Modulating the behaviour of pancreatic tumour cells

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Chapter 5: Whole snake venoms: Cytotoxicity, anti-metastatic and anti-angiogenic properties

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Abstract

Currently, biological and organic substances are globally screened for development of a new generation of therapeutics active against cancer. Amongst the snake venoms, candidates have been identified which exhibit promising properties. In this pilot study, snake venoms from different species (*Naja anulifera*, *Naja kaouthia*, *Ophiophagus hannah* and *Echis carinatus*) were investigated for their anti-malignant properties on pancreatic cancer cells. To this end, cells were incubated with different venoms and subsequently subjected to *in vitro* cell death and migration assays and *in vivo* dissemination and angiogenesis assays. For the *in vivo* assays, the cells after incubation and labelling were subjected to transplantation into the yolk sac of zebrafish embryos and monitored for motility and angiogenesis. Since there were few effects observed *in the vivo* assay, we soaked fish injected with untreated cells in the different venom solutions. The results showed that strong effects occur in *in vitro* samples treated with venoms derived from *Ophiophagus hannah* and *Echis carinatus*. Conversely, in the *in vivo* assays, venom derived from *N. naja* had the most beneficiary effects with respect to angiogenesis. These venoms might therefore be well-suited candidates for further studies.

Introduction

At present, biological and organic compounds are promising candidates for anti-cancer drugs. These include snake venoms which were shown to exhibit multiple therapeutic effects, despite their toxic properties. Snake venoms, which are toxic to most animals, can damage many tissues upon a snakebite, such as the central nervous system (Osipov A. and Utkin Y., 2012), the kidneys (Karthik S. and Phadke KD., 2004) and the blood (haemorrhage) (Kamiguti AS., 1996). Also, an innate immune response can be initiated in which the complement system is activated upon venom injection (Leon G., et al., 2011).

Currently, compounds derived from snake venoms are used for multiple therapeutic applications, such as drugs for cardiovascular purposes, anti-coagulants (Koh CY., and Kini RM., 2012) and anti-cancer drugs (Jain D1, Kumar S., 2012; Selistre-de-Araujo HS., et al., 2010; Markland FS, 2001). In this study, anti-malignant properties of snake venoms were tested on pancreatic tumour cells.

Tumourigenesis involves interactions of malignant cells with each other, neighbouring cells and their microenvironments, hereby often attracting surrounding vasculature to balance the oxygen supply with the demand of a growing tumour. The vascular network is further employed by metastasizing cancer cells for their long and short distance traveling to possible secondary metastatic sites. The importance to understand and prevent metastasis formation is emphasized by the fact that over 90% of all cancer related deaths are due to secondary tumours arising from metastasizing cancer cells (Jemal A., 2007). This is especially true for pancreatic cancer types which have a poor prognosis in 85-90% of the patients due to metastatic complications (Ganeh P., et al, 2007).

Zebrafish and their transparent embryos have been employed in several useful models for therapeutic drug research and preclinical studies (Dooley K. and Zon LI., 2000). High throughput screening (HTS) in zebrafish embryos has been established and is nowadays commonly used for different applications (Jones KS., et al., 2008; Miscevic F, et al, 2014; Pichler FB., et al., 2003). A number of unique features make this animal model very attractive: zebrafish are inexpensive to maintain, they breed in large numbers, develop rapidly *ex vivo*, and can be maintained in small volumes of water (Lieschke GJ., and Currie PD., 2007). Recently, the zebrafish and its transparent embryos have also come into view as a new model system to investigate tumour development, cancer cell invasion and metastasis formation (Nicoli S., et al., 2007).

Materials and methods

Animal care and handling

Tg(fli1:eGFP) zebrafish were kept at 28° C in tanks with day/night light cycles of 10 hours dark alternated with 14 hours light periods. The zebrafish were handled in compliance with Dutch animal care regulations and standard operating procedures. Zebrafish eggs were harvested at two hours post fertilization (hpf), followed by incubation at 28° C in egg water (sea salt, 60 µg/ml in tap water). The developing embryos were kept in incubators at constant temperatures dependent on experimental requirements.

Cell culture

The pancreatic cancer cell line (PaTu 8988t) was cultured at a temperature of 37^0 C and a CO_2 pressure of 5%. The PaTu 8988t cell line was isolated from liver metastases over a decade ago (Elsässer H.P., 1992). Their behaviour in an *in vitro* wound healing assay and in an *in vivo* zebrafish xenotransplantation assay were described in detail before (Marques I., 2009). PaTu 8988t cells were cultured in DMEM (Gibco) high glucose supplemented with 10% FCS (Gibco). ZF4 cells are zebrafish cells (Driever W., 1993) that were cultured in L15 medium (Gibco) enriched with 15% FCS (Gibco). Cells were incubated at 28^0 C, without CO_2 pressure.

In vitro observation of Cytopathic effects and CCID50 (dose that kills half the cells, by observation)

Cells were seeded at a concentration of 10^4 cells per well in 96 wells plates (Greiner) and subsequently incubated at the proper temperature and CO_2 saturation. DMEM (Gibco) was used as culture medium for PaTu 8988t cells and L15 medium (Gibco) was used for ZF4 cells. Venom was dissolved in Hank's salt solution (Gibco) at a concentration of 2 mg/ml and diluted with to a concentration of 0.1 mg/ml. Subsequently, this solution was passed through a 0,2 µm filter. Logarithmic venom dilutions of stock solution in PBS were brought onto the cells in the culture plates. Plates were then incubated for three days under the conditions mentioned in 2.2. During these three days, cells were photographed regularly. A representative set of photos is shown in Figure 1 CPE (lysis of the cells, blebbing, pyknosis etc.) was first determined using a microscope (Olympus). Data was confirmed with Trypan blue staining of randomized samples. The LD50 was calculated using the Reed and Muench method (Reed, L.J.and Muench, H., 1938) as was described before for other venoms (Lee K.H. et al., 2006).

In vitro *migration assay*

PaTu 8990t cells were grown in DMEM (Gibco), supplemented with 10% FCS (Gibco) in 24 well glass-bottom culture plates (Invitrosci) and incubated o/n at 37 ^{0}C and 5% CO_2 saturation. Before the addition of snake venom, PaTu 8990t cells were supplied with fresh medium without FCS and subsequently a scratch was made using a pipet tip (Eppendorf). A stock solution of 2mg/ml was made of the venom in Hanks salts (Gibco), subsequently, this solution was diluted ten times in PBS (Gibco) and filtered through a 200 nm filter (Millipore) using a syringe (BD). The highest dose rendered a final concentration of 0.5 mg/ml. At a ratio of 1:1 the venom in PBS and medium, the plate was incubated at 37 ^{0}C and 5%. CO_2 saturation until pictures of the scratch were taken under a Leica stereomicroscope at different times, shown in Figure 2. The gap was measured and closure was calculated in percentage to the initial gap (scratch).

In vitro *apoptosis assay*

All cell lines were screened for expression of annexin V on apoptotic bodies after exposure of the cells to snake venoms, using fluorescently labelled antibodies. Annexin V is detected using a fluorescent label: Mitochondrial Membrane Potential Apoptosis Kit, with Mitotracker™ Red & Annexin V Alexa Fluor® 488, for flow cytometry (Molecular Probes®). Cells were grown in glass-bottom multiwell plates (Invitrosci) until 80% confluence was reached. Cells were washed three times in PBS (Gibco, Bleiswijk, Netherlands) before they were incubated with kit components. After labelling and analysis, cells were fixed in PFA (Sigma, Zwijndrecht, Netherlands) 4% in PBS, pH 7,4 for fifteen minutes, followed by incubation for 1h in the antibody solution containing 0.1% BSA (bovine serum albumin) (Sigma, Zwijndrecht, Netherlands) in PBS at a 1:100 dilution. After removal of the antibody solution, cells were washed in PBS and incubated for 1h at RT with a fluorescently labelled secondary antibody.

Cytotoxicity (Lactate dehydrogenase)

PaTu 8988t cells and ZF4 cells were cultured in 96 wells plates (Greiner) as was indicated in 2.2, however, culture media were prepared again without FCS. Cultured cells were incubated with snake venoms inducing CPE and subsequent LDH release. The measurement was performed using a LDH Cytotoxicity Assay Kit (Pierce Protein Biology Products). The reactions were performed according to manufacturer's protocol. In brief: The LDH released into the medium was transferred to a new plate and mixed with reaction Mixture. After a 30 minute room temperature incubation, absorbance was measured at 490 nm and 680 nm using a plate-reading spectrophotometer (Tecan) to determine LDH activity. Each measurement was done in four-fold. The last two rows of each plated contained background- positive- and negative (plain media) controls. Results were statistically analysed using a T-test to determine significance of the results.

Cell tracker application for in vivo *experiments*

Cells were plated in 24-well plates (Greiner) at a seeding density of 4,0 *10^4 cells and incubated for 48 hours at 37^0 C and 5% CO_2. Snake venom dilutions were added, and the cells were incubated again at 37^0 C and 5% CO_2 for 24 hours. After detaching the cells with trypsin (Gibco), two washing steps were done with DPBS (Dulbecco's PBS, Gibco), after which the cells were transferred to 1.5 ml Eppendorf tubes and centrifuged 5 min, at 1500 rpm. Then, the cells were stained according to manufacturer's protocol after which the cells were suspended in 25µl DPBS for injection into the embryos.

Cell transplantation of PaTu 8988t cells into zebrafish embryos

After dechorionation, 2 dpf zebrafish embryos were anesthetised with Tricaine methanesulfonate (TMS, MS-222, Sigma). Using a manual injector (Eppendorf, Celltram), the cell suspension was loaded into an injection needle (15 µm internal- and 18 µm external diameter (Oxford apparatus) and cells were injected in 48 hpf Tg(fli1:eGFP) zebrafish embryos. After injection, the embryos were incubated at 35^o C and checked for cell presence at 2 hours post transplantation (hpt). Embryos with fluorescent cells outside the implantation area at 24 hpt were excluded from further analysis. All other embryos were incubated at 35^o C for analysis during the following days.

Tumour cell-induced angiogenesis

The tumour-cell induced angiogenesis assay was in general performed as was previously described (Nicoli S., Presta M., 2007; Nicoli S., Ribatti D., 2007). The 48 hpf zebrafish embryos were dechorionated and anesthetised using 0.042 mg/ mL Tricaine (Sigma). Then, the embryos were positioned on a 1. 8% agarose dish for injections. Cells were stained as described above, then suspended in 20 µl 12. 0 mg / ml Matrigel® solution (Cultrex R Basement Membrane Extract, R&D systems, Minneapolis, USA) and kept on ice for a short period until injection. The CellTram Oil injector (Eppendorf, Hamburg, Germany) was loaded with an injection capillary (Harvard apparatus) which was filled with 5 µl of the cell suspension in Matrigel®, and embryos were injected as described in more detail in previous studies (Nicoli S., Protocols 2007; Nicoli S., Cancer Res 2007;Vlecken and Bagowski, 2009). For statistical analysis, a student t-test was performed which showed that all changes observed were significant (with p-values <0.01).

Imaging

Light-microscopy images of pancreatic cells, an MZ16FA stereomicroscope (Leica) and a DFC 420C camera (Leica) were used. Injected zebrafish embryos (which were incubated in egg-water at 35°C for 24h before screening) were photographed using a Bio-rad confocal microscope equipped with a

1024ES lens (Zeiss) and a Krypton/Argon laser. The data obtained were processed using ImageJ software and Photoshop (Adobe). Annexin V labelled cells were analysed using confocal laser scanning microscopy (Zeiss Observer) and processed afterwards using Photoshop (Adobe).

Results

In vitro *venom titration on PaTu 8988t cells and ZF4 cells: Cytopathic effects (CPE) and CCID50*

The pancreatic cancer cells and the zebrafish cells were incubated with snake venoms, which induced CPE (lysis) within 4 hours after application (Figure 1). Viable cell densities decreased the first day after which the populations started to grow again. Compared with the untreated control cells, treated cell samples were showing significantly more CPE. Also, it can be seen in Figure 1 that there is a dose dependent effect in the degree of CPE. As can be seen in photo A: cells treated with a low concentration of venom, do not proliferate as one would expect, however, cells shown in D and E have proliferated as well or better than the controls. Cells with the highest concentrations of venom detach and will likely die or change.

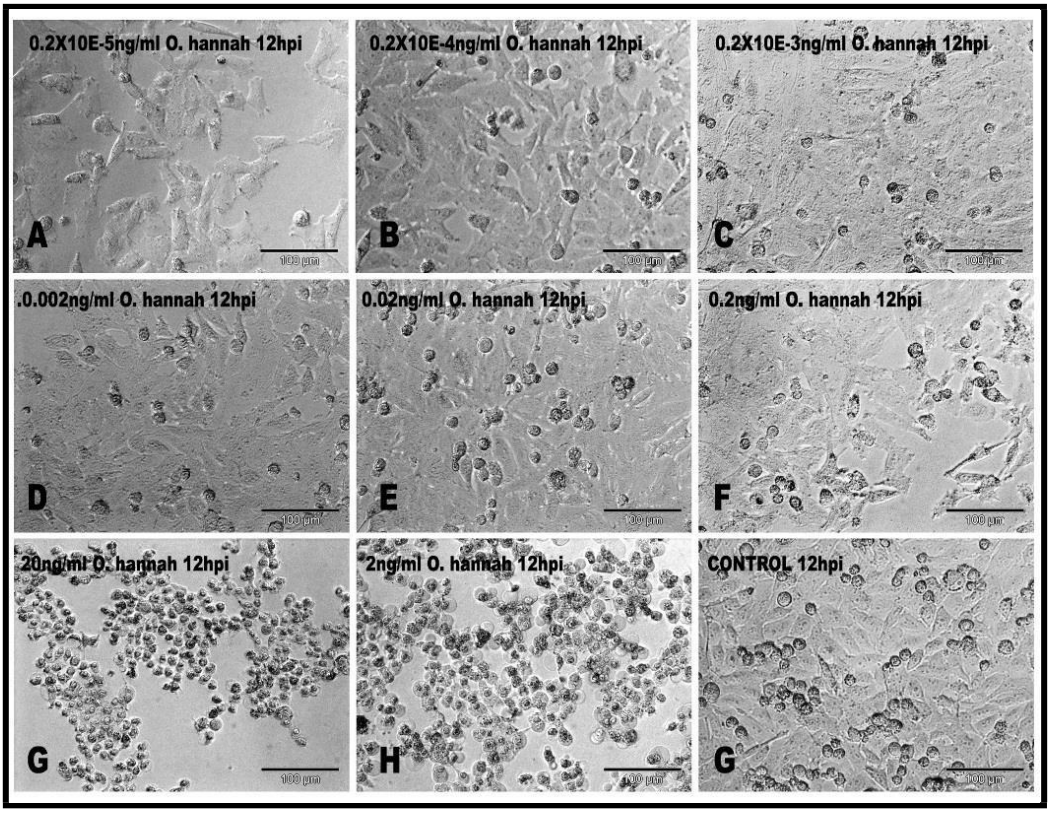

Figure 1: CPE titration after venom treatment. PaTu 8988t cells were treated with king cobra venom and a Trypan blue stain was done to confirm the results. As is shown, many cells have detached from the wells, however not all of those are really dead.

In vitro *migration of pancreatic tumour cells treated with snake venoms.*

Venom treated PaTu 8990t cells tend to migrate slower compared to the cells in the control group. The latter form already after 24 hours a layer with a confluence of 50% while there is not much migration visible at this time in the venom treated cells. However, the amount of CPE, was increased in the venom treated cells. The lower doses, shown in the first column, exhibit a delayed migration pattern, compared with the control cells. After 96 hours, the lowest dose shows to have reached the same level the controls reached after 24 hours. In the medium dose, half of the cells died after 96 whereas in the highest concentration half of the cells were dead after 60 hours and most of the cells were dead after 96 hours. Also, the integrity of the scratch was compromised.

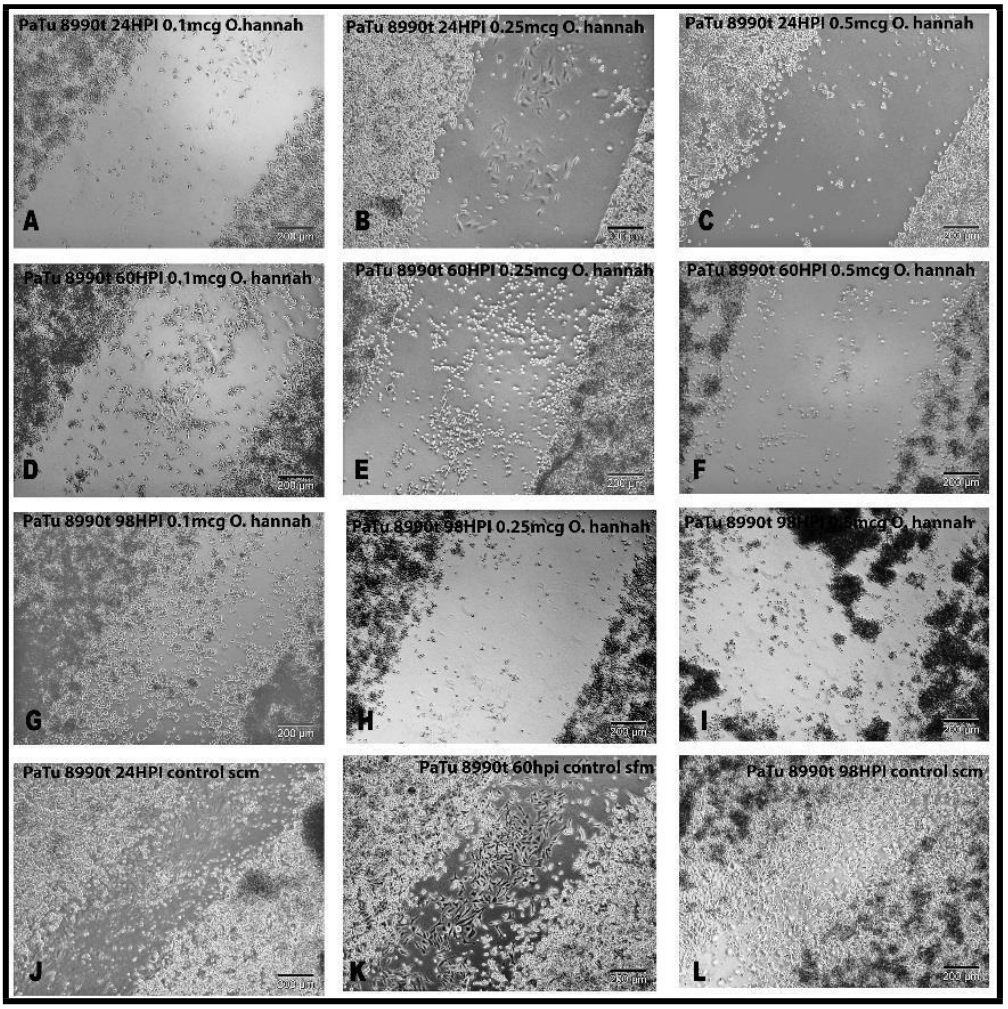

Figure 2: Cell migration of PaTu 8990t cells treated with venom of *Ophiophagus hannah*. Lyophilized *O. Hannah* venom was added to PaTu 8990t cells. In A, D and G 0.1 µg of the venom was added to the cells and in a final concentration of 10E-4 the cells were incubated. In B, E and H, the cells were incubated in a final concentration of 2.5 X10E-4. In the last column a dilution of 5X10E-4 was maintained. The cells were scored at three different times: at 24 hours post infection (24hpi), 60hpi and 98 hpi and the results in time are shown per row. Normal migration of PaTu 8990t cells after the application of a scratch is depicted in J, K and L.

Apoptosis caused by snake venom

To investigate whether apoptosis takes place in PaTu 8988t cells after addition of snake venoms immunocytochemistry was performed using an Annexin V antibody, a representative selection is shown for PaTu 8988t cells in Figure 3. Control cells have intact mitochondria and they lack the Annexin V signal (green), whereas cells treated with a low concentration of venom (Second photo, D10000) show apoptotic activity. Cells treated with moderate-high concentrations of venom (Third photo, D1000) show less apoptotic bodies and lysed mitochondria are visible. In the last photo (D100), a high concentration of venom was added and there is no apoptotic activity visible in the sample, although mitochondria lysed to a higher degree than is shown in photo C.

Formation of apoptotic bodies is limited to cells infected with moderate and low concentrations of snake venoms. Also, the integrity of mitochondria proved to be dependent on the concentration of snake venom. Thus, there is a dose related appearance of apoptotic bodies and cell disintegration, mitochondrial lysis and necrosis are observed in the high concentrations of snake venoms.

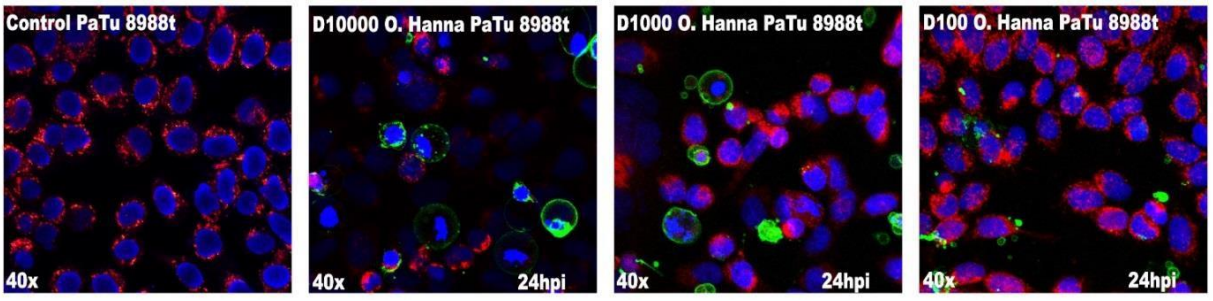

Figure 3: Apoptosis in cells treated with venom of *Ophiophagus hannah* (King cobra). Annexin V is shown using a green fluorescent label while the mitochondria are shown in red Mitotracker™ Red & Annexin V Alexa Fluor® 488, for flow cytometry (Molecular Probes®). In the control picture, PaTu 8988t cells do not show apoptotic

bodies whereas the lower concentrations venom show apoptosis. The degree of mitochondrial integrity decreases when the administered dose increases.

Cytotoxicity (Lactate dehydrogenase (LDH))

Lactate dehydrogenase measurement using PaTu 8988t cells and ZF4 cells treated with snake venoms. LDH is an enzyme that is found in damaged tissues in nearly all organisms. It converts lactate to pyruvate and vice versa (and NADH to NAD^+). LDH is abundant when tissues are damaged or, in cell culture, when cells are dying.

We used a colorimetric assay to quantitatively measure lactose dehydrogenase (LDH) released into the media from damaged cells as a biomarker for cellular cytotoxicity and cytolysis. An enzymatic reaction that results in a red Formazan product can be measured spectrophotometrically.

As is shown in Figure 4, both cobra venoms do not generate an expected dose dependency, whereas viper and king cobra venoms exhibit a response which is dose dependent. Doses with pronounce CPE, are much higher for zebrafish cells then for human pancreatic cancer cells.

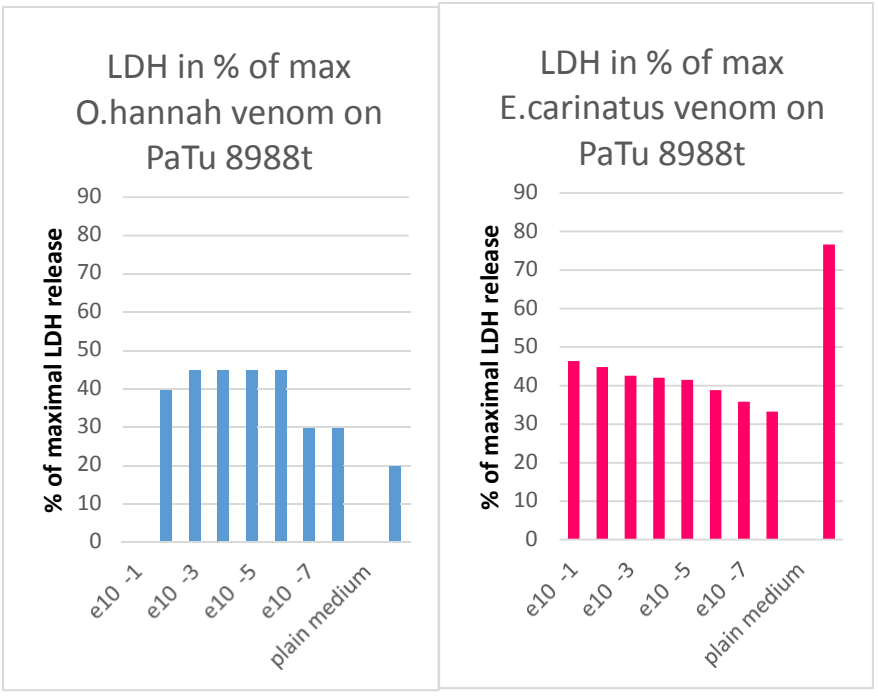

Figure 4: LDH (percentages with respect to LDH max = positive control) released by PaTu 8988t cells treated with snake venoms

The graphs above show LDH production as a reaction on the administered dose of snake venom. In the left graph, measurements with spent medium containing king cobra venom can be seen. The plot on the right also shows a dose response with spent medium containing venom from the Saw-scaled viper. With both venoms dose dependency was observed. For a positive control, three wells with cells were lysed to obtain the LDH-max values and plain medium without cells was used to generate negative controls.

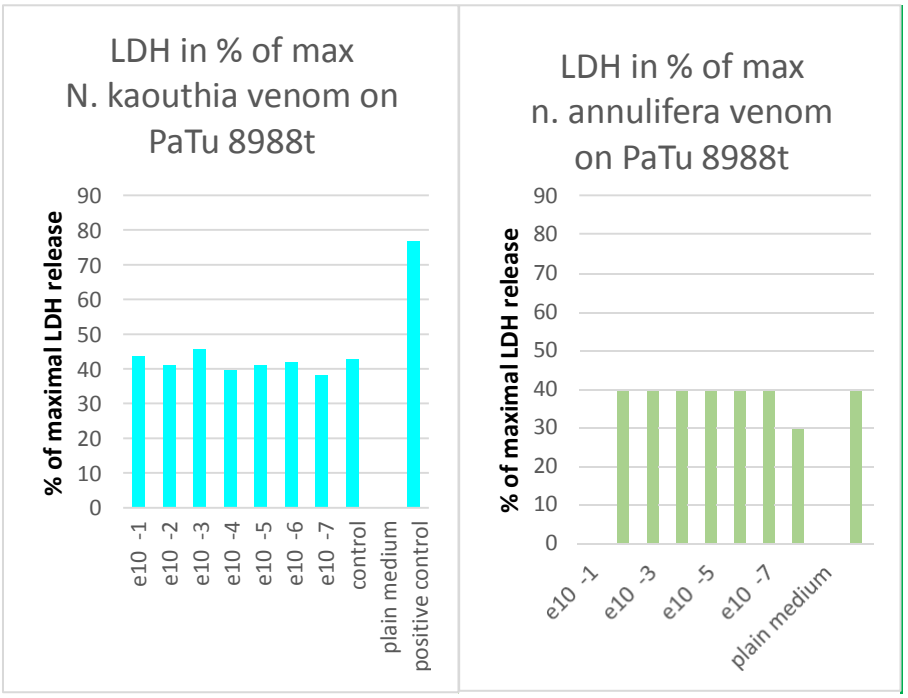

Figure 5: LDH measurements on PaTu 8988t cells treated with snake venoms from Naja species. The graphs how LDH production as a reaction on the administered dose of snake venom. In the left graph, measurements on spent medium treated with Monocled Cobra venom are shown showing that there is no dose dependency observed for these venoms. The plot on the right also shows the results of a dose response experiment on spent medium containing venom from the Snouted Cobra. No dose dependency was observed for these samples.

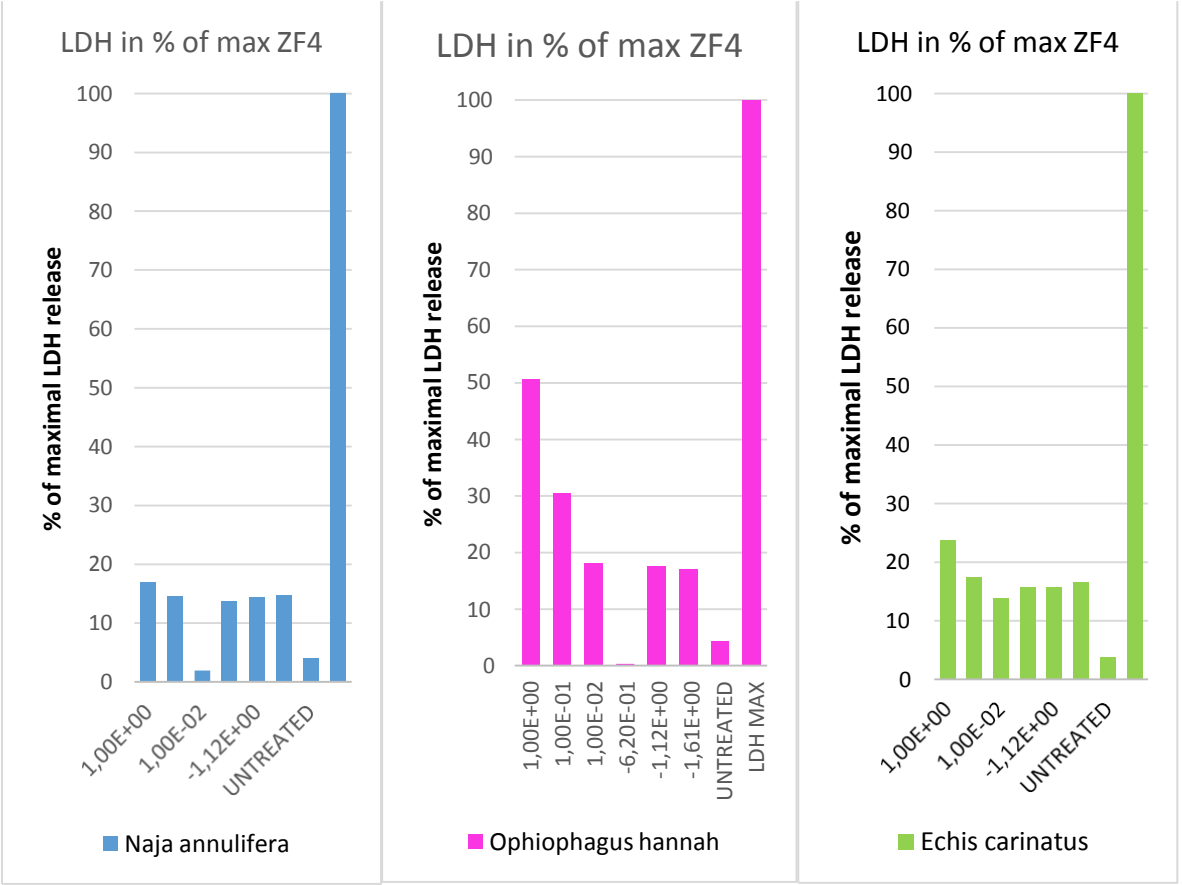

Figure 6: LDH measurements on ZF4 cells treated with snake venoms from different species. In ZF4 cells, the venom of *O. Hannah* generates the expected dose dependent response, whereas the other two (*E. carinatus* venom and *N. annulifera* venom) did not.

Cobra venom exerts anti-metastatic properties on the behaviour of pancreatic cancer cells in vivo

To study the role of the snake venoms in metastatic behaviour, PaTu 8988t cells were treated and then labelled with a carbocyanine dye (CM-Dil) and ectopically transplanted in the yolk sac of 2 dpf zebrafish embryos of the transgenic zebrafish line, Tg(fli1:eGFP), which express GFP under the fli1 promoter (an early endothelial marker) and therefore exhibit a green fluorescent vasculature (Lawson N.D. and Weinstein B.M., 2002). As a negative control for the venoms, PBS was used.

The results (Figure 6 and Supplemental Table 1) showed that several venoms (e.g. *O. Hannah* venom and *E. carinatus* venom in this study) have an inhibitory effect on metastatic behaviour, however, not all venoms displayed such effects, for example, *N. annulifera and N. kaouthia* venoms did not cause

significant dose-related effects when applied to the cells. The data obtained in the migration assay *in vivo* differs from the *in vitro* data. Differences exist due to lack of availability of nutrients and growth factors present in an animal. Since no significant effects were observed when cells were treated and implanted into the embryo, a different approach was attempted: Embryos were injected with untreated cells and the fish with the cells was then soaked in an egg water-venom solution. Other venoms that were tested without success were venoms of the copperhead snake and from the rattlesnake. For the angiogenesis assay, the same preconditions as are described in this paragraph are applicable.

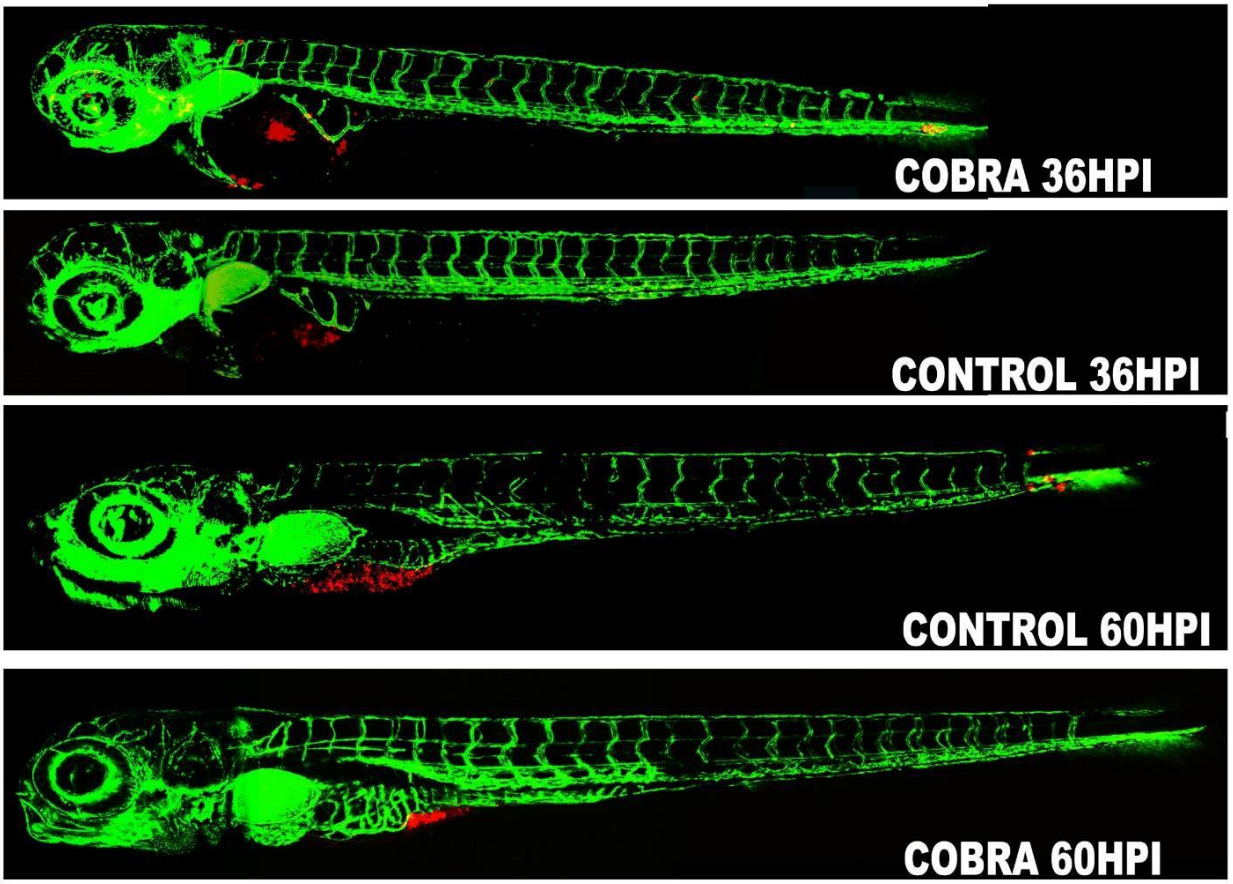

Figure 7: Anti-metastatic effects of cobra venom on fish with pancreatic cancer cell graft containing PaTu 8988t cells. The two control photos show embryos with a transplanted cell mass of which cells have spread via the vasculature. The zebrafish embryos that were treated with cobra venom after receiving the cell mass, did not show migration of malignant cells. (n=20 embryos per tested snake venom).

Treatment of snake venoms reduces tumour cell-induced angiogenesis

To study tumour angiogenesis *in vivo*, a method is available that is based on the injection of pro-angiogenic mammalian tumour cells into the perivitelline space of zebrafish embryos (Nicoli, S., Presta M., 2007). PaTu 8988t were injected into the perivitelline space of zebrafish embryos at 48h post-fertilization. These pro-angiogenic tumour cell grafts induced an angiogenic response originating from the developing sub intestinal-vessels. The results indicate shown in Figure 8 and Supplemental Table 1, that treatment with king cobra venom resulted in inhibition of angiogenesis.

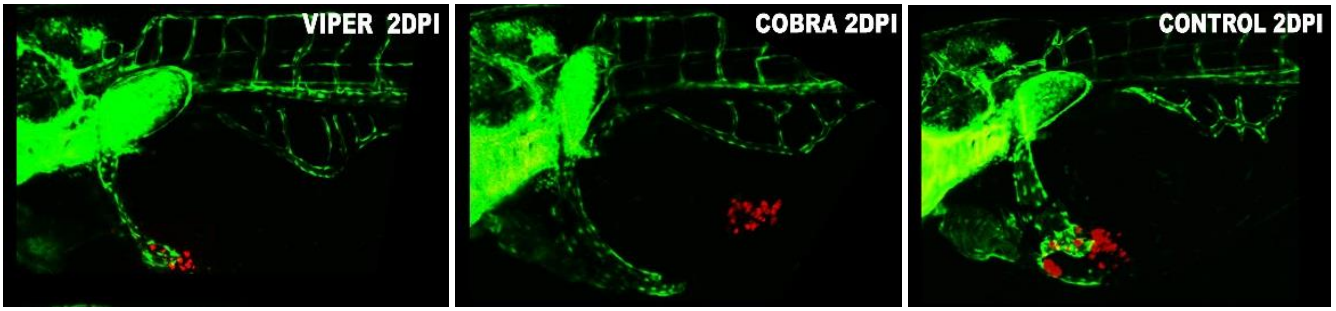

Figure 8: Anti-angiogenic properties of Cobra venom and Viper venom. The figure shows three confocal micrographs of PaTu 8988t cells (red) that were transplanted into 48 hpf fli zebrafish embryos (green). The cells were dispersed in Matrigel, because of which they stay ectopically in the embryos yolk. When Cobra venom was added to the fish carrying the cells, inhibition of angiogenesis was observed, however, treatment of the fish with Viper venom resulted in angiogenesis, like the controls. The magnification of the pictures is 20x. (n=20 embryos per tested snake venom).

Discussion

Because public demand continues to be strong and the incidence increased, investigation of candidate anti-cancer drugs is a part of this study. The studied compounds vary from different snake venoms to iridium containing complexes. The compounds that are observed to show an effect are followed up with additional experiments. Not only identification of compounds inhibiting malignant processes must be addressed but also ways to target the malignancies with these compounds must be explored.

Growth inhibition was shown for samples with very low doses, addition of high doses to the egg water in which the zebrafish resided, resulted in cell death (for 20 ng and 2 ng of added venom). However, the moderately higher doses appeared to induce enhanced proliferation. This phenomenon was reported before for venoms from *Bothrops* species which can activate cytokines (Luna K.P.O., et al., 2011; Mora R., et al., 2005) and venom from *Echis* species can cleave TNF (tumour necrosis factor) to its active form for example (VORONOV E., et al., 1999). Samples that received moderate doses did not differ much from control cells in the number or morphology of the cells. There might be some sort of equilibrium and deviating from it, could result in altered behaviour of the cells. Cells with high doses snake venom added to them appeared to detach and die. But not all of these cells were dead because upon seeding in fresh medium, cell proliferation was resumed. Damaging by for example snake venoms is known to bring about more mutations resulting in a higher survival rate, therefore prior to experienced trauma the cell and its descendants are not identical to the population that has grown after damage.

Apoptosis caused by snake venom was only induced in moderate doses. In higher doses cells almost lysed and in the controls, no apoptotic bodies could be discerned. This event could be due to the ability of tumour cells to block the apoptotic pathways (Cotter T. G., 2009). The 100x dilution must have been far too toxic for the cells to survive, because no apoptosis was observed, whereas mitochondrial integrity was clearly lost. Also, nuclei became brighter which also indicates that CPE takes place.

Cytotoxicity (LDH) is less severe in ZF4 cells compared to PaTu 8988t cells upon addition of the same dose of the same venom. This suggests that untransformed zebrafish cells are more resilient to snake venoms than tumour cells. This could be explained by a retention of defence mechanisms in untransformed cells that might be able to take action against damage.

Snake venoms were suggested to be effective against metastasis based on the presence of disintegrins (Selistre-de-Araujo H.S., et al., 2010).

Only indications for such an effect were observed when venom from *N. naja* was added to the fish water. Venoms of viperidae (rattlesnake and copperhead snake) did not exert any visible effects in any of the experimental settings used here. Snake venoms exhibit inhibitory properties on tumour cell-induced angiogenesis. This was only observed using venom of *N. naja* on PaTu 8988t cells. Viper venom slightly reduced the response the vasculature had on the cells, but vasculature was formed. Although, it was reported that viper venom contains disintegrins which act against processes such as tumour-induced angiogenesis (McLane M.A., et al., 1998; Markland F.S., 1998).

Snake venoms contain sometimes exosomes which can easily trigger an immune response in the victim, because these particles display MHC molecules on their surface (Ogawa Y., et al., 2008).

Conclusion

The results indicate that, if administered in low concentrations, snake venoms display promising anti-malignant properties. However, comparing the results obtained with different venoms it is obvious that venoms derived from the cobra species that were tested, did not have a strong impact on PaTu 8988t cells. Venoms from *Ophiophagus hannah* and *Echis carinatus* might be well suited candidate cancer inhibitors and will be used for more detailed studies wherein whole venom mixtures as well as fractions thereof will be screened.

In vitro obtained data is not interchangeable with data generated in *in vivo* assays. This underscores the aspect that the micro-environment of a living being cannot be imitated precisely in a culture flask. The nutrients and other resources which the cells have available in *in vivo* assays, is not present in culture media. Therefore, the response differs between the *in vivo* and *in vitro* assays.

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Supplemental Tables

Supplemental table 1:

Quantitation of embryos showing malignant processes

Russels viper	total	cells out	angiogenesis
0h	20	0	-
36h	16	>5 in 8 embryos	-
60h	13	>10 in 6 embryos, >5 in 1 embryo	-

Control	total	cells out	angiogenesis
0h	20	0	-
36h	19	>5 in 8 embryos	+ in 13 embryos
60h	19	>20 in 14 embryos, >10 in 2 embryos	++ in 12, +/- in 2 embryos

King cobra	total	cells out	angiogenesis
0h	20	0	-
36h	20	>5 in 2 embryos	-
60h	20	>10 in 2 embryos, <3 in 2 embryos	+/-- in 2 embryos