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RESEARCH ARTICLE

# The effects of selected Australian snake venoms on tumour-associated microvascular endothelial cells (TAMECs) *in vitro*

Emma Bateman[α,*], Michael Venning[α], Peter Mirtschin[β] and Anthony Woods[α]

[α]Sansom Institute, School of Pharmacy and Medical Sciences, City East Campus, University of South Australia, GPO Box 2471, Adelaide, South Australia 5001, [β]Venom Supplies, PO Box 547, Tanunda, South Australia 5352, *Present address: Mucositis Research Group, Centre for Personalised Cancer Medicine (CPCM), Centre for Clinical Research Excellence (CCRE) in Oral Health, Faculty of Health Sciences, University of Adelaide, Frome Road, Adelaide SA 5005, Australia

*Correspondence to: Emma Bateman, Email: emma.bateman@adelaide.edu.au, Tel: +61 8 8222 3547, Fax: +61 8 8222 3217

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## ABSTRACT

The effects of various viperid and elapid venoms on the cellular biology of tumour-associated microvascular endothelial cells (TAMECs) were determined in the current study using cells isolated from a rat mammary adenocarcinoma. Previous studies to determine the effects of snake venoms on endothelial cells *in vitro* have in the main been performed on either human umbilical vein endothelial cells (HUVECs), bovine aortic endothelial cells (BAECs) or endothelial cell lines. These cell populations are accessible and easy to maintain in culture, however, it is well established that endothelial cells display vast heterogeneity depending upon the local microenvironment of the tissue from which they are isolated. Vascular targeting agents have been isolated from a variety of snake venoms, particularly from snakes of the *Viperidae* family, but it is yet to be established to what extent the venoms from Australian elapids possess similar vascular targeting properties. The present study used endothelial cells (ECs) isolated from the microvasculature of a rat mammary adenocarcinoma to determine the effects of a panel of snake venoms, including viperid venoms with known apoptotic activity and elapid venoms (both exotic and indigenous to Australia), on endothelial morphology and viability, paying specific attention to apoptotic responses. Three of the five Australian snake venoms investigated in this study elicited significant apoptotic responses in ECs which were in many ways similar to responses elicited by the selected viperid venoms. This suggests that these Australian elapids may possess vascular targeting components similar to those found within viperid venoms.

**KEYWORDS:** Tumour-associated endothelial cells, snake venom, endothelial apoptosis, Australian elapids, anti-angiogenesis

## INTRODUCTION

The search for anti-cancer therapies constitutes one of the largest fields of research at present. The current focus of these investigations is directed towards less toxic, biologically-driven therapies for solid tumours in lieu of traditional chemotherapy, radiotherapy, or a combination of both. One process by which neoplastic cells may be effectively killed with limited physiological effects is via the destruction of the tumour blood supply. Tumour growth beyond 1-2mm$^3$ in size is contingent upon an adequate blood supply, to provide oxygen and nutrients and to remove catabolites (Folkman, 1971; de Waal et al, 2005).

The majority of solid tumours require development of a nascent vascular supply. This is achieved through the process of angiogenesis. Tumour angiogenesis has been shown to be essential for the expansion, survival and metastasis of solid

tumours; furthermore, it has also been shown that abrogation of this tumour angiogenesis is sufficient to initiate regression of the neoplastic tissue (Folkman, 1971).

Although derived from host endothelial cells (ECs), tumour-associated ECs are physiologically and immunologically distinct from the normal vasculature and as such, present a unique targeting moiety for anti-neoplastic therapy (Bar et al, 2012; Klotz et al, 2012). Since the nascent tumour vasculature is comprised almost exclusively of endothelial cells, targeting these cells to induce an apoptotic response is sufficient to cause regression of tumour-associated microvessels, which in turn induces regression of the neoplastic mass (Bar et al, 2012; Klotz et al, 2012). Past and ongoing studies have sought to identify and characterise various agents potentially capable of initiating this apoptotic response and it has been demonstrated that the venoms of particular snake species possess components capable of such a vascular targeting mechanism (Yeh et al, 2000; Swenson et al, 2007; Ramos et al, 2008). These components include disintegrins, snake venom metalloproteases (SVMPs), phospholipase $A_2$s ($PLA_2$s) and l-amino acid oxidases which are capable of initiating EC apoptosis in different ways.

Disintegrins are small peptides derived from the proteolytic cleavage of SVMP classes II, III and IV (found primarily within viperid venoms) and contain tripeptide sequences similar to that within many extracellular matrix (ECM) components. Those primarily known to inhibit endothelial cell-mediated angiogenesis are those containing arginine-glycine-aspartic acid (RGD), lysine-threonine-serine (KTS), or arginine-threonine-serine (RTS) sequences (Marcinkiewicz et al, 2003; Olfa et al, 2005). Integrins on EC surfaces (for example, integrins $a_vb_3$ and $a_1b_1$) bind to these motifs within many ECM components, however, disintegrins also containing these sequences will preferentially bind to the membrane-bound integrin. Since disintegrins are small peptides (5-9kDa), they cannot provide the conformational support normally supplied by the solid, fixed ECM which allows for endothelial elongation. Since endothelial cells intrinsically require an elongated cellular shape, the rounding which occurs due to disintegrin binding causes ECs to undergo apoptosis (Juliano et al, 1996; McGill et al, 1997; Yeh et al, 2001; Marcinkiewicz et al, 2003; Olfa et al, 2005; Kikushima et al, 2008; Brown et al, 2009).

Found primarily within viperid venoms, but also Australian elapid venoms (Birrell et al, 2007), SVMPs elicit apoptosis through degradation of the extracellular matrix (ECM) underlying endothelial cells (Wu et al, 2003; Sanchez et al, 2010), which induces shape changes and prevents EC adhesion, giving rise to apoptosis.

l-amino oxidase (LAO) is an enzyme found in many snake venoms (Zuliani et al, 2009; Guo et al, 2012) that catalyses the stereospecific oxidative deamination of l-amino acids, generating the corresponding α-ketoacids, ammonia and hydrogen peroxide ($H_2O_2$) in the process (Zhang et al, 2003). $H_2O_2$ is a reactive oxygen species that causes apoptosis of endothelial cells by inducing oxidative damage to DNA, proteins and lipid membranes (Du et al, 2002). Endothelial cells are particularly susceptible to LAO effects (Ahn et al, 1997; Masuda et al, 1997; Torii et al, 2000).

Phospholipase $A_2$ is also ubiquitous within snake venoms and, like LAO, can have a digestive function. There have been several reports of haemorrhage caused by $PLA_2$s (Francis et al, 1991; Francis et al, 1993; Francis et al, 1995; Uma et al, 2000), however, the exact mechanisms are not entirely clear. It is though that $PLA_2$s can cause endothelial cell apoptosis via the liberation of arachidonic acid and subsequent stimulation of sphingomyelinase and production of ceramide (Taketo et al, 2002).

Studies into the haemorrhagic and vascular targeting capabilities of snake venoms have been routinely performed on ECs isolated from non-malignant tissues, and with snake venoms from species exotic to Australia. Given the unique properties of Australian snake venoms and a lack of data on their effects on endothelial cells, this study was performed to determine to what extent Australian elapid venoms possess vascular targeting properties on ECs. Endothelial cells from tumour and control tissue were successfully isolated and maintained for the purposes of low-scale screening studies to determine whether the effects of Australian elapid venoms were synonymous with adhesion inhibition and apoptosis. Specific apoptosis detection systems were employed to confirm apoptotic cell death, and analysis of data obtained from a viability assay allowed for comparisons between venom effects on the two cell populations; these analyses also allowed for determination and comparison of patterns of venom effects on endothelial cells.

## MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich unless otherwise specified. Appropriate animal ethics approval was obtained from IMVS (now SA Pathology) Animal Ethics Committee, with experiments conducted in accordance with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training (2004).

### Isolation and characterisation of tumour-associated microvascular endothelial cells (TAMECs)

A mammary adenocarcinoma was transplanted and passaged in Dark Agouti rats; after 8 days, whole tumours were excised and processed for culture of TAMECs as previously described (Nilsson, 2000). Briefly, tumour tissue was freed of connective tissue and dissociated mechanically before being digested enzymatically in a collagenase/dispase solution, followed by incubation with 0.25% trypsin/EDTA. The tissue digest was centrifuged and resuspended in complete growth medium (Nilsson, 2000). The cell suspension was then filtered through a 100μm nylon cell strainer to remove large tissue fragments (Becton Dickinson); the resultant filtrate was added to gelatinised culture flasks and allowed to settle. To prevent phenotypic drift of the tumour-derived EC population, cultures were fed every 24-48hrs with tumour-conditioned medium (TCM). Adherent TAMECs were separated via their preferential adhesion to the gelatin substrate. Cultures were passaged no more than twice and immunohistochemistry (IHC) was used to detect and monitor cell adhesion molecule expression.

**Isolation and characterisation of brain-derived microvascular endothelial cells (BMVECs)**

Microvascular endothelial cells were isolated and cultured from non-malignant tissue to represent a control EC population. The following method was adapted from several different methods in order to achieve pure brain-derived microvascular endothelial cell (BMVEC) cultures (de Angelis et al, 1996; Kis et al, 1999; Fischer et al, 2001; Lee et al, 2003). Briefly, entire brains from Dark Agouti rats were aseptically removed and dissociated into 1-2mm$^3$ fragments then incubated with collagenase/dispase. This digest was then centrifuged with the addition of 500µl bovine serum albumin (BSA; 25%, v/v solution in PBS) to encourage density-dependent separation of brain tissue components such as myelin, neurons and astrocytes from capillary fragments. The resultant capillary fragment pellet was resuspended in complete growth medium (CGM) and plated into gelatinised flasks and allowed to settle for 5-6hrs, after which time non-adherent cells were decanted. Brain-derived EC cultures were fed every 48-72hrs with CGM and passaged no more than twice to reduce phenotypic drift. Cell adhesion molecule expression was monitored via IHC.

**Immunohistochemistry (IHC)**

TAMEC and BMVEC cultures were incubated with a panel of antibodies intended to confirm their identity as endothelial cells. Antibodies were directed against the $\beta_3$ integrin subunit (Santa Cruz Biotechnology), E-selectin (Santa Cruz Biotechnology), Intracellular Cell Adhesion Molecule-1 (ICAM-1; Serotec), Platelet and Endothelial Cell Adhesion Molecule-1 (PECAM-1; Santa Cruz Biotechnology), Vascular Endothelial Cadherin (VE-Cadherin; Santa Cruz Biotechnology) and von Willebrand's Factor (vWF; DAKO). Antibodies to detect smooth muscle cells (alpha smooth muscle actin; DAKO) and fibroblasts (vimentin; Santa Cruz Biotechnology) were also used to identify potential contaminants. Cell cultures were fixed with methanol/acetone, washed in Tris-buffered saline (TBS) and processed through a standard streptavidin/biotin IHC technique.

**Snake venoms**

All venoms used were a generous donation from Peter Mirtschin (Venom Supplies, Tanunda) and are listed in Table 1. Venom collection was restricted to snakes from known populations, in order to eliminate the geographic intraspecific variation that is known to occur in some species. Lyophilised venoms were comprised of pooled milkings of several snakes from the same species over time, in order to further reduce intraspecific venom variation. Lyophilised venoms were reconstituted in phosphate-buffered saline (PBS) as 1mg/ml stock solutions (protein concentration was determined by the Bradford method of protein quantification (Bradford, 1976), with the subsequent addition of BSA at a final concentration of 0.1% (w/v) to convey protein stability. Complete growth medium was used as the diluent for all venom preparations to be applied to cell cultures. TAMEC and BMVEC cultures were grown in gelatinised 24-well plates (Nunc) and were incubated at 37°C with varying concentrations of venom and monitored for morphological changes every hour for six hours via phase contrast microscopy.

A range of concentrations (0.01µg/ml-1mg/ml) was used to screen for effects; an optimum concentration range was then selected for each venom. This range was selected to show a clear spectrum of venom effects. These screening assays were performed in triplicate on 10 separate occasions. Representative images of morphological changes were recorded where possible to best demonstrate these observations in both cell populations. Observations of cellular responses to venoms were also tabulated (Table 2) – this table presents data amalgamated from observations made during all 30 separate assays.

**CONTROLS**

Apoptotic controls were obtained by first coating cell culture plates/flasks with poly(2-hydroxyethylmethacrylate) (poly-HEMA). Poly-HEMA prevents adherence of adhesion-dependent cells (such as endothelial cells) to the substrate which gives rise to apoptosis (McGill et al, 1997). Cells were induced to undergo cytotoxic (non-apoptotic) cell death by repeated freezing and thawing, an experimental procedure often used to induce non-apoptotic cell death in an experimental (*in vitro*) setting (Kandil et al, 2005).

**Assessment of morphological changes**

TAMEC and BMVEC cultures were monitored for morphological changes characteristic of either apoptotic or cytotoxic cell death. Apoptotic cell death was characterized by cell shrinkage, loss of membrane processes and gradual acquisition of a spherical shape, cellular detachment from the gelatin substrate, nuclear condensation and fragmentation, cytoplasmic vacuolation, membrane blebbing and formation of apoptotic bodies (Sartorius et al, 2001). Cytotoxic (non-apoptotic) cell death was defined by rapid shrinkage or swelling of cells, rapid development of large cytoplasmic vacuoles, cytoplasmic granulation, nuclear pyknosis and cytolysis (Lomonte et al, 1999; Rivers et al, 2002). Morphological changes were recorded photographically and were also tabulated (Table 2).

**Fluorochromatic detection of apoptosis**

Apoptotic venom-treated endothelial cells were collected, washed in PBS and centrifuged. At this time, a 20-fold dilution of an acridine orange/ethidium bromide dye mixture (both at concentrations of 100µg/ml in PBS) was applied to washed cells, which were then incubated with the dye mixture for 5-10min before viewing under blue fluorescence (wavelength 420-495nm) with an Olympus BX60 fluorescence microscope (Olympus).

**Confirmation of apoptosis via gel electrophoresis**

Venom-treated TAMECs and BMVECs and apoptotic and cytotoxic controls were processed identically. After 6hr incubation with venom, detached cells were processed for isolation of DNA with the Wizard® Genomic DNA Purification Kit (Promega). Samples and markers were separated by electrophoresis (100mV for 1hr) in a 2% (w/v) agarose gel/Tris borate buffer (pH 7.4) and visualised under UV transilluminator after staining with ethidium bromide.

**Determination of cell viability by the mitochondrial function assay (MTT assay)**

TAMECs and BMVECs were grown to confluence in gelatinised 96-well plates (Nunc) and incubated with venoms

**Table 1.** Snake venoms used in this project

| Snake species, abbreviation used in this project, common and family name. **(Australian elapids are highlighted in bold type)** | Country/region of origin |
|---|---|
| *Agkistrodon bilineatus bilineatus*; AB; Common Cantil; Viperidae | Mexico, Central America |
| ***Austrelaps superbus*; Lowland Copperhead; AS; Elapidae** | **Australia – TAS, SA, VIC, NSW** |
| *Bitis arietans*; BA; Puff Adder; *Viperidae* | Sub-Saharan Africa |
| *Bitis gabonica*; BG; Gaboon Viper; *Viperidae* | Sub-Saharan Africa |
| *Bitis nasicornis*; BN; Rhinoceros Horned Viper; *Viperidae* | Central Africa |
| *Crotalus vegrandis*; CV; Uracoan Rattlesnake; *Viperidae* | Northeastern Venezuela |
| ***Hoplocephalus stephensii*; HS; Stephen's Banded Snake; *Elapidae*** | **Australia –QLD, NSW** |
| *Naja kaouthia*; NK; Monocellate Cobra; *Elapidae* | Indochina |
| *Naja melanoleuca*; Nmel; Forest Cobra; *Elapidae* | Tropical Africa |
| *Naja mossambica mossambica*; Nmoss; Mozambican Spitting Cobra; *Elapidae* | South eastern Africa |
| *Naja siamensis*; Nsi; Thai Spitting Cobra; *Elapidae* | Indochina |
| ***Notechis scutatus scutatus*; NS; Mainland Tiger Snake; *Elapidae*** | **Australia – TAS, NSW, VIC, SA** |
| ***Oxyuranus microlepidotus*; OM; Inland Taipan; *Elapidae*** | **Australia – SA and QLD** |
| ***Pseudonaja nuchalis*; PN; Gwardar or Western Brown Snake; *Elapidae*** | **Australia – NT, WA, SA** |
| *Vipera latatsi*; VL; Snub-nosed Viper; *Viperidae* | Spain |

TAS (Tasmania), SA (South Australia), VIC (Victoria), NSW (New South Wales), QLD (Queensland), NT (Northern territory)

**Key to species abbreviations:** AB; *Agkistrodon bilineatus bilineatus,* AS; *Austrelaps superbus,* BA; *Bitis arietans*, BG; *Bitis gabonica*, BN; *Bitis nasicornis*, CV; *Crotalus vegrandis,* HS; *Hoplocephalus stephensii,* NK; *Naja kaouthia,* Nmel; *Naja melanoleuca*, Nmoss; *Naja mossambica mossambica,* Nsi; *Naja siamensis,* NS; *Notechis scutatus scutatus,* OM; *Oxyuranus microlepidotus,* PN; *Pseudonaja nuchalis,* VL; *Vipera latatsi*

**Table 2.** Venom effects on TAMECs

| Snake species | Concentration (μg/ml) | | Cellular changes at optimum concentration | | | | | | | | | | | | Primary mode of cell death |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Range | Optimum | CC/F | NP | AB | Sh | Sw | R | D | MB | V(s) | V(l) | CG | CL | |
| **AB** | 0.1-100 | 1 | +++ | - | +++ | +++ | + | +++ | +++ | +++ | + | - | - | - | A |
| **AS** | 0.1-100 | 10 | +++ | - | +++ | + | + | ++ | ++ | + | ++ | + | - | + | A |
| **BA** | 0.01-10 | 1 | +++ | - | +++ | ++ | + | +++ | +++ | +++ | ++ | + | - | + | A |
| **BG** | 0.1-100 | 10 | +++ | + | ++ | ++ | + | ++ | +++ | +++ | + | - | - | + | A |
| **BN** | 0.1-100 | 10 | ++ | - | ++ | ++ | ++ | ++ | ++ | ++ | + | - | - | + | A |
| **CV** | 0.1-100 | 1 | ++ | + | ++ | ++ | + | +++ | +++ | ++ | + | + | + | + | A |
| **HS** | 0.1-100 | 10 | +++ | - | ++ | ++ | ++ | ++ | +++ | +++ | ++ | - | - | + | A |
| **NK** | 0.1-100 | 10 | + | +++ | - | +++ | - | + | ++ | - | - | - | ++ | ++ | NA |
| **Nmel** | 0.01-10 | 5 | + | +++ | - | +++ | - | - | +++ | - | - | - | +++ | +++ | NA |
| **Nmoss** | 0.01-10 | 1 | + | +++ | + | +++ | - | - | +++ | - | - | - | ++ | +++ | NA |
| **Nsi** | 0.01-10 | 1 | - | +++ | - | +++ | - | - | ++ | - | - | - | ++ | +++ | NA |
| **NS** | 1-500 | 100 | ++ | + | ++ | - | +++ | ++ | ++ | ++ | ++ | ++ | - | ++ | NA |
| **OM** | 10-1000 | 500 | + | + | - | ++ | - | + | + | - | + | + | - | ++ | NA |
| **PN** | 10-1000 | 500 | ++ | + | ++ | ++ | + | ++ | ++ | ++ | +++ | + | ++ | ++ | NA |
| **VL** | 0.01-100 | 10 | +++ | - | +++ | +++ | + | +++ | +++ | +++ | + | - | - | + | A |

**Abbreviations: CC/F;** Chromatin condensation/fragmentation, **NP;** Nuclear pyknosis, **ABF;** Apoptotic body formation, **Sh;** Shrinkage, **Sw;** Swelling, **R;** Rounding, **D;** Detachment, **MB;** Membrane blebbing, **V(s);** Vacuolation (small), **V(l);** Vacuolation (large), **CG;** Cytoplasmic granulation, **CL**; Cytolysis, **+;** Weak response, **++;** Moderate response, **+++;** Strong response, **-;** Absence of response, **A;** Apoptotic, **NA;** Non-apoptotic

for 1-6 hrs. At every hour, 10µl of 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5mg/ml in PBS) was added to each well and incubated for 30min. Acid isopropanol was then added for 15min to solubilise the formazan crystals, after which time the absorbance was read at 570nm using a multiplate reader and associated software (Multiskan Ascent Software, version 2.4.1, Labsystems). The absorbance values of 'blank' wells (MTT plus medium; no cells) were subtracted from experimental and control values – the resultant values were then expressed as a percentage of negative control wells (untreated cells in growth medium). Assays were performed on 3 separate instances, all in triplicate; these results were plotted on a standard column chart as mean values ±1SEM (standard error of the mean).

**Statistical analyses of MTT assay results**

Comparisons between and within different venoms over time were made by one-way (stacked) analysis of variance (ANOVA). Comparisons between venom effects on TAMECs and BMVECs were made by using Student's two-sample *t*-test. In cases in which ANOVA showed a significant overall difference among the group means, Tukey's *post hoc* test was used to determine whether the mean value of one particular group differed significantly from another specific group. Probability values of ≤ 0.05 were considered significant.

## RESULTS

**Morphological and immunohistochemical characterisation of endothelial cells**

Endothelial cells were successfully isolated and cultured from malignant and control tissues and grown on gelatin-coated plastic. Pure endothelial cultures reached confluence approximately 14-21 days post-seeding, depending on initial plating density. Both TAMEC (Figure 1A) and BMVEC (Figure 1B) cultures displayed the characteristic 'cobblestone' arrangement of endothelial cells, with individual cells appearing elongated and spindle-shaped. Immunohistochemical characterisation confirmed endothelial cell identity and allowed for the monitoring of cell adhesion molecule (CAM) expression profiles over subsequent passages. In this study, CAM profiles of TAMECs and BMVECs were consistent over 3-4 passages, however, cultures used in venom screening studies were passaged only twice. Both TAMECs and BMVECs stained positive with antibodies directed toward vWF (Figure 1C and 1D), $\beta_3$ integrin subunit, E-selectin, ICAM-1, PECAM-1 and VE-Cadherin (data not shown), which confirmed their identity as endothelial. There were differential staining patterns between TAMECs and BMVECs, which indicated up-regulation of the $\beta_3$ integrin subunit and down-regulation of E-selectin, ICAM-1,PECAM-1 and VE-Cadherin on TAMECs compared to control ECs (BMVECs). Antibodies directed towards smooth muscle actin and vimentin failed to detect these CAMs which indicated that there was no contamination of cultures with either fibroblasts or smooth muscle cells.

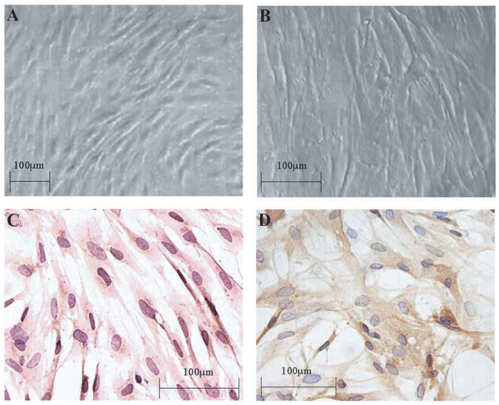


Figure 1. Morphological and immunohistochemical characterisation of endothelial cells. A magnification bar representing 100mm is included within each image. **A.** Phase contrast micrograph showing confluent TAMEC monolayer. Note swirling, cobblestone pattern. **B.** Confluent BMVEC culture, phase contrast. **C.** Positive TAMEC immunostaining for vWF. D. Positive BMVEC immunostaining for vWF.

**Apoptotic cell death and associated cellular changes**

Endothelial cells undergoing apoptotic cell death manifested a number of characteristic morphological changes. In the first instance, cells grown on poly-HEMA retracted cytoplasmic processes until they became rounded and eventually detached from the gelatin substrate. After this time, cells shrank, developed cytoplasmic vacuoles and formed a number of small, evenly-sized vesicles on the cell surface (zeiosis). Chromatin became condensed and fragmented and large membrane blebs then appeared on the cell surface which gradually formed into apoptotic bodies. These were gradually dispersed throughout the culture medium (Figure 2A).

**Non-apoptotic (cytotoxic) cell death and associated cellular changes**

Cellular changes associated with non-apoptotic cell death were distinct from apoptotic changes (Figure 2B). Cells undergoing cytotoxic cell death underwent rapid shrinkage upon incubation, whereby cells shrank to approximately half of their original size after only 1-2hrs. Some cells undergoing cytotoxic cell death, however, swelled rapidly and eventually ruptured. Evidence of lysis included presence of membrane fragments (which retained some degree of translucency), nuclear material (appearing as dense, dark aggregates) and cytoplasmic contents (which made the culture medium appear cloudy). Rapid condensation of nuclear material into dense, pyknotic packages was also a common feature, however, fragmentation of this nuclear material into evenly-sized aggregates (as would occur during apoptosis), did not occur in cells undergoing cytotoxic cell death. Cytoplasmic granulation was also common in freeze/thawed cells, as was a retention of a basic spindle-shape – loss of cellular processes and rounding did not typically precede detachment in the instance of cytotoxic cell death (Figure 2B).

**Effects of snake venoms on endothelial cell morphology**

Endothelial cultures incubated with snake venoms were monitored over 6hrs for morphological changes associated with both apoptotic and non-apoptotic death. A qualitative summary of venom-induced changes is presented in Table 2. Optimal concentration ranges were determined to be 0.1µg/ml to 100µg/ml (in 10-fold dilutions) for the majority of the venoms, however, some venoms were effective at a lower range (for example, *Bitis arietans, Naja melanoleuca, N.mossambica* and *N.siamensis* at a range of 0.01-10µg/ml),

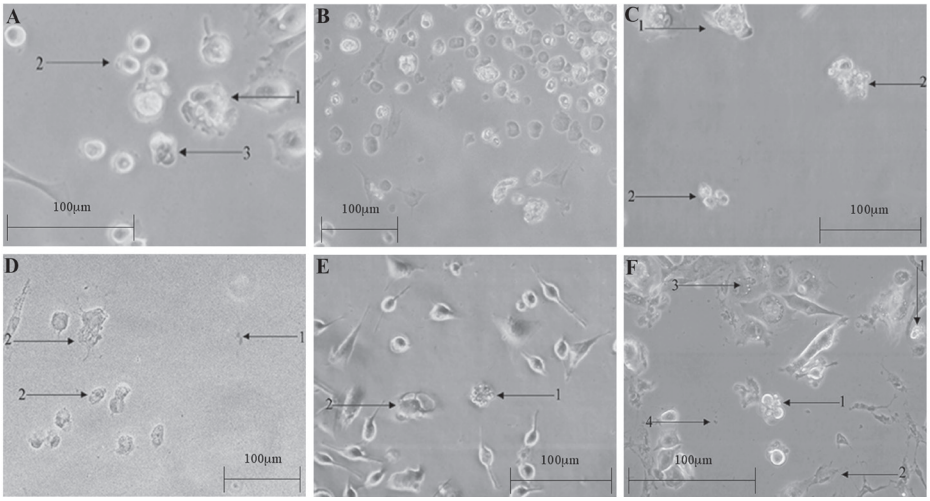


**Figure 2.** Selected venom effects on TAMECs. A magnification bar representing 100mm is included within each image. **A.** TAMECs after 3 hours plating on poly-HEMA. TAMECs retracting cellular processes (arrow 1) and becoming shrunken and rounded (arrow 2). One cell is clearly undergoing mid-apoptosis (arrow 3) with condensed, fragmented nucleus and development of apoptotic bodies. **B.** TAMEC culture 5 hours after freeze/thawing. All cells are detached and irregularly shaped. Many TAMECs have undergone cytolysis. The medium is cloudy, with cellular contents and membrane fragments dispersed throughout. **C**: TAMECs treated with 1µg/ml *Bitis arietans* venom for 6 hours. Few adherent, swollen TAMECs with cytoplasmic vacuoles (arrow 1). Clusters of apoptotic cells and apoptotic bodies visible (arrows 2). **D.** TAMECs treated with 1µg/ml *Naja mossambica* venom for 6 hours. Most cells have become detached and lysed – the culture medium is very cloudy and cellular contents are visible (arrow 1). Cells remaining attached to the substrate are shrunken, with very granular cytoplasm and irregular shapes (arrows 2). **E.** TAMECs treated with 10µg/ml *Austrelaps superbus* venom for 4 hours. TAMECs are becoming rounded and detached. Cell in centre (arrow 1) is undergoing mid/late apoptosis – cell is divided into evenly-sized and –shaped membrane-bound fragments. Some adherent cells have developed cytoplasmic vacuoles (arrow 2). **F.** TAMECs treated with 10µg/ml *Hoplocephalus stephensii* venom for 4 hours. TAMECs are becoming rounded and detached; a proportion of these cells are apoptotic (arrows 1). Most adherent cells appear shrunken with granular cytoplasms and condensed nuclei (arrow 2); a small proportion of adherent cells are swollen with cytoplasmic vacuoles (arrow 3). Cytolysis is evident in cellular contents (arrow 4)

whereas others required a higher range for appreciable effects to be observed (for example, *N.scutatus* at 1-500µg/ml and *O.microlepidotus* and *P.nuchalis* at 10µg/ml-1mg/ml). Venoms of viperid snakes (*Agkistrodon bilineatus* (AB), *Bitis arietans* (BA; Figure 2C), *B.gabonica* (BG), *B.nasicornis* (BN), *Crotalus vegrandis* (CV) and *Vipera latatsi* (VL) induced morphological changes synonymous with apoptosis (Table 2). However, endothelial cells treated with cobra venoms (*Naja kaouthia* (NK), *N.melanoleuca* (Nmel), *N.mossambica* (Nmoss; Figure 2D) and *N.siamensis* (NSi)), displayed morphological changes which were consistent with non-apoptotic cell death (Table 2).

Australian elapid venoms *Austrelaps superbus* (AS; Figure 2E) and *Hoplocephalus stephensii* (HS; Figure 2F), induced cellular changes which were most similar to those associated with application of viperid venoms or growth on poly-HEMA; that is, TAMECs and BMVECs gradually lost cellular processes, became rounded and detached and developed vesicles on the cell surface, followed by nuclear condensation and fragmentation, membrane blebbing and gradual dissolution into apoptotic bodies (Table 2). However, in the case of *Oxyuranus microlepidotus* (OM) venom, effects on ECs were comparable to those elicited by cobra venoms (Table 2) and freeze/thawing; in these cases, cells swelled (or shrank rapidly) upon venom application, developed cytoplasmic granules and gradually lysed, leaving clumps of membrane fragments and cellular contents dispersed throughout the culture medium. The venoms of *Notechis scutatus* (NS) and *Pseudonaja nuchalis* (PN) primarily elicited a non-apoptotic response in cell cultures, however, PN venom also elicited a significant apoptotic response in both TAMECs and BMVECs, whereby non-apoptotic and apoptotic cell death in response to PN venom occurred at an estimated 60/40 ratio. Since the specific focus of this project was to identify Australian elapid venoms capable of eliciting an apoptotic response in ECs, PN venom (even though primarily non-apoptotic) was included in further tests designed to characterise apoptosis.

While both TAMECs and BMVECs underwent significant morphological changes in response to all venoms tested, TAMECs displayed more rapid and pronounced responses to the venoms than BMVEC cultures, especially in relation to apoptotic manifestations. This apparent differential response to venoms was further investigated via the MTT assay of cell viability.

**Fluorochromatic detection of apoptosis**

Venom-treated apoptotic cells stained with the acridine orange/ethidium bromide mixture displayed morphological changes similar to TAMECs grown on poly-HEMA (Figure 3B), which were characteristic of apoptosis. Condensed and fragmented apoptotic nuclear material was visualised as areas of bright yellow/green fluorescence, whereas normal chromatin appeared as diffuse areas of pale yellow fluorescence (Figure 3A). Areas of cytoplasm were detected via diffuse red/orange staining of RNA. Endothelial cells

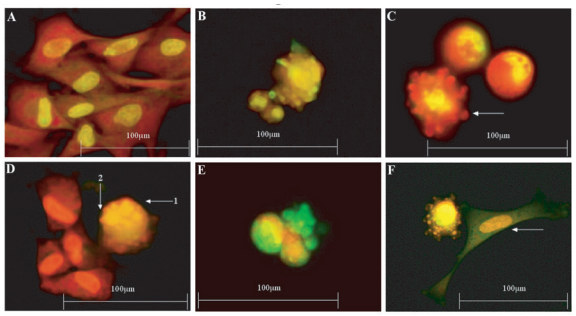


**Figure 3.** Fluorochromatic detection of apoptosis. A magnification bar representing 100mm is included within each image. **A.** Untreated, normal TAMEC culture stained with the fluorescent dye mixture. Note regularly-shaped central nuclei with pale, diffuse chromatin and homogenous cytoplasm. **B.** Single TAMEC undergoing apoptosis after plating on poly-HEMA. Note brightly stained, dense nuclear fragments and presence of numerous apoptotic bodies, as well as evidence of zeiosis (small membrane blebs). **C.** TAMECs treated with *Bitis nasicornis* venom undergoing early apoptosis; both are rounded, with dense, brightly stained, fragmented nuclei. TAMEC on the left (arrow) is undergoing mid-apoptosis, with evidence of zeiosis and membrane blebbing on the cell periphery. **D.** TAMECs treated with *Austrelaps superbus* venom in early/mid apoptosis (arrow 1) adjacent to adherent TAMECs, which are retracting their cellular processes. Notice pale, diffuse staining of nuclear material in contrast to the dense, brightly stained nuclear fragments of the apoptotic cell. Apoptotic bodies are beginning to form on the upper left side of the cell membrane (arrow 2). **E.** TAMEC undergoing mid/late apoptosis after being treated with *Hoplocephalus stephensii* venom – cell has fragmented and is packaged into apoptotic bodies. **F.** TAMECs treated with *Pseudonaja nuchalis* venom. TAMEC in mid/late apoptosis adjacent to an adherent cell (arrow).

grown on poly-HEMA (Figure 3B) or treated with venoms possessing apoptotic activity (Figure 3C, 3D, 3E and 3F) displayed nuclear condensation and fragmentation, zeiosis and membrane blebbing.

**Gel electrophoresis of venom-treated DNA**

Gel electrophoresis of DNA isolated from venom-treated TAMECs and BMVECs allowed for the further confirmation of apoptosis in cultures treated with venoms capable of eliciting an apoptotic response. TAMECs and BMVECs treated with AB, AS, BA, BG, CV, HS, PN and VL venoms displayed the characteristic 'ladder' pattern upon electrophoresis of isolated DNA (not shown). The DNA of TAMECs and BMVECs treated with NK, Nmel, Nmoss, Nsi, NS and OM venom, however, yielded a 'smear' pattern upon electrophoresis (not shown), which is associated with non-apoptotic cell death. (Kandil et al, 2005)

**Determination of cell viability by the MTT assay**

A one-way, stacked ANOVA model was used to compare cell viability decreases in response to venom application, both within individual venoms over time and between the fifteen different venoms studied. It was observed that cobra venoms generally elicited rapid, consistent decreases in cellular viability, with approximately 50% of cells being killed within the first two hours, suggesting a rapid 'cytotoxic' death (Figure 4; species NK, Nmel, Nmoss and NSi). Viability decreases elicited by viperid venoms, however, seemed to undergo a lag period, with the most significant decreases occurring after two to three hours (P=0.0001, Figure 4; particularly species AB, BA, BG and BN), which may correlate with apoptosis observed in venom-treated cultures 1-2hrs after cell detachment. The venoms from Australian elapids AS, HS and PN yielded viability patterns that were found to be more similar in terms of viability decreases to viperid venoms than to cobra venoms, whereas OM and NS viability patterns bore more similarity to those of the cobra venoms tested (Figure 4).

When comparing venom effects on viability of TAMECs and BMVECs via Student's *t*-test, TAMECs displayed more marked decreases in viability than BMVECs treated with the same venoms; on treatment with viperid venoms AB, BA, BG, BN, CV and VL, as well as AS, HS and PN venoms, TAMECs showed viability decreases of a higher magnitude over the 6hr period compared to BMVECs (P=0.0001). TAMECs treated with cobra venoms NK, Nmel, Nmoss and Nsi, and Australian elapid venoms NS and OM also displayed significantly lower cell viability compared to BMVECs, however, these differences between the viability decreases were less distinct (P=0.01).

Similar observations were made by studying venom effects on cell morphology, whereby TAMECs appeared to be more susceptible than BMVECs to cytocidal effects of both viperid and cobra venoms, but particularly viperid venoms.

## DISCUSSION

*In vitro* bioassays are valuable systems for screening pharmaceutical or biological agents, as they are inexpensive, reliable and homogenous and present less of an ethical concern than *in vivo* bioassays (Oliveira et al, 2002; Plant, 2004). Primary cell isolates from specific organs of interest are considered to be 'gold standards' for *in vitro* bioassays which are to be extrapolated to *in vivo* studies (Fischer et al, 2001; Plant, 2004). Studies into the effects of anti-angiogenic agents on tumour vessels will therefore carry greater weight if endothelial cells isolated from the tumour model of interest are used in initial screening studies.

Since one of the eventual aims of this ongoing study was to ascertain the *in vivo* effects of Australian snake venom components on the vasculature of a specific tumour model (Dark Agouti Mammary Adenocarcinoma; DAMA), cells used for the *in vitro* screening tests were successfully isolated and cultured from the vasculature of this same tumour model.

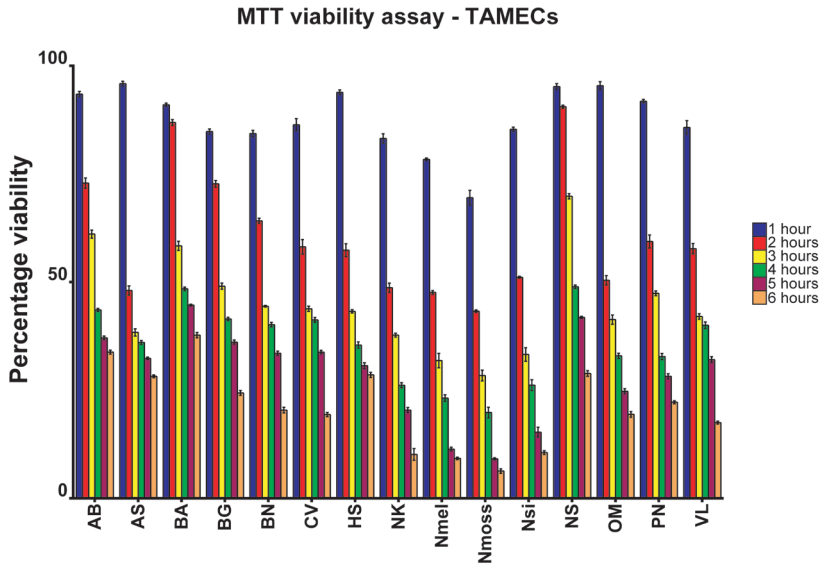


**Figure 4.** Column chart showing results from MTT viability assay for venom-treated TAMECs. Columns represent mean percentage viability at 1h, 2h, 2h, 4h, 5h and 6h of venom incubation for each venom. Error bars represent ±1 SEM (standard error of the mean).

This cell type (tumour-associated microvascular endothelial cells; TAMECs) fulfilled the requirements to achieve specificity and the entire culture process was appropriate in relation to cost, time and resource provisions.

The particular experimental model for determination of morphological changes was developed based on other screening studies (Araki et al, 1993; Borkow et al, 1994; Lomonte et al, 1999; Rivers et al, 2002) and proved most useful to characterise the effects elicited by different snake venoms. Venom effects on TAMEC morphology were observed over a six-hour period with the consideration that most physiological venom effects in bite victims occur rapidly, particularly those concerned with vascular targeting mechanisms. It was therefore decided that *in vitro* studies should be carried out over a time period which reflected these rapid effects. One of the principle aims of this study was to detect a rapid and sustainable apoptotic response in tumour-derived endothelia and this was achieved by application of low to moderate concentrations (0.01-100µg/ml) of viperid and selected Australian elapid venoms over a six-hour time period.

The experimental model used in the present study allowed for the grouping of different venom types on the basis of the morphological changes elicited by these venoms. As such, it was demonstrated that generally, viperid venoms caused detachment and subsequent apoptosis of TAMECs, whereas cobra venoms elicited a rapid cytotoxic effect on ECs. It was also demonstrated that the venoms of selected Australian species (*Austrelaps superbus* and *Hoplocephalus stephensii* in particular) induced similar morphological changes to those induced by viperid venoms. Other Australian elapid venoms (*Oxyuranus microlepidotus* in particular) initiated cell death characteristic of the non-apoptotic cell death induced by cobra venom. Furthermore, the venoms of *Notechis scutatus* and in particular, *Pseudonaja nuchalis,* were capable of eliciting both an apoptotic and a cytotoxic death response in TAMECs.

Statistical analysis of results obtained by a cell viability assay support these observations, such that venoms were able to be grouped according to their effects on EC viability. The MTT assay demonstrated that cobra venoms elicited cell death rapidly, a finding which correlates directly with observed morphological changes. It is unclear whether this rapid cell death is due to specific targeting of cell surface molecules, or whether cytolysis is elicited through indirect means, such asshifts in osmotic balance through insult to membrane integrity. Cell death elicited by viperid venoms, however, does not follow the same rapid pattern. Morphological studies showed that cell death generally occurred following cell detachment from the gelatin substrate, usually after two to three hours of venom incubation. Similar observations were made with the MTT assay, whereby a 'lag' period in cell death was observed and which may correlate to apoptosis of ECs following inhibition of cellular attachment.

Patterns obtained upon application of venoms from Australian snake species were comparable in some ways to those of the viperid venoms and not exotic elapids, to which Australian elapids are more closely related. In the current study, statistical analysis of MTT results suggests that the venom of *Austrelaps superbus*, in addition to venoms from *Hoplocephalus stephensii* and *Pseudonaja nuchalis*, bear more similarity in the pattern of effects on cell viability with the viperid venoms tested, than with the cobra venoms tested, which was also observed morphologically.

Statistical analyses also suggested that TAMECs were more susceptible to venom effects when compared to BMVECs, particularly in the case of viperid and selected Australian elapid venoms (AS, HS, NS and PN). It is known that many venoms from snakes of the *Viperidae* family cause apoptosis in ECs within angiogenic vessels, including those within tumours. Results from the current study correlate to some extent with these established findings. Venoms from exotic elapids (cobras) used in the present study were found to kill

ECs via non-apoptotic means and while there was a significant difference between TAMEC and BMVEC responses to cobra venoms, these differences were on the whole less distinct than those observed upon application of viperid and AS, HS, NS and PN venoms. These findings may suggest that these venoms target moieties differentially or constitutively expressed on TAMECs to elicit cell death, or, it may simply be related to different cytotoxic and apoptotic thresholds of the EC populations.

It must be re-established that this is a preliminary study with a specific focus on initiating an apoptotic response in TAMECs. Further studies using different *in vitro* systems and biochemical tests are required to provide information on the complete range of actions of each of the venoms studied.

## CONCLUDING REMARKS

These experiments demonstrated that selected Australian elapid venoms have effects on the cellular biology of endothelial cells that are in some cases synonymous with adhesion inhibition and apoptosis. It was also established that the effects of certain Australian snake venoms on endothelial cells bear some similarity to effects elicited by exotic viperid snake species. Differential responses to these venoms were observed between tumour-derived and control endothelial cells, which may suggest differential targeting of specific cell surface moieties or varying apoptotic thresholds.

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## STATEMENT OF COMPETING INTERESTS

Peter Mirtschin of Venom Supplies donated the venoms used in this project. Other authors have not declared any competing interests.

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