Inhibition of hemangioma development in a syngeneic mouse model correlates with bcl-2 suppression and the inhibition of Akt kinase activity.

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Abstract

Background Hemangiomas are benign vascular tumors that are characterised by excessive angiogenesis. While there is no definitive treatment for these tumors, several angiogenesis inhibitors, including bleomycin, have been employed in their treatment. To better understand the mechanism of bleomycin in accelerating haemangioma regression, we have investigated the effects of the drug on hemangiogenesis using a previously described mouse hemangioma model.

Materials and Methods The effects of bleomycin were tested in mice injected with endothelioma cells to induce hemangioma development. At termination, mouse tissue samples from bleomycin-treated and control mice were stained with hematoxylin and eosin for histological examination. Bcl-2, flk-1 and vWF expression were studied by immunofluorescence microscopy. Hematological analysis was undertaken using a hemo counter. Akt activity was analyzed in tissue homogenates and endothelioma cells using ELISA. Also, caspase activity was analysed in endothelioma cells by ELISA.

Results Bleomycin inhibited tumor growth *in vivo* in a dose-dependant manner. Our findings also revealed that bleomycin inhibited Akt activation and suppressed bcl-2. *In vitro* bleomycin increased caspase activation.

Conclusion Our observations reveal possible mechanisms for the inhibitory effects of bleomycin on hemangiogenesis, and raise the possibility that inhibition of Akt activation and suppression of bcl-2 may provide therapeutic targets for the treatment of hemangiomas.

Key words hemangioma, bleomycin, Akt, bcl-2, caspase

Introduction

Hemangiomas are benign tumors of the vasculature frequently encountered in children [1-2]. In neonates, these tumors are characterized by rapid proliferation of endothelial cells during the first few months of postnatal life followed by slow spontaneous involution [1-3]. Although the pathogenesis of hemangioma formation is poorly understood, angiogenic growth factors and steroid hormone influences have been postulated to underlie the abnormal proliferation of endothelial cells that characterise hemangioma development. [4-6]. In spite of the fact that the tumors are benign, some patients present with serious complications and as a result require treatment.

Current modalities of treatment of hemangiomas have been associated with serious side effects [7-9]. For instance, systemic corticosteroids, which are the first-line treatment for aggressive, life- or function-threatening lesions, exhibit side effects such as Cushingoid appearance, hypertension, insomnia, and growth retardation [7]. Interferon alpha, which has shown success rates of up to 80%, can lead to the development of spastic diplegia [8-9]. As a result, this has limited the use of this form of therapy for life-threatening hemangiomas in infants.

A number of antineoplastic drugs, including vincristine, cyclophosphamide and bleomycin, have been employed to treat hemangiomas of infancy [10-12]. The successful use of intralesional bleomycin to treat vascular lesions in infants has been well-documented [13-15]. In addition, bleomycin has been shown to inhibit angiogenesis in cultured human hemangioma biopsies [16]. More recently, we reported on the antiangiogenic and pro-apoptotic effects of this drug in bovine microvascular endothelial (BME) cells [17].

The antiangiogenic and antitumor effects of bleomycin have not previously been evaluated in an animal hemangioma model. One of the models cited in the literature as being useful for studying vascular tumors and tumor-associated angiogenesis is based on the inoculation of mice with endothelial cells transformed by the polyoma middle T (Pym T) oncogene [18-22].

The polyoma virus, a DNA tumor virus, induces neoplasms in a wide range of tissues in rodents, and has been used for many decades to study tumor development [23-24]. The middle T antigen of murine polyoma virus rapidly transforms endothelial cells, leading to the formation of vascular tumors in newborn mice [25].

In vitro, endothelioma (End.) cells established from such tumors grow as immortalized cell lines and can be used to induce secondary tumors in mice [26]. Using this model, we sought to determine the effects of bleomycin on tumor growth *in vivo*, and to elucidate the drug's mechanism of action. Bleomycin inhibited vascular tumor development in mice, and this was accompanied by a recovery from tumor-induced thrombocytopenia and anaemia. Both bcl-2 and Akt were suppressed in drug-treated mice. Bleomycin also suppressed bcl-2 and induced the activation of caspase-3 and -9 in immortalised endothelioma cells.

Materials and Methods

Cell culture

The endothelioma cell line, sEnd.2, derived from Pym T induced vascular tumors in the skin of the thorax of C57BL6 mice, was grown in Dulbeco's Modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat inactivated fetal calf serum (Invitrogen, USA), 10 nM glutamine (Invitrogen, CA, USA), and 1% penicillin-streptomycin (Highveld Biologicals, Sandringham, SA) [20]. The cell line was maintained in a 37 ^{o}C incubator in a humidified atmosphere containing 5% CO_2.

Effects of bleomycin on tumor development

Female C57BL6 mice (8-12 weeks old) were housed at the University of Pretoria Biomedical Research Centre (UPBRC). The mice were given standard commercial food (EPOL) and water *ad libitum*. Animal experiments were conducted according to a protocol approved by the University of Pretoria Animal Use and Care Committee (project 16/2005).

Tumors were induced based on a previously described method [20]. Mice were preloaded with 0.6-1.2 mg/kg bleomycin intraperitoneally (i.p.) on day one; 2 x 10^6 mouse endothelioma (sEnd. 2) cells were injected subcutaneously into the flanks of the mice to induce tumor growth. The animals were injected with bleomycin at doses of 0.6-1.2 mg/kg i.p. every three days. The dose range was based on reviewed literature and an initial study undertaken on 20 mice. During our investigation, the mice were monitored every second day for tumor formation and the time taken for the onset of the tumors was recorded. Tumour size was measured using callipers. The observation period was up to 15 days following tumor induction.

Histological Analysis

At termination, tumors were removed and fixed in buffered 4% paraformaldehyde and embedded in paraffin using standard procedures. Five µm sections were cut and stained with hematoxylin and eosin. Sections were viewed under a Zeiss Axio microscope and images attached to a digital camera (Carl Zeiss, Ferndale, South Africa).

Hematological analysis

At termination, blood samples were collected into heparinised tubes for haematological analysis. The red cell count, platelet count, hematocrit percentage and haemoglobin levels were determined using a hemocounter, Cell-Dyne 3700 (Abbott, IL, USA).

Immunofluorescence

Deparaffinised tumor sections were incubated with 5% normal blocking serum in PBS. After removal of blocking serum, the slides were incubated with primary mouse monoclonal antibodies directed against mouse bcl-2, vWF and Flk-1 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by FITC-conjugated anti-mouse antibody or Alexa fluor 350-conjugated anti-rabbit antibody. Slides were mounted in fluorescent mounting medium (Dako Cytomation) and sealed. Image acquisition was performed on an Olympus Cell^R system attached to an IX 81 inverted fluorescence microscope equipped with an F-view-II cooled CCD camera (Soft Imaging Systems). Using a Xenon-Arc burner (Olympus Biosystems GMBH) as light source, images were acquired using 360 nm, 472 nm or 572 nm excitation filters.

For fluorescent microscopy, cells were seeded onto heat-sterilized coverslips in 6-well plates at a density of 30 000 cells. After 24 hours, cells were exposed to 0-20 µg/ml bleomycin (the dose was chosen based on cell viability studies). After 24 hours, the medium was removed and cells were fixed in cold methanol, washed three times in PBS and incubated in blocking serum.

Cells were then incubated with a monoclonal antibody directed against mouse bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by FITC-conjugated IgG antibody. The cells were observed under a CFL40 Axiovert microscope, and images were captured using a Zeiss digital camera attached to the microscope (Carl Zeiss, Johannesburg, SA).

Assay for Akt kinase activity

Sectioned tissue samples from control and drug-treated mice were placed in centrifuge tubes containing lysis buffer. The cytosolic fractions were extracted following the procedures which are described by the manufacturer (Stressgen, MI, USA). For in vitro studies, cultured sEnd.2 cells were incubated with 0-20 µg/ml bleomycin. After 24 hours of exposure to bleomycin, cells were washed twice with PBS, centrifuged at 1000*g* for 5 min at 4°C, and resuspended in extraction buffer for 30 min. The activity of Akt kinase was assayed using an enzyme-linked immunosorbent assay (ELISA) kit according to instructions of the manufacturer. Briefly, microtiter wells were soaked in 50µl/well kinase assay buffer. Aliquots of 30 µl sample, purified active PKB, inhibitor diluent or dilution buffer were added to triplicate wells.

Ten µl of diluted ATP were added and the plate incubated for 90 min. Aliquots of anti-phosphate specific substrate were added and the plate was incubated at room temperature. Subsequently, 40 µl/well of secondary antibody solution was added and the plate incubated at room temperature for 30 min. After washing, tetramethylbenzidine (TMB) was added and the plate was incubated at room temperature for 45 min. Aliquots of 20 µl/well of acid stopping solution were added and the absorbance was read at 450 nm using the ELx 800 Universal Microplate Reader (Bio-Tek instruments Inc, Weltevreden, SA). The assay was repeated twice.

Cell Viability Assay

Mouse-derived endothelial tumour cells (End.2 cells) were grown in D-MEM and exposed to bleomycin (0.001 - 100 µg/ml) or vehicle for 24 - 48 hours. The effect of bleomycin on cell proliferation was determined using the crystal violet stain.

Caspase Activity

Caspase-3 and caspase-9 activity was measured using commercially available kits according to the manufacturer's instructions (BioVision, Mountain View, USA). In brief, control and drug-treated sEnd.2 cells were lysed in caspase lysis buffer, followed by centrifugation at 16,000 x g for 15 minutes. The supernatant was mixed with assay buffer in the presence of 200 mM substrate. Samples were incubated at 37 °C for 4 hours and absorbance was read at 405 nm using the ELx 800 Microplate Reader (Bio-Tek instruments Inc, Weltevreden, SA).

Data Analysis

Statistical analyses were performed using either the Student's *t* test or analysis of variance with the Bonferroni method. For animal studies the analysis was repeated using the Welch test and the non-parametric Mann-Whitney test. Each value was expressed as the mean ± SEM unless stated otherwise. Differences were considered significant when the calculated *p* value was <0.05.

Results

Tumor development

The Pym T model was used to test the effect of bleomycin on haemangioma growth in mice and to elucidate its mechanism of action. All mice in the control group developed tumors at the site of injection 5-7 days after inoculation with sEnd.2 cells (Fig. 1a). Tumors were observed less frequently in mice treated with bleomycin at a dose of 0.6 mg/kg, with 60% of the mice developing tumors compared to 100% in the control (Fig. 1a). At higher doses (0.9 mg/kg and above), there was complete inhibition of tumor growth (Fig. 1a). In the group of mice treated with 0.6 mg/kg bleomycin, the tumors that developed were smaller in diameter (average 20.4 mm) compared to control mice (average 28.8 mm) (Fig. 1b).

Histology analysis and Bcl-2 staining

Histological analysis of the vascular tumors that developed in the control group revealed large blood-filled cavities (Fig. 1c left). Although blood-filled cavities were also observed in tumor sections of bleomycin treated mice, these cavities were reduced in size (Fig. 1c right). We investigated bcl-2 expression, which has previously been correlated with increased vascularity in various tumors. Bcl-2 staining was observed in sections of tumor-bearing mice (blue fluorescence) (Fig. 1d left). This antiapoptotic protein was barely detectable in tissue sections of bleomycin-treated mice (Fig. 1d right).

Hematological analysis

Vascular tumors such as hemangiomas have been associated with abnormal haematological parameters. Haematological analyses was therefore undertaken to investigate whether blood parameters were affected in tumor bearing mice, and to determine the effect of treatment on these factors. The mean hematocrit percentage was reduced significantly in control mice injected with sEnd.2 cells (20.4%) compared to healthy mice injected with saline only or baseline (38%) (Fig. 2).

In healthy mice (baseline), the red cell count (RCC) was 13.86 X 10^6 cells/ µl of blood, and the hemoglobin concentration was 118.4 g/L. The RCC and the hemoglobin concentration were markedly decreased in untreated tumor bearing mice, with values of 5.8 X 10^6 cells/ µl and 78 g/L respectively (Fig. 2). The platelet count was also markedly decreased in control mice (297.16 X 10^3 cells/ µl) (Fig. 2). In bleomycin treated mice, the hemoglobin concentration, the RCC and the hematocrit percentage and platelet count were increased significantly compared to control mice (Fig. 2). The platelet count was increased, and in mice treated with 1.2 mg/kg bleomycin the platelet count was 569.2 X 10^3 cells/ µl.

vWF and Flk-1 immunofluorescence

To better understand the mechanism of bleomycin action, we investigated the presence of the endothelial cell marker, von Willebrand Factor (vWF), tissue samples of mice bearing sEnd.2-induced vascular tumors. We also investigated the expression of fetal liver kinase-1 (Flk-1), also known as vascular endothelial growth factor receptor-2, in the same samples. Both vWF (blue fluorescence) and Flk-1 (green fluorescence) were detected in samples of control tumor-bearing mice, while staining for these markers was reduced in samples of bleomycin-treated mice (Fig. 3a).

Akt kinase activity

Akt activity was measured using an ELISA kit which is based on the use of a polyclonal antibody that recognises phosphorylated Akt. There was increased Akt kinase activity in the tissue homogenates of control tumor-bearing mice compared to normal skin homogenates, indicating a possible role of Akt in tumor development in this mouse model. The levels of Akt kinase activity were lower in homogenates of tissue sections harvested from bleomycin-treated mice (Fig. 3b).

In vitro Cell Viability Assay

In this study we evaluated the effects of bleomycin on sEnd.2 cell growth. Accordingly, we demonstrated that bleomycin induced a decrease in sEnd.2 cell numbers in a dose-dependant manner, with an IC_{50} of approximately 9.2 µg/ml. We also investigated the combined effects of bleomycin and zVAD fmk on cell growth. The reduction in the percentage of viable cells was partially prevented in cells treated with bleomycin and zVAD fmk (Fig. 4a). Based on these observations, a dose range of 5-20 µg/ml bleomycin was chosen for further *in vitro* studies.

Caspase activity

We also investigated the possible effect of bleomycin on caspases. Caspases are a family of cysteine proteases which play a role in the execution of apoptosis. We observed that there was increased activation of caspase-3 in bleomycin treated endothelioma cells when compared to control cells (Fig. 4b). Increased activation of Caspase-9 was also observed in these drug-treated cultures. A significant increase in caspase-3 and caspase-9 activation was not observed in cells exposed to a combination of bleomycin and the broad spectrum caspase inhibitor zVAD fmk (Fig.4b).

Bcl-2 localization and Akt activity in cultured sEnd.2 cells

Bcl-2 was expressed in control saline-treated endothelioma cells. There was decreased bcl-2 localization in bleomycin-treated cells (Fig. 4b). However, bleomycin induced a marginal decrease in activated Akt in cultured sEnd.2 cells (Fig. 4c).

Discussion

In humans, the beneficial effects of intralesional bleomycin in the treatment of hemangiomas have been widely reported [11-15,27]. However, bleomycin can induce a debilitating side effect, namely, pulmonary fibrosis, and there is thus concern over the use of the drug to treat benign tumors in pediatric patients. Understanding the mechanism of action of bleomycin in inducing hemangioma regression might enable the development of effective and safer therapies.

In this study, sEnd.2 cells were used to induce vascular tumors in mice and this occurred with 100 % frequency. According to Williams *et al.* (1989), endothelioma cells expressing the middle-T antigen are the primary cause of Pym T-induced hemangiomas, and the continuous presence of these cells sustains the tumor [28]. Furthermore, previous studies have shown that injection of primary non-transformed endothelial cells did not induce observable lesions [28]. In the present study, the development of sEnd.2 cell induced hemangiomas was reduced in mice treated with bleomycin in a dose-dependent manner.

To better understand the mechanism of bleomycin in restricting hemangioma development in mice, immunofluorescence analysis of sections of sEnd.2 cell-induced tumors were undertaken. This revealed the presence of the endothelial cell marker vWF in the tumors. The sections also stained positively for Flk-1 and bcl-2. Interestingly, there was a reduction in Flk-1 and bcl-2 staining in tumor sections of bleomycin-treated mice. Fetal liver kinase-1 is a receptor for the proangiogenic ligand vascular endothelial growth factor (VEGF) [29]. Increased expression of this receptor has been reported to correlate with an increase in bcl-2 expression in patients with metastatic colon cancer [29]. Also, Perrone *et al.* (2004) reported a direct correlation between increased microvascular density and increased bcl-2 expression [30]. In this study, sEnd.2 cell induced tumors that developed in mice treated with bleomycin were characterised by decreased bcl-2 staining. In vitro, cells exposed to bleomycin stained poorly for bcl-2, while control sEnd.2 cells stained positive for bcl-2.

Bleomycin has been shown to induce a dose-dependent decrease in bcl-2 protein, however, the mechanism by which bleomycin exerts this effect remains unclear [32].

According to published reports, bcl-2 overexpression by tumors promotes their survival by further stimulating angiogenesis [31]. In addition, studies undertaken by Fontanini *et al.* (1998) have revealed that bcl-2 controls the development of tumor angiogenesis in non-small cell lung carcinoma, with putative mediation by VEGF [33]. Therefore, the suppression of bcl-2 by bleomycin makes this marker an attractive target for hemangioma therapy.

The bcl-2 family is the best characterised protein family involved in the regulation of apoptotic cell death, and it consists of pro-apoptotic and anti-apoptotic members [34,35]. Bcl-2 is one of the most studied anti-apoptotic members of the bcl-2 family and has been shown to suppress caspase activation [31,35].

Further investigation of the effects of bleomycin on sEnd.2 cells revealed that bleomycin induces the activation of caspase-3 and -9. Indeed bcl-2 has been shown to promote the existence of the inactive form of caspase 3, namely procaspase 3 [35]. A correlation between the down-regulation of bcl-2 and Akt has also been reported in human breast cancer cells undergoing apoptosis [36]. We therefore sought to investigate the possible role of Akt in tumor growth.

Akt, also known as serine/threonine protein kinase B, is a modulator of various physiologic events such as cell survival and cellular metabolism. Akt has also been implicated in tumor progression as a mediator of cell proliferation and an inhibitor of apoptosis [37]. In addition, in a previous study, the toxin melittin induced an increase in caspase-3 activation which was associated with the down-regulation of bcl-2 and Akt phosphorylation in human leukemic U937 cells [38]. Further, in another study, vascular endothelial growth factor was shown to induce the activation of the PI3K/Akt pathway [39].

It has also been shown that suppressing Akt activity leads to increased endothelial cell apoptosis and inhibits angiogenesis in the presence of proangiogenic factor, fibroblast growth factor-2 (FGF-2) [40]. In the current study, decreased levels of Akt activity were measured in tumor homogenates of bleomycin-treated mice. It is however worth noting that bleomycin induced a marginal decrease in Akt activity *in vitro*. Indeed it is apparent from our observations that Akt is activated in these vascular tumors, and that the inhibition of tumor growth correlates with the inhibition of Akt activation. However, the relationship between the expression of bcl-2, Akt and Flk-1, as well as their association with tumorigenesis in this hemangioma model, warrant further investigation, especially given that the decrease in Akt activity observed *in vivo* was not mimicked *in vitro*.

Our observations also revealed that sEnd. cell-induced tumors were characterised by abnormal hematological features, such as thrombocytopenia and a low red cell count. Previously, kaposiform haemangiomas (KHE), which are vascular tumors similar to hemangiomas, have been associated with thrombocytopenia [41]. According to Enjolras *et al.* (1997), severe thrombocytopenia is a constant feature in KHE patients in which tumors are associated with the Kasabach-Merritt syndrome (KMS); the presence of platelets inside the lesions appears to sustain the growth of the tumor [41]. Restoration of platelets to the circulation may promote tumor regression. On the other hand, anaemia is one of the complications observed in some patients with hemangiomas [29]. In the present study bleomycin prevented haemangioma-bearing mice from developing both thrombocytopenia and anemia. The mechanism by which bleomycin prevents thrombocytopenia and anemia is however unclear.

In summary, our findings provide evidence that bleomycin inhibits hemangioma development *in vivo* in a mouse model, and that tumour inhibition correlates with a decreased Akt activity and reduced bcl-2 expression. Given that previous studies have shown of the role of bcl-2 in promoting angiogenesis a, namely future studies should explore the role of this marker as a potential target for hemangioma therapy.

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Figure legends

Figure 1.

Bleomycin inhibits tumor growth in mice inoculated with sEnd. 2 cells.

The incidence of tumor development (a) and the size of the tumors (b) were reduced in bleomycin treated mice compared to control mice. n = 5 mice per group, * $P < 0.05$

(c) Light microscopic features of sEnd.2 cell-induced vascular tumors. Histology of tissue samples from a control mouse showing a vascular tumor with a large blood-filled lumen (asterisk); the hemorrhagic lumen is reduced in size in a mouse treated with 0.6 mg/kg bleomycin. Bar = 20 µm (d) bcl-2 immunofluorescence micrographs of tissue sections from control and bleomycin-treated mice. Bar = 0.1 mm.

Figure 2.

Blood parameter values in untreated and bleomycin-treated mice.

At termination, blood samples were collected from mice. Hematological analysis was undertaken using a Cell-Dyne 3700 hemocounter. Values are shown as mean ± SEM. n = 5; *$P < 0.05$ compared to control mice; $^{\S}P < 0.05$ compared to healthy mice (baseline).

Figure 3.

Analysis of vWF and Flk-1 expression and Akt kinase activity in tissue sections of control and bleomycin-treated mice.

(a) Deparaffinised sections of tumors were processed for the analysis of vWF and Flk-1 using fluorescence microscopy as described under Materials and Methods. Representative photographs are shown. Bar = 0.1 mm. (b) Bleomycin inhibits Akt kinase activity in mouse tissue sections. Cytosolic fractions were extracted from tissue sections and the activity of Akt kinase was assayed using an ELISA. Three wells were analysed per treatment; the assay was repeated. *$P < 0.05$ compared to control; $^{**}P < 0.05$ compared to healthy mice (baseline).

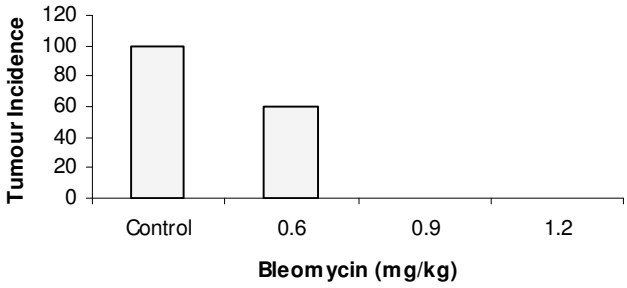
Figure 4.

Bleomycin decreases cell survival and increases the activity of caspase-3 and caspase-9. (a) dose-dependent response curves for the effects of varying doses of bleomycin with or without zVAD fmk. Cell viability was determined in control and drug treated sEnd.2 cells using crystal violet staining. The assay was repeated three times; three wells were analysed per concentration. (b) Cytosolic lysates were prepared from saline- or bleomycin-treated sEnd.2 cells. Caspase-3 and caspase-9 activation was determined using commercially available ELISA kits. Data shown are representative of two separate experiments. Three wells were analyzed per treatment dose. Values are mean ± S.D. *$P < 0.05$; ** $P < 0.001$

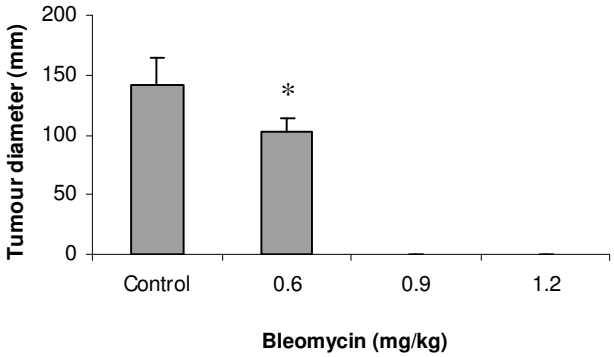
Figure 5.

The effects of bleomycin on bcl-2 expression and Akt activity in sEnd.2 cells. Representative images of bcl-2 localization in (a) sEnd.2 cells treated with saline and (b) 20 µg/ml of bleomycin. Cells were seeded on coverslips and treated with bleomycin or vehicle for 24 hours. The cells were then subjected to immunofluorescent staining with a antibcl-2 antibody and visualized with FITC-conjugated IgG. Bar 20 µm (c) Cells were prepared as discussed under Materials and Methods. Akt activity was measured using ELISA. Data are expressed as the mean ± S.D. of triplicate wells. The experiment was repeated twice.

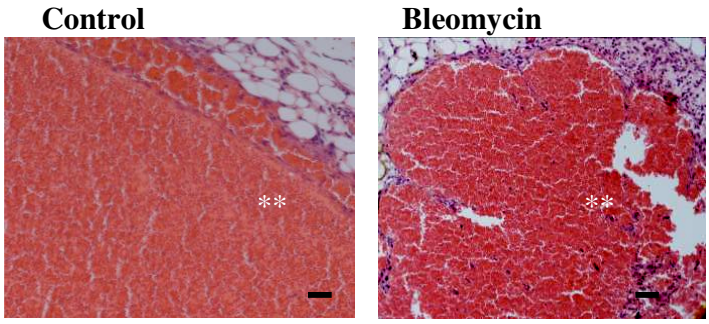
a


Tumour Incidence
120
100
80
60
40
20
0
Control
0.6
0.9
1.2
Bleomycin (mg/kg)

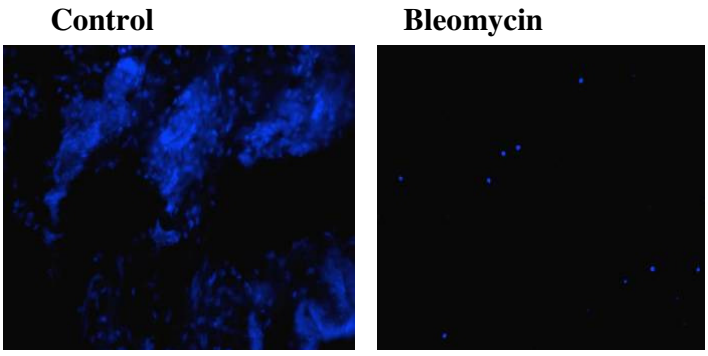
b

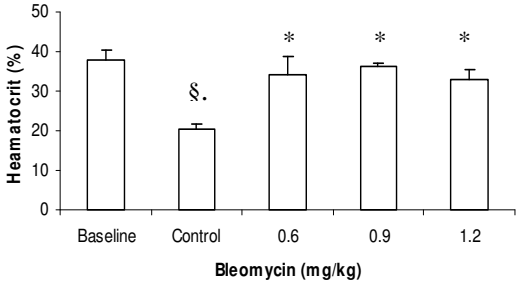

Tumour diameter (mm)
200
150
100
50
0
*
Control
0.6
0.9
1.2
Bleomycin (mg/kg)

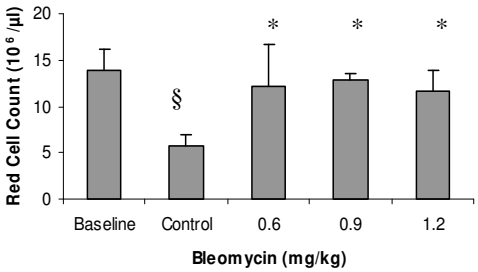
c

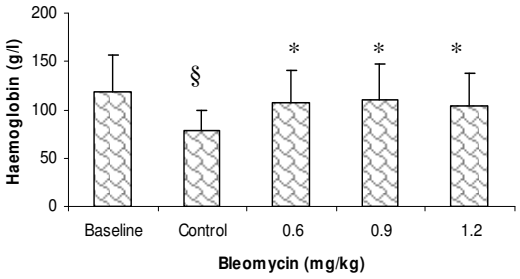

Control
Bleomycin
**
**

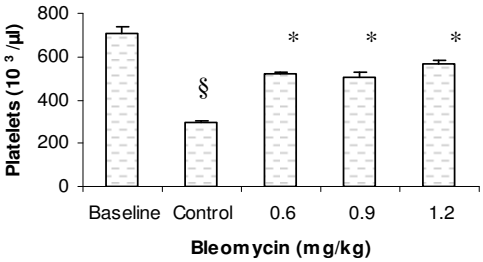
d


Control
Bleomycin

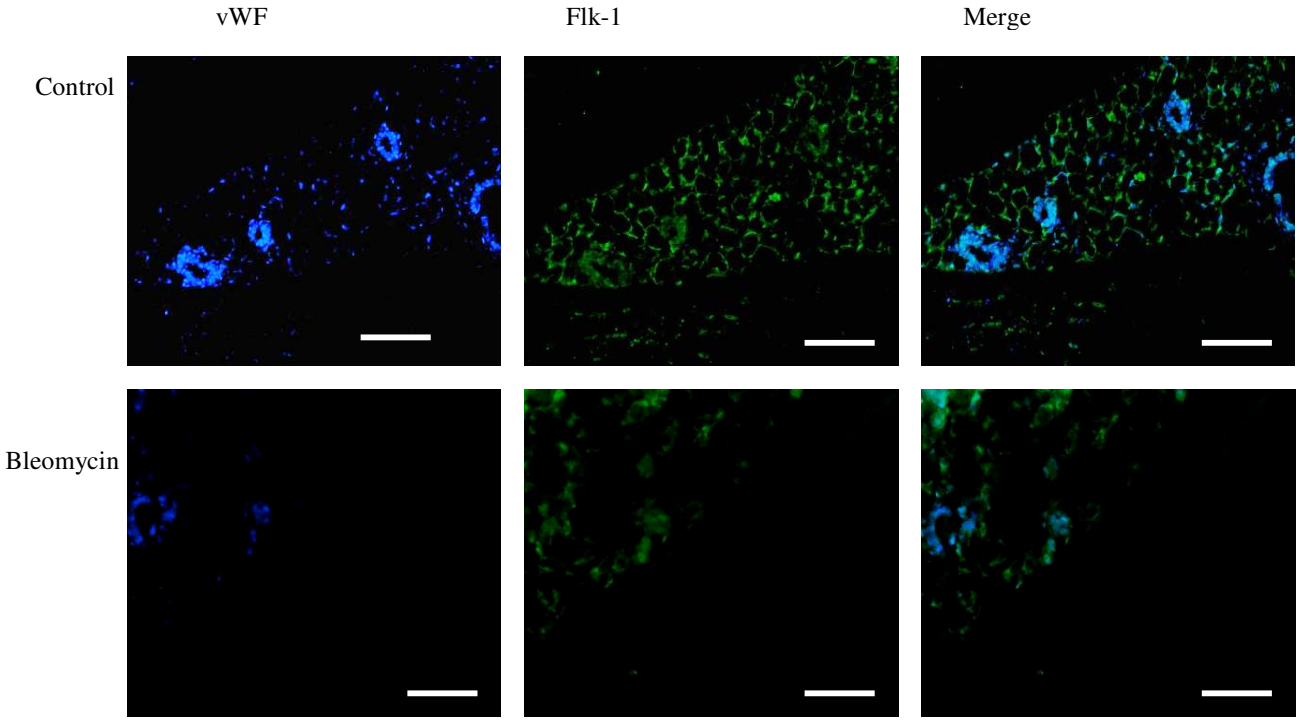

Heamatocrit (%)
50
40
30
20
10
0
§.
*
*
*
Baseline
Control
0.6
0.9
1.2
Bleomycin (mg/kg)


Red Cell Count (10 6 /µl)
20
15
10
5
0
§
*
*
*
Baseline
Control
0.6
0.9
1.2
Bleomycin (mg/kg)

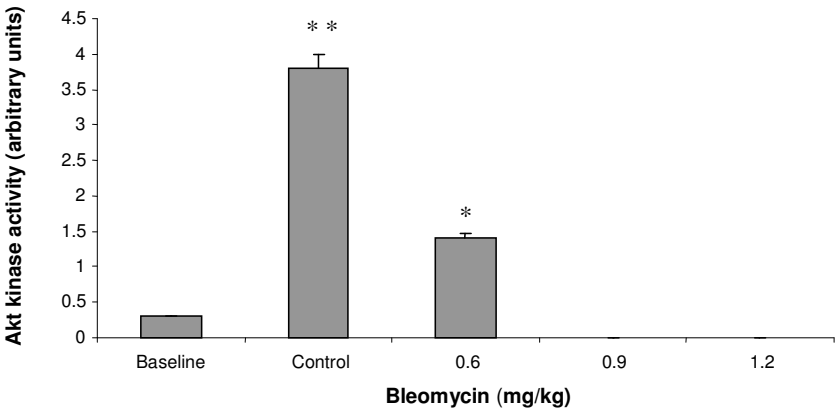

Haemoglobin (g/l)
200
150
100
50
0
§
*
*
*
Baseline
Control
0.6
0.9
1.2
Bleomycin (mg/kg)


Platelets (10 3 /µl)
800
600
400
200
0
§
*
*
*
Baseline
Control
0.6
0.9
1.2
Bleomycin (mg/kg)

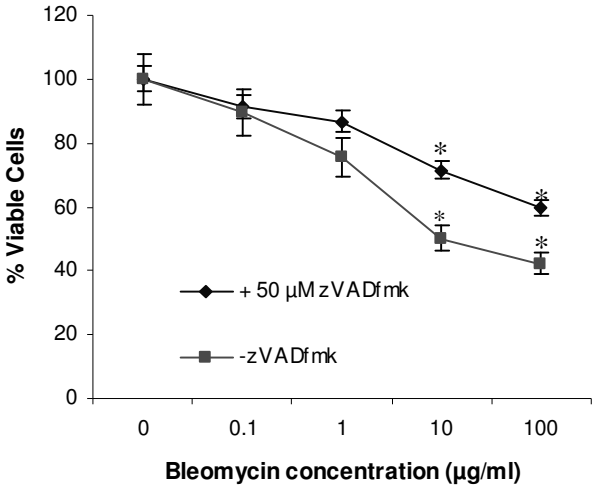
a


vWF
Flk-1
Merge
Control
Bleomycin

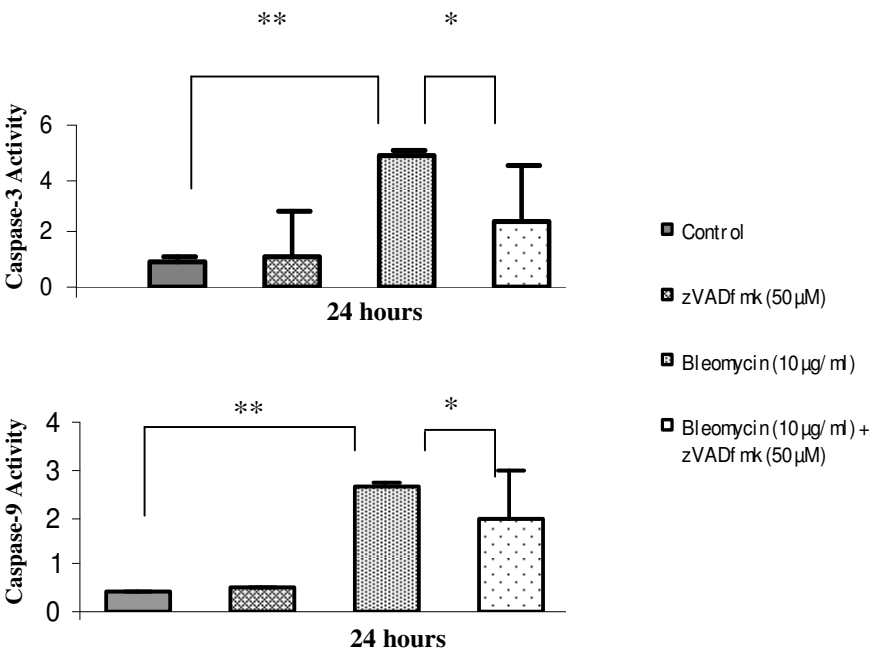
b

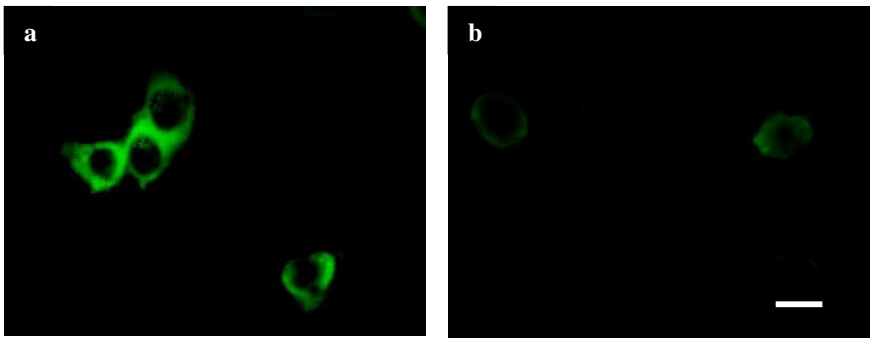

Akt kinase activity (arbitrary units)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
* *
*
Baseline
Control
0.6
0.9
1.2
Bleomycin (mg/kg)

a

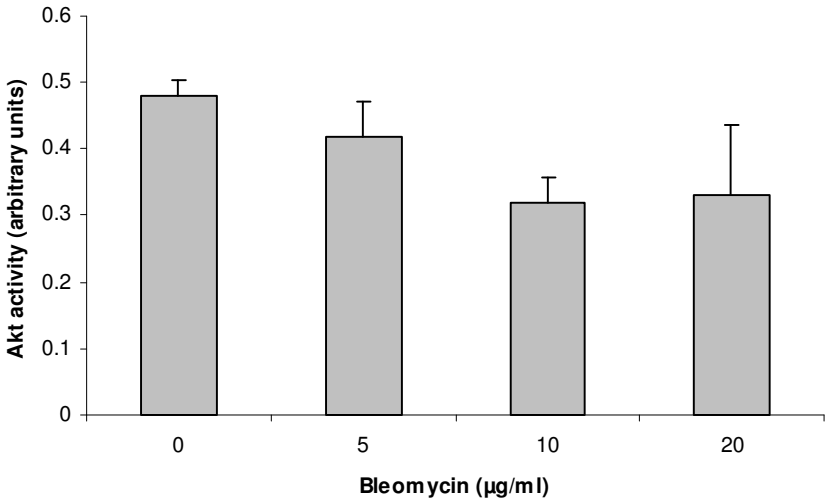

120
100
80
60
40
20
0
% Viable Cells
+ 50 µM zVADfmk
-zVADfmk
0
0.1
1
10
100
Bleomycin concentration (µg/ml)
*
*
*
*

b


**
*
Caspase-3 Activity
6
4
2
0
24 hours
**
*
Caspase-9 Activity
4
3
2
1
0
24 hours
Control
zVADfmk (50µM)
Bleomycin (10µg/ml)
Bleomycin (10µg/ml) + zVADfmk (50µM)


a
b

c


0.6
0.5
0.4
0.3
0.2
0.1
0
Akt activity (arbitrary units)
0
5
10
20
Bleomycin (µg/ml)