech Ltd. They were collected using Venomtech's optimised methodology to maintain maximum venom yield and quality. Snakes were 'milked' using a voluntary bite protocol, whilst invertebrates were milked using anaesthesia and mild electrical stimulation. Following extraction, the protein concentration of the whole venoms were determined by measuring absorbance at 280nm using a DS11 spectrophotometer (DeNovix, USA) blanked against HPLC grade H_2O. Venoms were then freeze dried or frozen at -20^{o}C until required.

2.2 Mammalian cell culture

MDA-MB-468 (ATCC, USA) were cultured in 25cm^3 culture flasks and Dulbecco's modified Eagle's medium (DMEM) (Gibco, UK). The DMEM media was supplemented with L-Glutamine (1%), penicillin-streptomycin (1%) and Foetal Calf Serum (10%). Cells were incubated at 37°C, 95% air and 5% CO_2. This cell line was authenticated using STR profiling in March 2018 (100% match to the MDA-MB-468 profile on the Cellosaurus database, ref CVCL_0419) and the work reported here was carried out in the same year. Cell growth was maintained using standard sub-culturing procedures.

2.3 Venom assay and cell lysis

MDA-MB-468 cells were plated out in six well plates and grown to 90% confluency before being treated with a 1:50- 1:1 000 000 serial dilution of each whole venom in DMEM media. Cells were incubated with venom for two hours at 37°C, 5% CO_2. The media containing the venom was then replaced with fresh media and EGF was added to a final concentration of $1x10^{-7}$M to all wells except the negative control. After 5 mins cells were washed with 2mM EGTA/PBS pH 7.4 and lysed using a RIPA buffer cocktail containing protease inhibitor cocktail, phosphatase inhibitor cocktail and EDTA. Cell lysates were then transferred to micro centrifuge tubes and centrifuged at 13000 rpm, 4°C for 10 mins. Once cell debris had pelleted, the supernatant was collected and mixed with 5x reducing sample buffer, heated to 100°C and stored at -20°C.

2.4 SDS PAGE and Western Blotting

20µl of each cell lysate were analysed on 9% and 12%, 0.75 mm polyacrylamide gels electrophoresed using a mini protein tetra cell (BioRad) and a Tris/Glycine/SDS buffer (0.025M Tris, 0.192M glycine, 0.1% SDS, pH8.3) at 70V until samples entered the gel and then at 170V till the dye front reached the end of the gel. Gels were then either stained for one hour using Coomassie blue dye or transferred to nitrocellulose via semi-dry blotting using an Invitrogen semi-dry western blotter for 45-60 minutes at 15V.

For Western blotting, the nitrocellulose membranes were incubated for one hour in blocking buffer (5% w/v Marvel non-fat milk powder solution in phosphate-buffered saline (PBS) 0.1% v/v Polyoxyethylenersorbitan monolaurate (Tween20)). Membranes were then washed 3 times for 10 min in PBS Tween20 and incubated overnight using a mouse monoclonal PY20 antibody (Sigma, UK) at 1.5µg/ml. Anti-β actin antibody (mouse, monoclonal AC-15 clone. Sigma) was used at a concentration of 3.5µg/ml as a loading control. After washing with PBS blots were incubated in rabbit anti-mouse-HRP antibody 1/20,000 for 1h. Blots were washed again using PBS and Enhanced

Chemiluminescence (ECL) reagents were mixed in equal volumes and applied directly onto the blots. ECL signals were developed using a Chemidoc (BioRad,UK).

2.5 Human Phospho-RTK Array

MDA-MB-468 cells were grown in 25cm^2 flasks until 90% confluent. Cells were treated with *A. geniculata, C. vegrandis, D. viridis, H. swammerdami, N. naja* or *P. liosoma* venoms for two hours at dilutions of 1:100, 1:1000, 1:10 000, 1:50, 1:10 000, 1:150 respectively. Cell lysates were then collected as described above and analysed according to the manufacturer's instructions using a Human Phospho-RTK Array Kit (R&D Systems).

Kinome array images were analysed using the ImageLab 5.2.1 analysis software (BioRad, UK). Lanes and pairs of dots were identified, allowing for the production of a pixel intensity from each duplicate pair of receptor dots. A template showing the RTK location on the blot is located in Appendix A, Table A.1). A fold change in intensity was created by dividing the intensity of each venom treated receptor by the intensity generated from the positive control of each receptor (No venom, +EGF). Changes in receptor phosphorylation were then displayed graphically as a fold-change in intensity, relative to the positive control receptor intensity levels. RTK's displaying a two fold change were considered to be affected by the venom treatment.

3 Results and Discussion

3.1 Changes in receptor phosphorylation and expression profiles in response to whole venom

The following optimised concentrations of venom were used (see Appendix B, Figure B.1) for these experiments 1/10,000 dilution (0.025mg/ml) of *N.naja* venom, 1/150 dilution (1.5mg/ml) of *P.liosoma* venom, 1/50 dilution (2mg/ml) of *H.swammerdami* venom, 1/10,000 dilution (0.025mg/ml) of *D.viridis* venom, 1/1,000 dilution (0.065mg/ml) of *C.durissus vegrandis* venom, 1/100 dilution (2.5mg/ml) of *A.geniculata* venom.

Changes in the phosphorylation and/or expression of 49 members of the receptor tyrosine kinase (RTK) family, were analysed in response to two hour incubation with venom. The arrays assess the changes in the expression/phosphorylation of representative members from most of the diverse sub-families of RTKs. Two-fold changes in receptor expression/phosphorylation in response to the venom were considered biologically interesting and both increases and decreases were observed (Appendix C, Table C.1).

The majority of the tested RTKs decrease in response to *C.durissus vegrandis* venom (See Table D.1., Appendix D). It is possible that *C.durissus vegrandis* does indeed have a pan kinome effect but further investigation would be required to confirm this. Even though protein levels in the Coomassie gel did not show a reduction from cytotoxicity, the comprehensive reduction in RTK signal could be due to proteolytic cleavage of the cell surface receptors but this is below the level detectable on the Coomassie gel. It is well known that Viperidae venoms contain more proteases than the other species tested. The majority of RTK's were also reduced in response to treatment with *P.liosoma* venom. However upon further follow up investigations with Coomassie gel analysis of the treated cell lysates produced for the kinome arrays, it was determined that the selected dose of *P.liosoma* venom appeared to have caused a large degree of cytotoxicity (Appendix E, Figure E.1). Due to these high levels of observed cytotoxicity, all changes in the expression/phosphorylation of the RTKs treated with this venom were deemed inconclusive and subsequently disregarded. There is still the

possibility that *P.liosoma* venom may have an effect on some of the 49 members of the RTKs provided on the array membranes at an appropriately selected sub-lethal dose, but this cannot be confirmed from these results.

The consistent protein level in all other lysates supports the changes detected, further in-depth discussion of some of the observed changes in RTK sub-family members and the implications of these changes to cancer therapy will be further discussed.

Figures 1-3 display changes in phosphorylation of the RTKs in response to the venoms. Two-fold threshold in phosphorylation/expression limits were set to triage effects compared to $1x10^{-7}$M EGF stimulated cell receptor levels. All receptor changes were displayed in a table for ease, with those over threshold highlighted (Appendix D, Table D.1).

The majority of the changes in phosphorylation levels were detected in the Ephrin and Epidermal growth Factor Receptor gene families. Although there are no previously published reports linking venoms to the Ephrin pathways, there is previous evidence of Chinese cobra cardiotoxin III from *Naja atra* venom reducing EGFR signalling (Tsai *et al*., 2016).

3.2 All venoms tested affected ephrin receptors

Eph receptors and their binding ligands, ephrins, constitute the largest sub-family of receptor tyrosine kinases. Eph receptors play a fundamental role in cell signalling pathways involved in animal development (Castaño, 2008). Binding of ephrins, to Eph receptors results in the bi-directional stimulation of the eph/ephrin signalling axis. Over-expression of ephrins and eph receptors can result in tumorigenesis, promoting tumour growth, survival, angiogenesis and metastasis (Surawska, 2004; Pasquale, 2010). Eph receptors can act as both suppressors and promotors of tumours in different contexts (Genander and Frisen, 2010). Many of the family members are clinically relevant

and tractable targets for intervention in human breast cancer (Brantley-Sieders *et al.*, 2011; Kaenel, Mosimann and Andres, 2012). Receptors of this subfamily are attractive targets for antiangiogenic therapy (Mosch *et al.*, 2010). This family's signalling activities in cancer appear to be complex (Pasquale, 2010) and therefore since some of the venoms target members of this subfamily, they could be used to understand these pathways further.

A

B

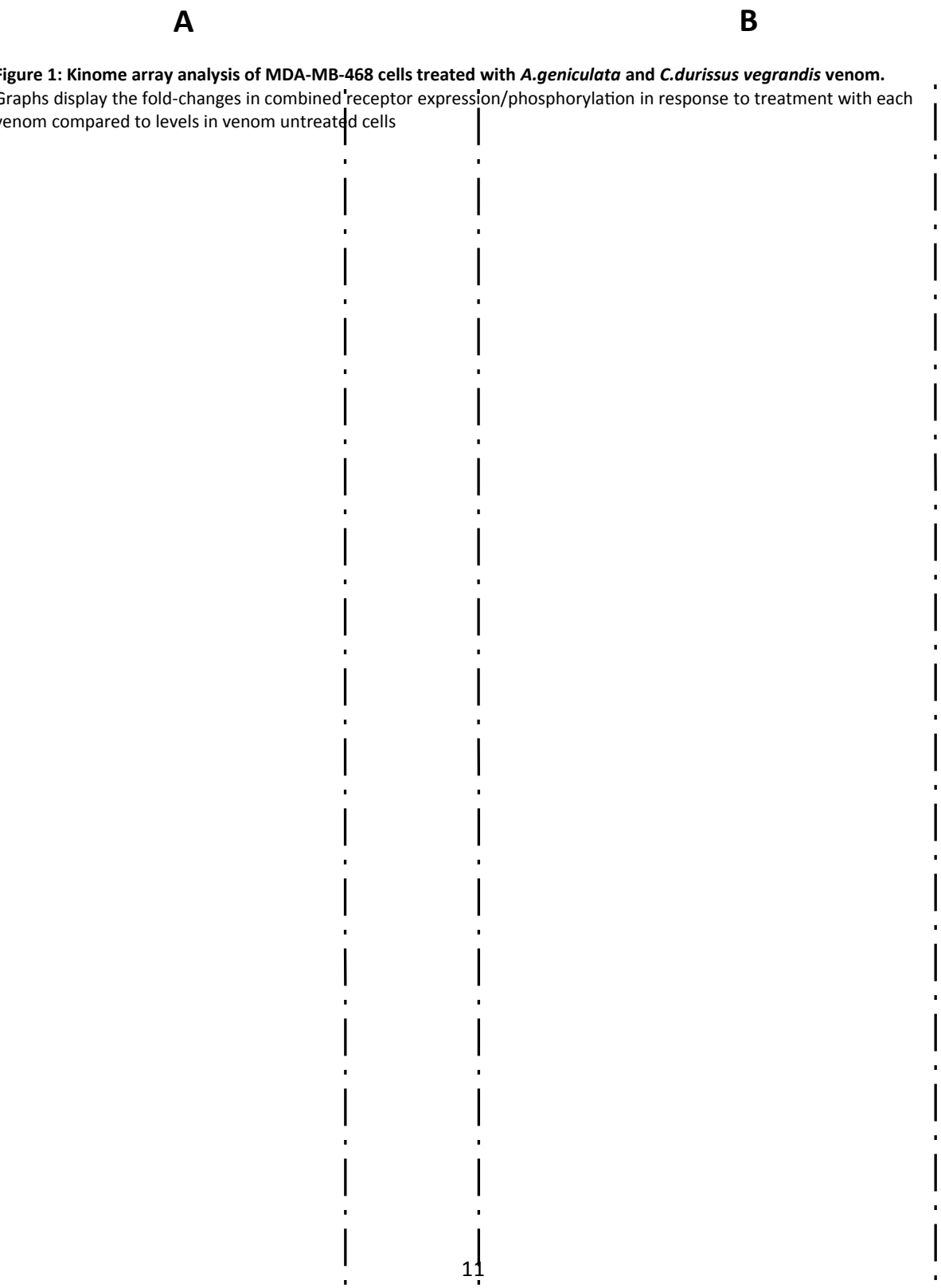

Figure 1: Kinome array analysis of MDA-MB-468 cells treated with *A.geniculata* and *C.durissus vegrandis* venom. Graphs display the fold-changes in combined receptor expression/phosphorylation in response to treatment with each venom compared to levels in venom untreated cells

Figure 2: Kinome array analysis of MDA-MB-468 cells treated with *D.viridis* and *H.swammerdami* venom.
Graphs display the fold-changes in combined receptor expression/phosphorylation in response to treatment with each venom compared to levels in venom untreated cells

Figure 3: Kinome array analysis of MDA-MB-468 cells treated with *N.naja* venom.
Graphs display the fold-changes in combined receptor expression/phosphorylation in response to treatment with each venom compared to levels in venom untreated cells

EphA1 over-expression has been identified in gastric cancer (Yuan, 2008), prostate cancer and a sub-set of colon, liver, lung, melanoma and mammary carcinomas (Robinson, 1996; Kao, 2003; Herath, 2006). EphA2 receptor over-expression has been identified in oesophageal, gastric, prostate and breast cancer (Easty *et al*., 1999; Miyazaki *et al*., 2003; Zelinski *et al*., 2001; Yuan *et al*., 2009; Nakamura *et al*., 2005; Xu *et al*., 2005; Gokmen-Polar *et al*., 2011; Petty *et al*., 2012, Huang *et al*., 2014, Tsouko *et al*., 2015) as well as lung cancer, where high EphA2 levels predicts metastatic outcome (Kinch, 2003). EphA3 mutations are the most commonly occurring Eph receptor change, identified in lung, hepatocellular, colorectal, glioblastoma and melanoma (Bae *et al*., 2009; Balakrishnan, 2007). These mutations appear to confer impaired kinase function, suggesting that EphA3 may function as a kinase-dependent tumour suppressor, which is disrupted by somatic cancer cell mutations (Lisabeth *et al*., 2012). High levels of EphA4 expression have been linked to poor patient survival in gastric cancers (Miyazaki *et al*., 2013), promotes cell proliferation and migration of human glioma cells (Fukai *et al*., 2008), promotes cell growth in human pancreatic ductal adenocarcinoma (Iiizumi, *et al*., 2006) and increased levels of metastasis in colorectal cancer (Oshima *et al*., 2008). EphA5 receptor has been shown to be downregulated in breast cancer (Fu *et al*., 2010) and EphA6, whilst shown to be downregulated in colorectal and kidney cancer (Hafner *et al*., 2004), has been shown to be upregulated in breast cancer (Zhou *et al*., 2018). Higher expression of EphA7 is detected in breast and glioblastoma cancers (Brantley-Sieders, 2011; Wang *et al*., 2008) and a reduced expression level in colorectal and prostate cancer (Wang *et al*., 2005; Guan *et al*.,

2009). EphA10 receptor has been shown to be upregulated in breast cancer including triple negative types, prostate and colorectal cancer (Nagano, 2014a and 2014b; Li, 2017).

Reductions in EphA2 occurred in response to *N.naja* venom (Figure 3), EphA6 in response to *A.geniculata* (Figure 1a) and *D.viridis* (Figure 2a) venoms, and EphA7 in response to *A.geniculata* venom. Over-expressions of EphA1 and A2 have been identified in a variety of cancers, including breast cancer, making *N.naja* venom attractive for further investigation. Whilst EphA4 receptor does not appear to be commonly upregulated in breast cancer, its upregulation in gastric, pancreatic, colorectal and glioma cancers are indicative of metastasis and poor patient prognosis. *A.geniculata* and *D.viridis* venoms would however be interesting to follow up for down-regulation of EphA6, which has been shown to be over-expressed in breast cancer, where it is indicative of poor patient prognosis (Zhou *et al*., 2018). Similarly *A.geniculata* venom shows potential for containing interesting targeting molecules for EphA7 receptor, which has also been shown to be overexpressed in breast cancer and glioblastoma (Brantley-Sieders, 2011; Wang, 2008). The search for novel targets for triple negative breast cancer makes EphA10 a viable target for novel targeted therapies, several venoms were close to threshold and related species might contain useful molecules.

Loss of EphB1 receptor tyrosine kinase has been linked to the progression of aggressive cancer types including acute myeloid leukaemia (Kampen *et al*., 2015), gastric, colorectal and ovarian cancer (Sheng *et al*., 2007; Wang *et al*., 2013) and renal cell carcinoma (Zhou *et al*., 2014). Both upregulations and downregulations in the expression of EphB2 have been linked to the progression of cancer. EphB2 has been shown to be over-expressed in highly aggressive breast cancer types, where it has been shown to regulate multiple functions including autophagy, apoptosis and invasion (Chukkapalli *et al*., 2014) and to result in poor survival in patients with ovarian cancers that present with high levels of EphB2 (Wu *et al*., 2006). Whilst reduced EphB2 expression has been linked to liver metastasis in colorectal cancer (Oshima *et al*., 2008). Like EphB2, EphB3 has been found to be both

upregulated and downregulated in the progression of cancer. EphB3 has been shown to be both up and downregulated in non-small cell lung cancers (Li *et al*., 2012; Ji *et al*., 2011). Over-expression of EphB3 has been linked to the suppression of colon cancer tumour growth (Chiu *et al*., 2009) and alternatively has been linked to stimulation of cell migration and metastasis in papillary thyroid cancer (Li *et al*., 2017). EphB4 receptor increases and decreases have both been linked to breast cancer progression (Xiao *et al*., 2012). EphB4 has been shown to supress breast cancer tumorigenicity through an Abl/crk pathway (Noren *et al*., 2006) and to acts as a survival factor in breast cancers (Kumar *et al*., 2006). The extracellular domain of EphB4 has also been shown to induce angiogenic responses in endothelial cells and its expression on the surface of breast cancer cells has been shown to promote angiogenesis by activating EphB2 reverse signalling, increasing tumour growth (Noren *et al*., 2004; Noren *et al*., 2007). EphB4 has been shown to be expressed in other cancers, with its upregulation shown in colon cancer (Stephenson, *et al*., 2001), bladder cancer (Xia *et al*., 2006) and ovarian cancer (Kumar *et al*., 2007). Finally, the expression levels of EphB6 in cancer cells has been linked to their progression and development, with losses in EphB6 expression often indicative of a more aggressive cancer type. Downregulations in EphB6 receptor have been seen in breast cancer cells, where it has been found to interact with c-Cbl to function as a tumour suppressor and to prevent tumour cell invasiveness (Fox & Kandpal, 2009; Truitt *et al*., 2010; Fox & Kandpal, 2006; Fox & Kandpal, 2004). EphB6 expression has been shown to partially supress epithelial-to-mesenchymal transition in triple negative breast cancer cells and to reduce tumour drug-resistance to DNA-damaging drugs, resulting in better chances for recurrence-free survival in patients with higher EphB6 expressing tumours (Toosi *et al*., 2018). Silencing or mutations of EphB6 expression in early stage non-small cell lung cancer has been found to be associated with the development of distant metastases and a more aggressive cancer type. It is thought, like with breast cancer, that EphB6 confers tumour suppression in NSCLCs (Yu *et al*., 2010; Bulk *et al*., 2012). Reduced gene expression of EphB6 in neuroblastoma, melanoma and prostate cancer are indicative of poor patient prognosis, amongst other cancer types (Tang *et al*., 2000; Tang *et al*., 2004;

Mohamed *et al*., 2015). Alternatively, EphB6 receptor over-expression in colorectal cancer has been linked to the promotion of tumour cell proliferation, migration and invasion (Xu *et al*., 2016).

EphB type receptors EphB4 and B6 were reduced following *A.geniculata* venom treatment (Figure 1a). Greater than 2-fold increases in EphB1 and B3 were observed in response to treatment with *H.swammerdami* venom (Figure 2b). Losses of EphB1 receptor have been shown in literature to be indicative of highly aggressive cancer types and poor patient prognosis. *H.swammerdami* venom is likely to contain possible therapeutic tools for this target. Treatment with this venom resulted in a 2-fold increase in EphB1 combined expression/phosphorylation levels, which could be useful for restoring EphB1 expression levels in cancers where, EphB1 acts as a tumour suppressor and had been downregulated. Both up and downregulations in EphB2 expression levels have been observed in cancer. Dosing with *H.swammerdami* venom resulted in a greater than 2-fold increase in the combined expression/phosphorylation of EphB3. A component from this could have potentially interesting implications for the treatment of cancers where EphB3 is a known tumour suppressor and has been downregulated in expression to circumvent this, such as has been shown to be the case in colon cancer tumours (Chiu *et al*., 2009). Both EphB4 and EphB6 receptors were reduced in response to treatment with *A.geniculata* venom (Figure 1a). Whilst both receptors have been shown to be up and downregulated in cancer types, EphB4 upregulations are more commonly implicated in poor cancer prognosis, whilst EphB6 downregulations are more indicative of poor patient outcome. Treatment with both these venoms resulted in downregulations in both receptors expression/phosphorylation levels. These changes could prove beneficial for the treatment of ErbB4 over-expressing cancers, including breast, bladder, ovarian and colon cancer. Whilst EphB6 downregulations are less likely to be useful in a therapeutic capacity for the treatment of cancer, as loss of EphB6 expression appears more common in tumorigenesis, there are cases of cancer that do appear to express over-expression of EphB6, such as colorectal cancer. Currently in clinical trials are

cancer drugs that target the kinase activity of EphA2 and EphB4 but there is still a lot to learn about this receptor network (Buckens *et al*., 2020).

3.3 Effects of venoms on EGFR family member phosphorylation levels

Some, but not all, of the investigated venoms reduced detectable phosphorylation above threshold in EGFR family members. Interestingly, from the changes observed in the EGFR family members, there appears to be both selective and multiple targeting of like-receptor types. Selectivity is interesting from complex mixtures and hints at a single active component.

No significant reductions were seen in EGFR expression/phosphorylation in response to venom using the kinome arrays. However due to the over-expression of EGFR in the MDA-MB-468 cells used as a model for these experiments, it was considered possible that changes may be occurring but are undetectable due to saturation of the EGFR antibody binding capacity of the blots (see the EGFR dots in Appendix C, Figure C.1). Follow up Western blot analysis was undertaken on lysates previously produced for kinome array analysis to determine if changes to EGFR expression or phosphorylation could be observed using an alternative/less sensitive method (Figure 4a). Anti-β actin analysis confirmed that all samples were loaded with a similar amount of total protein, with the exception of the *P.liosoma* treated cell lysate, previously shown in Coomassie gel analysis to be lacking protein due to toxicity (see Appendix E, Figure E.1). Western blot analysis of EGFR phosphorylation (pY) and expression levels (EGFR) showed that there were detectable differences in phosphorylation in response to the different venoms, previously undiscernible in kinome arrays because of oversaturation (Appendix C, Figure C.1). Thus the differences are likely due to the degree of sensitivity of the two analyses. Kinome arrays are highly sensitive to low levels of receptors or small changes in their expression level. MDA-MB-468 cells express EGFR at such a high level that there is a very strong probability that even in low levels (like with the *P.liosoma* lysate) there was enough receptor present to saturate the preloaded antibody available on the blot for binding.

Figures 4b and 4c display the percentage phosphorylation and percentage receptor expression respectively and reveal that reductions in EGFR receptor phosphorylation levels of nearly 70% occurred in response to treatment with both *A.geniculata* and *N.naja* venom. A combination of changes in the overall expression and phosphorylation level of EGFR were seen in response to treatment with *C.durissus vegrandis* venom, with calculated reduction in expression of 60% and reductions in the detectible phosphorylation levels of nearly 95%. EGFR expression and phosphorylation levels post-dosing with *H.swammerdami* venom revealed that there appears to be EGFR breakdown in response to treatment. A detectable phosphorylated band of around 75k Da was observed when looking at phosphorylation levels of EGFR on the Western blot (Figure 4a), with reductions in detectible EGFR phosphorylation of 75% also observed. Overall EGFR expression levels

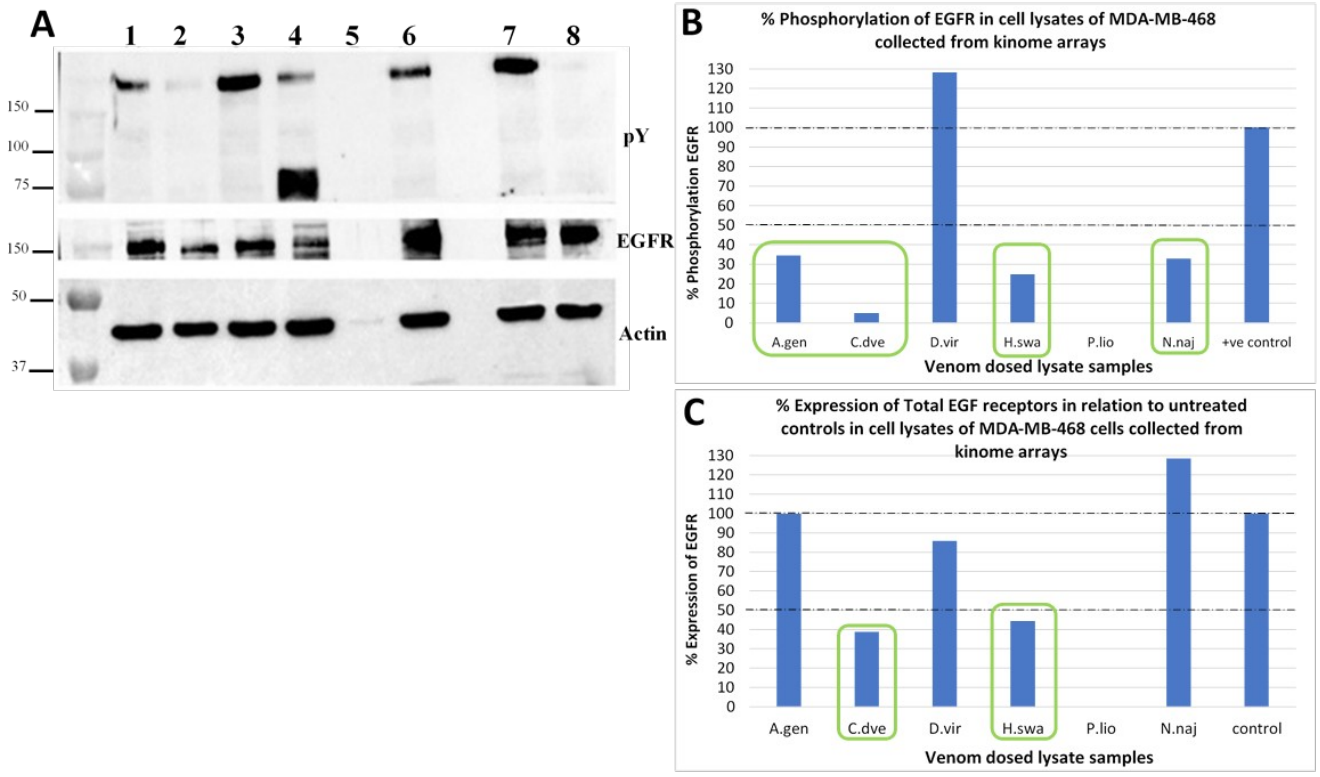

Figure 4: Western Blot analysis of EGFR phosphorylation and expression levels in MDA-MB-468 cells post whole venom treatment.

Lysates produced for kinome array analysis were analysed via western blot to determine changes in expression (EGFR) and phosphorylation (pY). Anti-β actin was used as a loading control to validate any observable changes. All pY and EGFR bands were normalised against actin bands before % phosphorylation was calculated relative to the +ve control band for each blot.

5041: *A.geniculata*, 2: *C.durissus vegrandis*, 3: *D.viridis*, 4: *H.swammerdami*, 5: *P.liosoma*, 6: *N.naja*, 7: +EGF only (+ve Control),
5058: -Venom, -EGF (-ve Control)
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508are also considerably lower compared to the untreated EGFR expression level controls, with around
50955% reductions in total EGFR protein. There appears to be a slight EGFR expression reduction of 15%
510in response to *D.viridis* treatment, however despite this, treatment with this venom still appears to
511have resulted in nearly a 30% increase in EGFR phosphorylation levels.

512

513Reductions in EGFR expression or phosphorylation in response to treatment with four of the
514selected venoms, including two distinctly diverse snake species, one scorpion and a theraphosid
515venom warrants further investigation. EGFR over-expression and signalling have been shown to be
516important in the development, progression and metastasis of a large number of cancer types
517including triple-negative breast cancer (Foley *et al*., 2010; Ueno *et al*., 2011; Davis, 2014; Park, 2014;
518Nakai, 2016; Ali, 2017; Liang, 2017), colorectal cancer (Koustas, 2017; Zhao *et al*., 2017; Huang *et al*.,
5192017; Huang *et al*., 2018) and lung cancer (Liu *et al*., 2017; Nukaga *et al*., 2017; Singh, 2018)
520amongst many others. EGFR is now well established as a target for treatment in a wide variety of
521cancers, with high-level EGFR in primary tumours correlating with highly aggressive basal-like
522phenotypes and poor patient prognosis. Due to its over-expression in many cancer types, the
523inhibition of EGFR phosphorylation has been shown to be a successful strategy in the fight against
524cancer (Ono and Kuwano, 2006). However, development of resistance to current cancer therapies
525through further EGFR mutation or EGFR-pathway circumvention is a common occurrence
526(Pietrantonio *et al*., 2017; Lim, 2018; Liu, 2018; Yu, 2018), often linked to patient relapse. Reduction
527in EGFR expression and phosphorylation levels seen in response to *C.durissus vegrandis*, *N.naja* as
528well as scorpion *H.swammerdami* and theraphosid *A.geniculata* venoms could open up a large pool
529of venom-derived biological molecules, from a large diverse population of species, which may

possess novel mechanisms and binding sites for the targeted suppression of EGFR and treatment of EGFR-expressing cancers where prior treatment has failed due to therapy resistance.

HER2 over-expression has been shown to be linked to the induction and progression of a variety of cancer types including, prostate (Day *et al*., 2017), colon and colorectal (Takegawa *et al*., 2017; Pirpour Tazehkand *et al*., 2018; Siena *et al*., 2018), gastric and oesophageal (Gerson *et al*., 2017; Wu *et al*., 2017), ovarian (Luo *et al*., 2018) and predominantly occurring in the HER2+ breast cancer subtypes (Baselga *et al*., 2017: Loibl & Gianni, 2017; Pondé, 2018). A two-fold reduction was observed in HER2 phosphorylation in response to treatment with *N.naja* (Figure 3) and *H.swammerdami* (Figure 2) venoms. Whilst prognosis for patients presenting with HER2-positive breast cancers has greatly improved thanks to advances in targeted therapies, resistance through HER3 up-regulation is a common problem. Oncogenic HER3 mutations have emerged as new therapeutic targets for the treatment of breast, ovarian, lung, prostate and other cancer subtypes (Jaiswal *et al*., 2013; Schardt *et al*., 2017). Targeting of HER3 has also been shown to sensitise head and neck squamous cell carcinomas by increasing tumour sensitivities to Cetuximab, reducing HER3 activity and preventing HER2/HER3 dimerisation occurrence (Wang *et al*., 2017). HER3 has been shown to play fundamental roles in cancer, both independently and in conjunction with other RTKs to circumvent therapeutic suppression, making it, like other ErbB receptor kinases a key target for therapeutic development. Reductions in combined HER3 phosphorylation/expression levels were observed in response to *A.geniculata* (Figure 1a) and *N.naja* (Figure 3), suggesting that these venoms could contain useful components for the treatment of HER3 over-expressing cancers. Activating mutations in HER3 have been identified in both HER2+ and ER+ breast cancer types, with HER2 and HER3 co-expression in breast cancer commonly observed (Mishra, 2018; Mishra, 2018). Studies have shown upregulation in HER3 expression by HER2 as a mechanism involved in therapy-resistance (Yang, 2017; Li, 2018; Lyu, 2018). Treatment with *N.naja* venoms resulted in the suppression of both HER2 and HER3, suggesting the propensity for these venoms to contain useful components effective

against HER2/HER3 co-expressing cancers and HER2+ breast cancer subtypes which have upregulated HER3 overcoming therapeutic suppression.

H.swammerdami venom produced a two-fold increase in HER4 phosphorylation. Given that both up-regulations and down-regulations in HER4 expression and signalling have been linked to the development and progression of cancer. The observed increase in expression of HER4 following treatment with *H.swammerdami* venom could have useful implications in for finding compounds to restore normal receptor levels. Cancers known to down-regulate HER 4 expression include renal (Thomasson *et al.*, 2004), papillary carcinoma (Kato *et al.*, 2004) high-grade gliomas (Andersson *et al.*, 2004) and breast cancer (Wang *et al*., 2016). HER4 expression levels have been shown to be both down- and up-regulated in triple-negative and non-triple-negative breast cancer types respectively (Ansarin *et al*., 2018).

Pan-HER treatments, targeting EGFR, HER2 and HER3 simultaneously have been developed in recent years in an attempt to overcome the development of resistance to antibody-based therapies through target circumventing (Iida *et al.*, 2016) and small molecules tyrosine kinase inhibitors (Geuna *et al.*, 2012; Solca *et al.*, 2012; Mancheril, Aubrey Waddell & Solimando, 2014). Aptamer based therapies that also target the three receptors in breast cancer are also being used in an attempt to eliminated the problem of resistance development and therapy failure (Yu *et al.*, 2018). Treatment with *N.naja* venom displayed this same Pan-HER targeting ability, causing reductions in the combined phosphorylation/expression levels of EGFR, HER2 and HER3. *A.geniculata* venom, whilst not targeting all HER receptors showed dual targeting of EGFR and HER3.

Whilst all other tested venoms resulted in a change in at least one of the ErbB receptor family members, *D.viridis* venom had no detectible effects on the members of this receptor sub-family,

thus the effects observed are likely to be specific responses to the venoms. HER3 and HER4 receptors have been detected at the neuromuscular junction and in association with nicotinic acetylcholine receptors (Zhu *et al*., 1995), a known venom target, thus this may explain the evolutionary targeting of venom components to these receptors.

Other effects of whole venoms on receptor tyrosine kinases

FGFR1 phosphorylation level was dramatically downregulated in cells dosed with *A.geniculata* venom, with a lesser effect seen on FGFR2 (Figure 1). The amplification of FGFR1 is linked to 21% of lung adenocarcinomas (Dutt *et al*., 2011), as well as being observed in prostate (Devilard *et al*., 2006), ovarian (Cole *et al*., 2010), lung (Jiang *et al*., 2015; Heist *et al*., 2012) and oral squamous cell carcinomas (Freier *et al*., 2007). FGFR1 upregulation has been found to occur in 10% of cases in oestrogen-dependent breast cancer (Gru & Allred, 2012), where it is linked to the promotion of cell proliferation. Gene amplification of FGFR1 has been shown to occur in breast cancer (Turner *et al.*, 2010). 12% of endometrial cancers have been identified as positive for FGFR2 gene mutations (Dutt *et al*., 2008) as well as cases of lung squamous cell and cervical cancers (Liang *et al*., 2013). FGFR2 gene amplifications or missense mutations are more commonly occurring than FGFR1 mutations in cancer, with amplifications found in 10% of gastric cancers (Katoh & Katoh, 2009) and 2% of breast cancers (Heiskanen *et al*., 2001; Bai *et al*., 2010). Even though the cells were dosed with whole venom it suggests further study is warranted on the components responsible for the effect of Theraphosidae venoms on FGF receptors. Other effects produced by *A.geniculata* venom include reductions in TrkA, TrkC, Tie2 and HGFR.

Neurotrophin receptor signalling through tropomyosin receptors kinases (Trk) A, B and C has been linked to the development of certain types of cancer. Whilst rare in most cancers, Trk fusions with other proteins are well-established oncogenic-driver events in papillary thyroid carcinoma, glioblastomas and secretory breast carcinomas. As well as Trk fusions, amplifications and alternative splicing events have been described as drivers in cancer pathogenesis (Lange and Lo, 2018). Trk A

over-expression has been linked to enhanced growth and metastatic propensity of breast cancer cells (Lagadec *et al*., 2009; Demont *et al*., 2012), with downregulation of Trk A expression by small interference RNAs (siRNAs) shown to abolish metastatic potential and increase chemosensitivity in them (Zhang, 2015). In neuroblastoma tumours, variable expression of Trk B in conjunction with brain-derived neurotrophic factor (BDNF) are indicative of poor patient prognosis, whilst high-level expression of Trk A and Trk C are associated with a good prognosis (Lucarelli *et al*., 1997; Yamashiro *et al*., 1997; Sugimoto *et al*., 2001; Thiele & McKee, 2009; Tanaka *et al*., 2014). Trk B over-expression has also been identified in highly aggressive metastatic human pancreatic cancers (Sclabas *et al*., 2005). Crosstalk between EGFR and Trk B has been shown to enhance cell migration and proliferation in ovarian cancer cells (Qiu *et al*., 2006), whilst Trk B has been found to be a key regulator of PI3K and JAK/STAT signalling pathway activated metastasis and epithelial-mesenchymal transition in breast cancer cells (Kim *et al*., 2015). TrkB forms a complex with EGFR and Sortilin in exosomes and Sortilin is also deregulated in cancer (Wilson *et al*., 2016).

Neurotrophin effects were expected from *N. naja* venom as NGF has been identified in this species (Hogue-Angeletti *et al*., 1976) and other cobras *Naja kaouthia* (Katzir *et al*., 2003) and *Naja atra* (Lu *et al*., 2017). The published sequence of *Naja naja* venom NGF from only differs in two amino acids at the C terminal from the other two Aspartate (D) instead of from Glutamate (E) at position 103 and Threonine from Lysine at position 114 (Appendix F, Figure F.1). Although unlikely, these mutations may have an effect on trk phosphorylation especially if mutations in these RTK's are present in the MDA-MB-468 cells, but this requires further investigation.

Dendroaspis viridis venom has a dramatic effect on the insulin receptor, increasing total phosphorylation 4.5 fold from control. Diverse venoms from Cone snails (*Conus spp*.) have utilised insulin signalling in prey capture (Ahorukomeye *et al*., 2019) and famously a glucagon like peptide has been clinically utilised from *Heloderma suspectum* (Yap & Misuan, 2019). Therefore, this is

possibly a newly discovered mechanism in the mamba *Dendroaspis* viridis which warrants further investigation.

Dendroaspis viridis venom also had a dramatic effect on RET (REarranged during Transfection) proto-oncogene (c RET). Gain-of-function mutations and upregulation in the expression of c-RET receptor tyrosine kinase have been linked to the development of several cancer types. C-Ret expression is often observed in oestrogen receptor positive (ER+) breast cancer subtypes, where RET expression is induced by oestrogens and RET signalling enhances oestrogen-driven proliferation of the breast cancer cells. RET expression has been detected in primary breast cancer samples, with higher RET expression levels identified in ER+ tumours (Boulay *et al*., 2008; Morandi *et al*., 2011). RET mutations in the form of chromosomal rearrangements (inversion of balanced-translocation) involving the RET catalytic domain and leading to the creation of fusion RET/PTC oncogenes have been linked to papillary thyroid carcinoma (PTC) development (Mologni, 2011). Gain-of-function mutations in RET have also been linked to the development of medullary thyroid carcinoma (MTC) (Hedayati *et al*., 2016, Carlomagno, 2012; Phay and Shah, 2010). Both small molecule (Andreucci, 2016) and antibody-drug conjugate (Nguyen *et al*., 2015) therapies have been developed as potential therapies for the treatment of RET over-expressing breast cancer, with treatments proving effective against these RET over-expressing cancers. However, despite the evidence for RET as an oncogene promoting cancer development in thyroid cancer and pheochromocytoma, RET has been identified to potentially have a tumour suppressive role in colon cancer. RET methylation was found in 27% of colon adenomas and 63% of colorectal cancers, where these aberrant methylation events were found to correlate with decreased RET expression. Subsequent restoration of RET expression in these instances was found to result in apoptosis of colorectal cancer cells (Luo *et al*., 2013). The other major effect of *D.viridis* venom was the increase in hepatocyte growth factor receptor (HGF R/ c-MET). C-MET receptor tyrosine kinase has been identified as a key player in many cancers including breast (Yan *et al*., 2015), lung (Zucali *et al*., 2008), melanoma (Cheng *et al*., 2017), myeloma, (Moschetta *et al*., 2013) adrenocortical carcinoma (Phan *et al*., 2015), pancreatic cancer (Brandes *et*

al., 2015) and gastric cancer (Ha *et al*., 2013), with its over-expression indicative of poor patient prognosis and highly invasive tumour types (Cazet *et al*., 2010; Goetsch *et al*., 2013; Ho-Yen *et al*., 2014; Ho-Yen *et al*., 2015; Yan *et al*., 2015; Zhang *et al*., 2018). Within breast cancer subtypes high levels of c-MET protein in hormone-receptor (HR)+ breast cancers and phosphor-c-MET in HER2+ breast cancers were indicative of worse relapse-free survival and overall survival rates in patients. High levels of c-MET and phosphor-c-MET were identified in all breast cancer types are were indicative of poor patient prognosis (Raghav *et al*., 2012), with c-MET over-expressions also having been identified in triple-negative breast cancer cell lines, MDA-MB-468, HCC-1395, and MDA-MB-231, where it has been linked to EGFR-therapy resistance (Sohn *et al*., 2014). Thus even though the c-MET is over expressed in this cell line, it is not fully phosphorylated as further phosphorylation was stimulated by *D.viridis* venom. *A.geniculata* and *N.naja* venoms are expected to contain useful signalling molecules due to the down regulation detected.

Greater than two-fold reductions in the expression/phosphorylation were observed in Tie-2 receptor in response to all venoms, apart from *D.viridis,* (Figure 2) with the greatest specific reduction observed with *H.swammerdami* (Figure 2b) venom. Interestingly, no changes were observed in Tie-1 receptor in response to treatment with any of the 5 selected venoms. Given Tie-1 is an orphan receptor and depends on dimerisation with Tie-2 to promote its activation (Savant *et al*., 2015; Mueller & Kontos, 2016), it is possible that its expression and activity levels were unaffected by treatment with the venoms as there were no compatible binding molecules. Tie-2 mutation and upregulation has been linked to the development and progression of cancer, with increased expressions promoting the onset of angiogenesis in tumour microenvironments. Decreases in the expression or activation of this receptors in response to venoms, may prove beneficial in over-expressing tumour types, by delaying the triggering of angiogenesis. The lack of reduction in Tie-1 receptor expression/activity may also be beneficial, as Tie-1 is thought to play a role as a tumour

suppressor in Tie-2 over-expressing cancers cells, by reducing Tie-2 signalling activity upon their dimerisation together (Singh *et al*., 2012).

Proto-oncogene tyrosine-protein kinase (Mer) receptor is overexpressed in many human cancers, including various leukaemias and numerous solid tumours, including breast cancer (Linger *et al*., 2010). Mer upregulation has been linked to increased cancer cell aggression, where like Axl, it has been found over-expressed in metastatic breast cancer cells, relative to non-metastatic breast cancer cells (Tavazoie *et al*., 2008; Linger *et al*., 2010). Frequent over-expression of Mer receptor has been found to occur in human non-small cell lung cancer (NSCLC), where it has been shown to attribute to erlotinib small molecule resistance, in EGFR mutated cancer types (Xie *et al*., 2015). A study by Linger *et al*.,(2013), evaluating 88 human NSCLC tumours found that Mer and Axl receptor tyrosine kinases were over-expressed in 69% and 93% of the tumours tested respectively, when compared to the levels of these receptors in normal surrounding lung tissue. The study also found that Mer and Axl were frequently over-expressed and activated in NSCLC cell lines. They showed that inhibition of Axl and Mer receptor activities promoted apoptosis, prevented proliferation and enhanced the chemosensitivity of the cells (Linger *et al*., 2013). A similar study in astrocytoma found that inhibition of these receptors resulted in increased apoptosis and chemosensitivity (Keating *et al*., 2010). The two arachnid venoms tested in this study both reduced Mer phosphorylation levels and thus could contain useful components for cancer research.

4 Conclusion

This study provides the first kinome scale investigation of the effects of venoms on cancer cells *in-vitro*. The preliminary evidence identifies many previously unknown effects of venoms on receptor tyrosine kinases and demonstrates the effects reported from other venoms on RTK's maybe widespread throughout venomous taxa. Further work is required on fractionated venoms to identify the active components responsible for these effects, to understand their evolutionary origin and

therapeutic potential. It is clear through evolution that there is no selection pressure for venoms to affect cancer cells, however through this research we may uncover a greater understanding of the signalling pathways of venom components and their potential for therapeutic utility.

Additional information:

Authorship

Danielle McCullough, Cristina Atofanei and Emily Knight: Investigation, Carol M Trim and Steven A Trim: Conceptualization, Methodology and Supervision. All authors contributed to writing original draft preparation, review and editing.

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Competing interests

There are no competing interests

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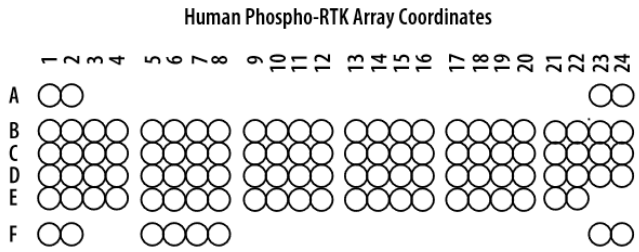
Appendix A

Coordinate	Receptor Family	RTK/ Control		Coordinate	Receptor Family	RTK/Control
A1, A2	Reference Spots	----------		D1, D2	Tie	Tie-2
A23, A24	Reference Spots	----------		D3, D4	NGF R	Trk A
B1, B2	EGF R	EGF R		D5, D6	NGF R	Trk B
B3, B4	EGF R	ErbB2		D7, D8	NGF R	Trk C
B5, B6	EGF R	ErbB3		D9, D10	VEGF R	VEGF R1
B7, B8	EGF R	ErbB4		D11, D12	VEGF R	VEGF R2
B9, B10	FGF R	FGF R1		D13, D14	VEGF R	VEGF R3
B11, B12	FGF R	FGF R2a		D15, D16	MuSK	MuSK
B13, B14	FGF R	FGF R3		D17, D18	Eph R	Eph A1
B15, B16	FGF R	FGF R4		D19, D20	Eph R	Eph A2
B17, B18	Insulin R	Insulin R		D21, D22	Eph R	Eph A3
B19, B20	Insulin R	IGF-IR		D23, D24	Eph R	Eph A4
B21, B22	Axl	Axl		E1, E2	Eph R	Eph A6
B23, B24	Axl	Dtk		E3, E4	Eph R	Eph A7
C1, C2	Axl	Mer		E5, E6	Eph R	Eph B1
C3, C4	HGF R	HGF R		E7, E8	Eph R	Eph B2
C5, C6	HGF R	MSP R		E9, E10	Eph R	Eph B4
C7, C8	PDGF R	PDGF Rα		E11, E12	Eph R	Eph B6
C9, C10	PDGF R	PDGF Rβ		E13, E14	Insulin R	ALK

C11, C12	PDGF R	SCF R		E15, E16	----------	DDR1
C13, C14	PDGF R	Flt-3		E17, E18	----------	DDR2
C15, C16	PDGF R	M-CSF R		E19, E20	Eph R	Eph A5
C17, C18	Ret	c-RET		E21, E22	Eph R	Eph A10
C19, C20	ROR	ROR1		F1, F2	Reference Spots	----------
C21, C22	ROR	ROR2		F5, F6	Eph R	EphB3
C23, C24	Tie	Tie-1		F7, F8	----------	RYK
				F23, F24	Control (-)	PBS

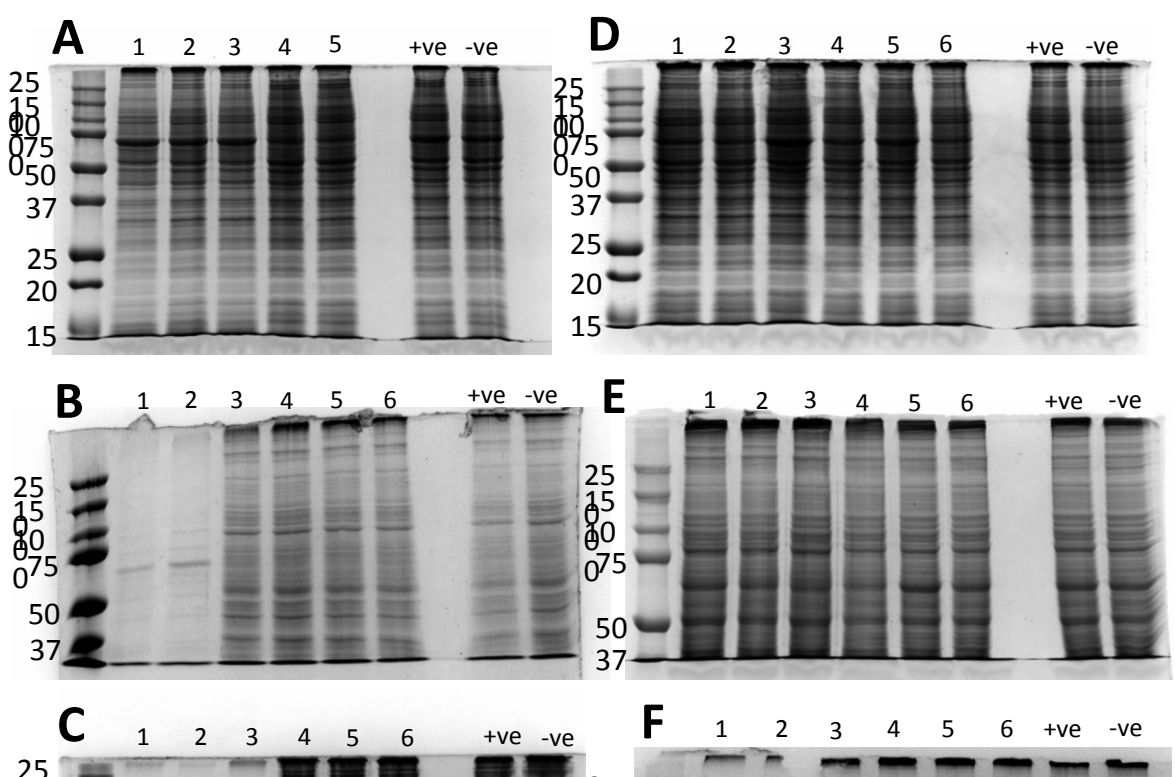
Table A.1 Human p-RTKs.

The table displays a list of each RTK probed for on the kinome array membranes, the sub-family of RTKs they belong to, and their relevant coordinate location on each blot.



Appendix B - Venom

The following SDS PAGE gels (Appendix B figure B.1) were used to optimise the concentrations of venom used to treat the cells in the kinome blots and Western blots of EGFR. The gels show the effect of the venoms on the whole proteome and which concentration the venoms become toxic to the cells reducing the protein available.



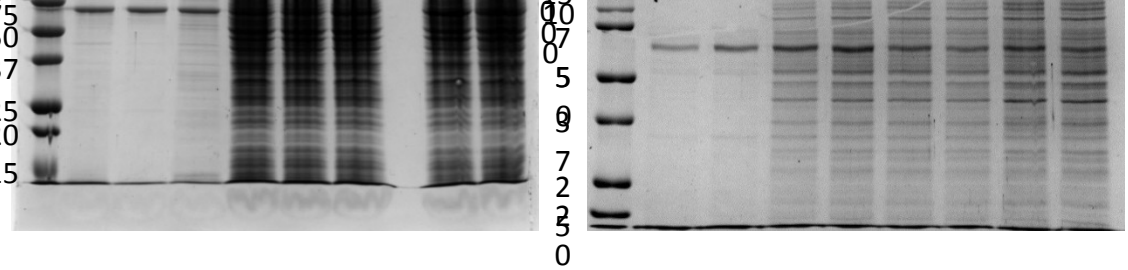


Figure B1: Coomassie Gel Analysis of Venom Toxicities

A. MDA-MB-468 cell lysates from cells treated with a serial dilution of whole ***A.geniculata*** venom lanes **1-5** (**1:**1/50, **2:**1/100, **3:**1/1000, **4:**1/10,000, **5:**1/100,000, respectively). **B.** MDA-MB-468 lysates from cells treated with a serial dilution of whole ***D.viridis*** venom lanes **1-6** (**1:**1/50, **2:**1/100, **3:**1/1000, **4:**1/10,000, **5:**1/100,000, **6:**1/1,000,000 respectively). **C.** MDA-MB-468 lysates from cells treated with a serial dilution of whole ***N.naja*** venom **1-6** (**1:**1/50, **2:**1/100, **3:**1/1000, **4:**1/10,000, **5:**1/100,000, **6:**1/1,000,000 respectively). **D.** MDA-MB-468 lysates from cells treated with a serial dilution of whole ***C.durissus vegrandis*** venom **1-6** (**1:**1/50, **2:**1/100, **3:**1/1000, **4:**1/10,000, **5:**1/100,000, **6:**1/1,000,000 respectively). **E.** Displays the Coomassie gel analyses of MDA-MB-468 lysates from cells treated with a serial dilution of whole ***H.swammerdami*** venom **1-6** (**1:**1/50, **2:**1/100, **3:**1/1000, **4:**1/10,000, **5:**1/100,000, **6:**1/1,000,000 respectively). **F.** MDA-MB-468 lysates from cells treated with a serial dilution of whole ***P.liosoma*** venom lanes **1-6** (**1:**1/50, **2:**1/100, **3:**1/1000, **4:**1/10,000, **5:**1/100,000, **6:**1/1,000,000 respectively). All positive controls were produced from MDA-MB-468 cells treated with $1x10^{-7}$M EGF (No venom, +EGF). All negative controls were produced from untreated MDA-MB-468 cells (No venom, -EGF).

Appendix C

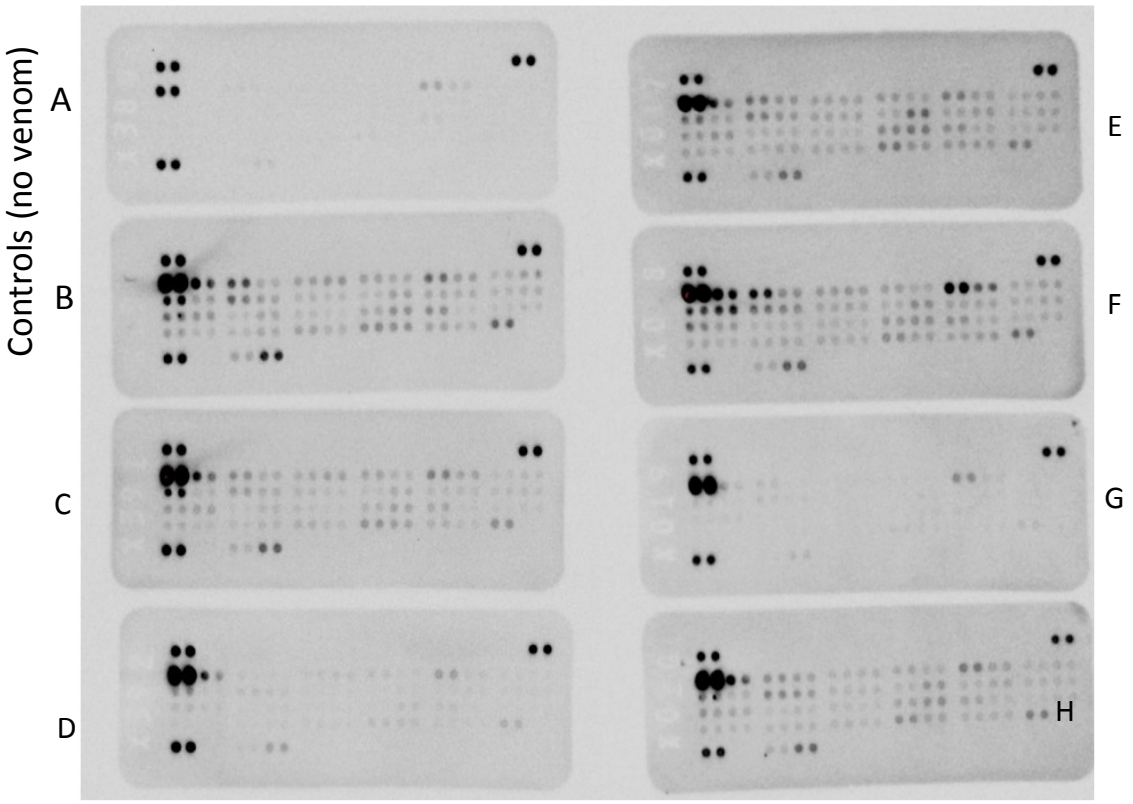


Figure C.1: Kinome array analyses of MDA-MB-468 cells treated with whole venoms
The kinome arrays show changes in the expression and activity of 47 receptor tyrosine kinases in MDA-MB-468 cells in response to the application of the six venoms of interest. All cells were treated with the venoms for two hours and subsequently stimulated with 1x10-7M EGF for five minutes except for the positive and negative controls **A**. Receptor expression and activity levels in untreated MDA-MB-468 cells which have not been stimulated with 1x10-7M EGF (Negative control). **B**. Receptor expression and activity levels in untreated MDA-MB-468 cells which have been stimulated with 1x10-7M EGF for five minutes (positive control). **C**. Receptor expression and activity levels of MDA-MB-468 cells which have been treated with a 1/10,000 dilution (0.025mg/ml) of *N.naja* venom. **D**. Receptor expression and activity levels of MDA-MB-468 cells which have been treated with a 1/150 dilution (1.5mg/ml) of *P.liosoma* venom. **E**. Receptor expression and activity levels of MDA-MB-468 cells which have been treated with a 1/50 dilution (2mg/ml) of *H.swammerdami* venom. **F**. receptor expression and activity levels of MDA-MB-468 cells which have been treated with a 1/10,000 dilution (0.025mg/ml) of *D.viridis* venom. **G**. Receptor expression and activity levels of MDA-MB-468 cells which have been treated with a 1/1,000 dilution (0.065mg/ml) of C.durissus vegrandis venom. **H** Receptor expression and activity levels of MDA-MB-468 cells which have been treated with a 1/100 dilution (2.5mg/ml) of *A.geniculata* venom.

Appendix D

	A. gen	*C.dve*	*D.vir*	*H.swa*	*N.naj*
EGFR	X	X	X	X	X
HER2	X	√	X	√	√
HER3	√	√	X	X	√
HER4	X	√	X	√	X
FGF R1	√	√	X	X	√
FGF R2 alpha	√	√	X	X	X
FGF R3	X	√	X	X	X
FGF R4	X	√	X	X	X
Insulin R	X	X	√	X	X
IGF-I R	X	√	X	X	X
Axl	X	√	X	X	X
Dtk	√	X	X	X	√
Mer	√	√	√	√	X
HGF R	√	√	√	X	√
MSP R	X	√	X	X	√
PDGF R alpha	X	√	X	X	X
PDGF R beta	X	√	X	X	X
SCF R	X	√	X	X	X
Flt-3	X	√	X	X	X
M-CSF R	X	√	X	√	X
c-RET	X	√	√	X	X
ROR1	X	√	X	X	√
ROR2	X	√	X	X	X
Tie-1	X	X	X	X	X
Tie-2	√	√	X	√	√
Trk A	√	√	X	X	√

Trk B	X	√	X	X	√
Trk C	√	√	X	X	X
VEGF R1	X	√	X	X	X
VEGF R2	X	√	X	X	X
VEGF R3	X	√	X	X	X
MuSK	X	√	X	X	X
Eph A1	X	√	X	X	X
Eph A2	X	√	X	X	√
Eph A3	X	√	X	X	X
Eph A4	X	√	X	X	X
Eph A5	X	√	X	X	X
Eph A6	√	√	√	X	X
Eph A7	√	√	X	X	X
Eph A10	X	√	X	X	X
Eph B1	X	√	X	√	X
Eph B3	X	√	X	√	X
Eph B2	X	√	X	X	X
Eph B4	√	√	X	X	X
Eph B6	√	√	X	X	X
ALK	X	√	X	X	X
DDR1	X	√	X	X	X
DDR2	√	√	X	X	X
RYK	√	√	X	X	X

Table D.1: Changes in RTK expression/phosphorylation state in response to treatment with whole venoms. The table displays 2-fold or greater reductions or increases in the combined phosphorylation/expression profile of each of 349 receptor tyrosine kinases in response to 5 whole venom treatments. Greater than 2-fold upregulations in green, greater than 2-fold downregulation in red.

Appendix E

Figure E.1: Coomassie gel analysis of total protein in MDA-MB-468 cell lysates after 2hr treatment with venoms. The Coomassie gel analysis of venom-treated MDA-MB-468 cell lysates shows that all cell lysates appear to have consistent levels of total protein, with the exception of the lysate produced from MDA-MB-468 cells treated with *P.liosoma* venom for two hrs. This lack of total protein can be attributed to cytotoxic at the dose of this venom selected for cell treatment.

Appendix F

```
P61898 NGFV_NAJAT            1   EDHPVHNLGEHSVCDSVSAWVTKTTATDIKGNTVTVMENVNLDNKVYKEYFFETKCKNPN        60
P61899 NGFV_NAJKA            1   EDHPVHNLGEHSVCDSVSAWVTKTTATDIKGNTVTVMENVNLDNKVYKEYFFETKCKNPN        60
P01140 NGFV_NAJNA            1   EDHPVHNLGEHSVCDSVSAWVTKTTATDIKGNTVTVMENVNLDNKVYKEYFFETKCKNPN        60
                                 ************************************************************

P61898 NGFV_NAJAT           61   PEPSGCRGIDSSHWNSYCTETDTFIKALTMEGNQASWRFIRIETACVCVITKKKGN           116
P61899 NGFV_NAJKA           61   PEPSGCRGIDSSHWNSYCTETDTFIKALTMEGNQASWRFIRIETACVCVITKKKGN           116
P01140 NGFV_NAJNA           61   PEPSGCRGIDSSHWNSYCTETDTFIKALTMEGNQASWRFIRIDTACVCVITKKTGN           116
                                 ******************************************:***********.**
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Figure F.1 Alignment of cobra Nerve Growth Factors (NGF). Although other neurotrophins are present in elapid snakes this alignment focuses on those of the same size across the species relevant to this manuscript.