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In Vitro and *In Vivo* Investigations into The Carbene Copper Bromide Anticancer Drug Candidate WBC4

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Abstract: The anticancer drug candidate 1,3-di(p-methoxybenzyl)-4,5-di(p-isopropylphenyl)-imidazol-2-ylidene copper(I) bromide (WBC4) was tested on the NCI 60 cancer cell panel in vitro. WBC4 showed very good activity against a wide range of human cancer cell lines inclusive renal cell cancer with an average GI50 value of 288 nM. This encouraged maximum tolerable dose (MTD) experiments in mice, where a MTD value of 10 mg/kg was determined with single injections to groups of 2 mice. In the following tumor xenograft experiment WBC4 was given at 5 and 10 mg/kg in 5 injections to two cohorts of 6 CAKI-1 tumor-bearing NMRI:nu/nu mice, while a control cohort of 6 mice was treated with solvent only. At the higher dose of 10 mg/kg WBC4 showed borderline toxicity leading to 2 mortalities, while a significant T/C value of 0.38 was observed on day 32. At the lower dose of 5 mg/kg WBC4 induced mild and reversible body weight loss with no toxic deaths. At this dose WBC4 showed an identical significant T/C value of 0.38 on day 32, when compared to the treatment group. Immunohistochemistry for the proliferation marker Ki-67 did not show significant changes due to WBC4 treatment in the animals. However, anti-angiogenic effects by WBC4 treatment were observed in CD31 immunohistochemistry. Here, significant reduction in microvessel number, area and ratio was determined in tumors treated with 10 mg/kg of WBC4.

Keywords: Anticancer drug, carbene-copper complex, NCI 60 cancer cell panel, CAKI-1 renal cell cancer, xenograft mouse model.

INTRODUCTION

Renal-cell carcinoma (RCC) is the most common malignant disease of the adult kidney, which accounts for approximately 3% of adult malignancies [1]. If not detected early, these cancers develop to an invasive adenocarcinoma, which have very limited treatment options and poor outcomes. New targeted compounds like Bevacizumab, Sunitinib, Sorafenib, Temsirolimus and others give a certain amount of hope to patients with advanced renal-cell cancer, since these compounds can block the VEGF or mTOR pathway and are therefore anti-angiogenic [2]. Nevertheless, these new drugs cannot cure advanced or metastatic renal cell cancer and give the patient only a few extra months of survival. These clinical facts suggest that new therapeutic regimens must be explored in the quest to develop an effective therapy for these metastatic or advanced forms of renal-cell cancer.

There is a significant unexplored space for chemotherapeutic coinage metal-based drugs [3] targeting difficult to treat cancers like RCC. A paper by Youngs and coworkers suggested already in 2008 that carbene-silver acetates derived from 4,5-dichloro-imidazole may have the stability and antitumoral activity to become anticancer drug candidates [4]. The idea was further pursued and led to the development of more lipophilic benzyl-substituted imidazole- and benzimidazole-derived carbene-silver, -gold, and -ruthenium complexes showing activity against the human renal cancer line CAKI-1 [5-19]. So far, the most promising derivative 1-methyl-3-(*p*-cyanobenzyl)benzimidazole-2-ylidene silver(I) acetate (SBC1) showed activity against CAKI-1 cells with an IC50 value of 1.2 μM [16], which is superior when compared to cisplatin. Due to its lipophilicity and suitable shape the anticancer drug candidate SBC1 is binding well to albumin, interacts with DNA *in vitro*, but failed to show an antitumoral effect *in vivo* [20]. Further synthesis led to 1,3-di(*p*-methoxybenzyl)-4,5-di(*p*-isopropylphenyl)-imidazol-2-ylidene copper(I) bromide (WBC4), which shows nanomolar activity with an IC50 value of 0.65 μM against CAKI-1 cells [21]; the structure of WBC4 is shown in Fig. (**1**).

This paper is now investigating the anti-proliferative effect of WBC4 against the full panel of NCI 60 cancer cell lines *in vitro* and its activity and toxicity in a CAKI-1 xenograft mouse model *in vivo*.

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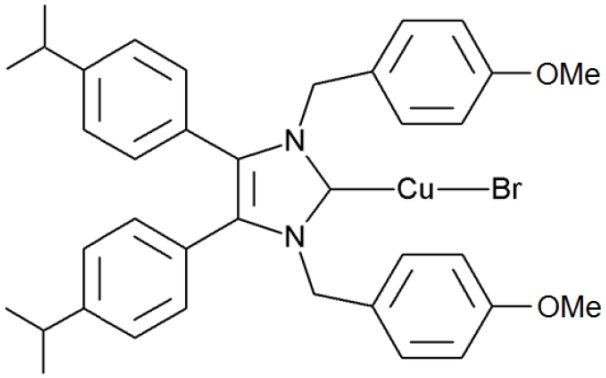


Fig. (1). Molecular structure of the carbene copper bromide compound WBC4.

MATERIALS AND METHODS

Cell Assays

The cell viability tests were performed on the US National Cancer Institute's 60 cancer cell panel (NCI 60) at Frederick, MD. For these tests WBC4 is formulated in DMSO:glycerol 9:1 and given to the cells at concentrations ranging from 10 nM to 10 μM. The growth inhibition assay allows for a 48 h incubation period with WBC4 solutions and uses Sulforhodamine B (SRB) to measure drug-induced cytotoxicity as described before [22]. The interpretation of the data is based on GI50 values, which are concentrations required to inhibit cell growth by 50%.

CAKI-1 Xenograft

In a first animal experiment the maximum tolerable dose (MTD) of WBC4 was determined in NMRI:nu/nu mice. WBC4 was dissolved in DMSO (final concentration 10%) and further diluted with saline, which resulted in a clear and stable solution. Male NMRI:nu/nu mice (n=2 mice per group) were treated with 5, 10 and 20 mg/kg intraperitoneally (i.p.) in single injections in order to determine the approximate MTD. In this experiment maximum body weight loss was determined.

In the second *in vivo* experiment 1 x 10^7 CAKI-1 cells (expanded *in vitro* in McCoy´s medium + 10% fetal bovine serum) were injected subcutaneously (s.c.) in a volume of 0.1 ml to male NMRI:nu/nu mice (n=6 mice per group) on day 0. When tumours were grown to a palpable size of around 0.1 cm^3 mice were randomised and treatment was initiated on day 7. For groups B and C the experimental anticancer drug WBC4 was injected into mice i.p. at doses of 5 or 10 mg/kg on days 7, 11, 15, 19 and 27, while the control group (group A) of mice was treated with the solvent only. Tumor size was measured with a caliper instrument. Tumour volumes, relative tumour volumes (relation to the first treatment day) and treated to control (T/C) values were calculated. Body weight and lethality of the mice were determined continuously during the experiments to estimate tolerability of the drug. Mice were sacrificed 5 days after their last treatment and their peritoneum was checked for possible signs of inflammation. Tumors were removed and snap frozen for immunohistochemical analyses.

The animal experiments were performed according to the German Animal Protection Law and with approval from the responsible authorities. The *in vivo* procedures were consistent and in compliance with the UKCCCR guidelines.

Ki-67 and CD31 Immunohistochemistry

Sections from snap frozen Caki-1 tumors (6 per group, thickness 5 μm) were fixed with 3.7% paraformaldehyde, blocked with H_2O_2 and goat serum. After incubation with the primary antibody (rat anti-mouse CD31, clone: MEC13.3, BD Pharmingen, Heidelberg, Germany), slides were incubated with a secondary HRP-labelled goat anti-rat antibody (Jackson ImmunoResearch, Hamburg, Germany), DAB substrate (Dako, Hamburg, Germany) and counterstained with hematoxylin. Evaluation of microvessel density was performed with AxioVision 4.5 (Zeiss, Jena, Germany). Vessels were labelled in six representative pictures of each tumor and quantified for microvessel size, number and ratio (microvessel area vs. total tumor area).

For the staining of the Ki-67 proliferation marker, fixation and blocking was performed according to the procedure used for CD31. The slides were incubated with the primary mouse anti-human Ki-67 antibody (Dako; clone: MIB-1). As secondary antibody the HRP-labelled anti-mouse antibody was used. For the staining the DAB substrate and for counterstaining hematoxylin was used (Dako). For evaluation of Ki-67 expression 6 pictures of each tumor were taken and the number of positive vs. negative cells was counted in three fields of view.

STATISTICAL ANALYSIS

Statistical evaluation for all experiments was performed with the One-way Anova test and Bonferroni-correction. The level of statistical significance was defined with a p-value of $p \leq 0.05$.

In Vitro Efficacy

WBC4 was tested against 5 leukemia, 8 NSC lung, 6 colon, 6 CNS, 8 melanoma, 7 ovarian, 8 renal, 2 prostate and 6 breast cancer cell lines. The concentrations that inhibited cell growth by 50% (GI50, as determined by a SRB assay after a 48 h incubation period) were generally in the upper nanomolar range with WBC4 exhibiting an average GI50 value of 288 nM. WBC4 exhibited the growth inhibition activity with GI50 values of lower than 200 nM against the leukemia cell line RPM18226, the lung cancer cell line HOP-92, the colon cancer cell lines COLO 205 and HCT-116 and the melanoma cell lines LOX IMVI and SK-MEL-5. Particularly interesting is the high activity against the prostate cancer cell line PC3 with a GI50 value of 183 nM and the breast cancer line MDA-MB-468, for which a GI50 value of 42 nM was determined. WBC4 showed the lowest activity against the multidrug-resistant ovarian cancer cell line NCI/ADR-RES with a GI50 value of 1.45 μM and the renal cell cancer cell line CAKI-1 with a GI50 value of 1.32 μM. The activity of WBC4 is high even against CAKI-1, which was chosen for the xenograft experiment. All cell test results of WBC4 are summarised in Table **1**.

Table 1. Overview on results obtained in cytotoxicity tests of WBC4 against the NCI 60 cancer cell line panel; efficacy is shown as growth inhibition 50% (GI50) values.

Cell Line	GI50 [M]	Cell Line	GI50 [M]
		NSC Lung Cancer	
		A549/ATCC	3.03E-7
Leukemia		HOP-62	3.43E-7
CCRF-CEM	3.47E-7	HOP-92	6.32E-7
HL-60(TB)	2.38E-7	NCI-H226	2.52E-7
MOLT-4	4.40E-7	NCI-H23	2.69E-7
RPMI-8226	1.46E-7	NCI-H322M	4.86E-7
SR	2.14E-7	NCI-H460	2.97E-7
		NCI-H522	2.29E-7
Colon Cancer		CNS Cancer	
COLO 205	1.65E-7	SF-268	3.88E-7
HCC-2998	3.10E-7	SF-295	2.62E-7
HCT-116	1.73E-7	SF-539	2.85E-7
HCT-15	5.70E-7	SNB-19	3.42E-7
KM12	2.99E-7	SNB-75	2.91E-7
SW-620	3.00E-7	U251	3.16E-7
Melanoma			
LOX IMVI	1.96E-7	Ovarian Cancer	
M14	2.88E-7	IGROV1	3.43E-7
MDA-MB-435	3.05E-7	OVCAR-3	2.99E-7
SK-MEL-2	3.33E-7	OVCAR-4	2.60E-7
SK-MEL-28	3.48E-7	OVCAR-5	2.61E-7
SK-MEL-5	1.45E-7	OVCAR-8	3.36E-7
UACC-257	2.80E-7	NCI/ADR-RES	1.45E-6
UACC-62	2.78E-7	SK-OV-3	2.16E-7
		Prostate Cancer	
		PC-3	1.83E-7
Renal Cancer		DU-145	3.64E-7
786-0	3.32E-7		
A498	2.23E-7	Breast Cancer	
ACHN	3.30E-7	MCF7	2.47E-7
CAKI-1	1.32E-6	MDA-MB-231/ATCC	2.26E-7
RXF 393	2.54E-7	HS 578T	4.35E-7
SN12C	3.45E-7	BT-549	2.92E-7
TK-10	3.18E-7	T-47D	2.15E-7
UO-31	5.74E-7	MDA-MB-468	4.23E-8

Table 2. Overview on results obtained in the CAKI-1 xenograft experiment. Male nude mice received subcutaneous tumour cell injections on day 0. Starting at palpable tumor size the mice were treated with WBC4 or solvent at days 7, 11, 15, 19 and 27. Tumor size in the treated group in relation to the control group (T/C) was measured as a therapeutic marker, while the number of deaths and the body weight change were used as toxicity parameters.

Group	Number	Substance	Treatment	Route	Dose	Opt. T/C	Deaths	BWC (%)
	of mice		[on day]		(mg/kg)	[on day]	[on day]	(max)
A	6	Solvent	7,11,15,19,27	i.p.			0/6	2

Table 2. contd…

Group	Number	Substance	Treatment	Route	Dose	Opt. T/C	Deaths	BWC (%)
	of mice		[on day]		(mg/kg)	[on day]	[on day]	(max)
B	6	WBC4	7,11,15,19,27	i.p.	5	0.38	0/6	-3
						[32]		
C	6	WBC4	7,11,15,19,27	i.p.	10	0.38	2/6	-3
						[32]	[14,21]	

WBC4 Mediated Growth Inhibiton on CAKI-1 Xenograft Tumors

In the mouse experiment for determination of MTD three groups of n=2 mice were treated with single doses of 5, 10 and 20 mg/kg of WBC4. The highest dose (20 mg/kg) led to two toxic deaths within one day after treatment, while the other mice recovered and the body weight loss was found to be reversible. From these investigations doses of 5 and 10 mg/kg were derived for a further experiment with an extended treatment period for the tumor-bearing mice.

In the CAKI-1 tumor xenograft experiment all tumors grew progressively and the tumors reached a palpable size of 0.075 cm^3 on day 7. Therefore, three groups of 6 mice each were treated at days 7, 11, 15, 19 and 27 intraperitoneally with solvent or WBC4 solution. The low-dose group (5 mg/kg/d) of treated mice showed a low and reversible body weight loss of 3% and all 6 mice survived the treatment. The high-dose group experienced a medium body weight loss of 3% as well, but 2 mice died on days 14 and 21. All parameters of the CAKI-1 xenograft experiment are shown in Table **2**.

As shown in Fig. (**2**), in both treatment groups B and C tumors grew slowly but steadily at almost the same rate until day 18 and reached mean tumor volumes of 0.089 cm^3 (group B) and 0.087 cm^3 (group C) after three injections, while the control cohort reached a significant higher tumor volume of 0.163 cm^3. On day 25 after 4 injections the tumors in the treated groups showed their highest volume of 0.121 cm^3 (group B) and 0.143 cm^3 (group C) when the control group exhibiting a value of 0.247 cm^3. After 5 injections this growth inhibitory effect increased reflected by tumor volumes of 0.113 cm^3 (group B) and of 0.112 cm^3 (group C). By contrast the solvent treated control group A reached a tumor volume of 0.294 cm^3 on day 32. This WBC4 mediated growth inhibition proved to be statistically significant ($p<0.001$, Fig. **2A**, **B**), which is also reflected by the T/C values of 0.38 for groups B and C on day 32 (Table **2**).

WBC4 Effect on Ki-67 Expression in CAKI-tumors

The expression of the proliferation marker Ki-67 was quantified by immunohistochemistry (Fig. **3A**) in all tumours (n=6) of each group. As shown in Fig. (**3B**) the ratio of Ki-67 positive cells in the solvent-treated tumors did not differ significantly from those determined in the WBC4 treated groups. This indicates that WBC4 treatment did not influence Ki-69 expression in this CAKI-1 tumor model.

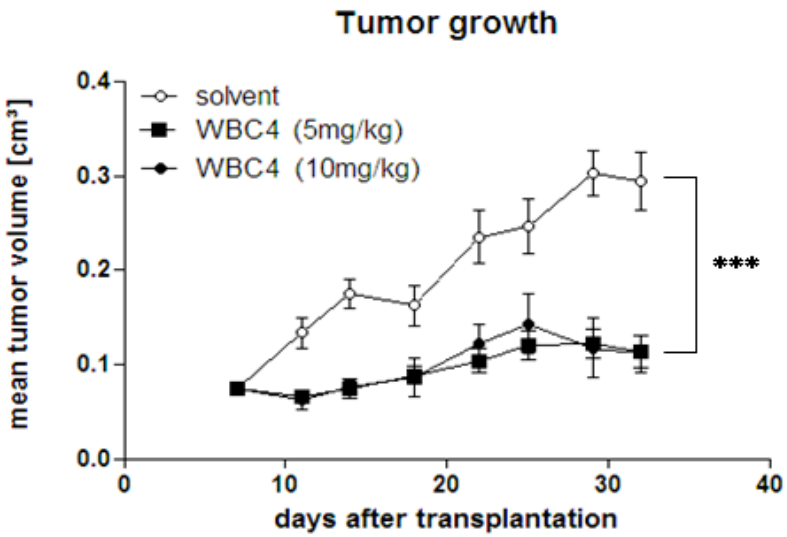
WBC4 Effect on CD31 Expression in CAKI-1 Tumors

The microvessels (mv) within the control and WBC4 treated tumors were analysed by the specific CD31 staining (Fig. **4A**). The CD31 positive brown-stained mv were quantified and counted by computer-based analysis in the 6 tumors of each group (Fig. **4B**). A distinct occurrence of vascularisation was determined in tumors of the control cohort, with a mean mv number of 96. The treatment of mice in groups B (5 mg/kg) and C (10 mg/kg) with WBC4 induced a decrease in the number of microvessels to 90 and 69 respectively. The reduction seen in group C is statistically significant ($p<0.001$) with respect to group A. Analysis of mv area revealed significant ($p<0.01$) reduction from 3.8×10^4 in the control group A to 2.0×10^4 in group C. Similarly, mv ratio significantly ($p<0.01$) decreased from 2.6% in the control group to 1.4% in WBC4 treated group C. This indicates anti-angiogenic activity of WBC4 at high-dose treatment in the CAKI-1 tumor model, particularly reflected by the significant reduction in mv number and area. In fact similar anti-angiogenic activities have been observed in titanocene-treated tumors [23-25].

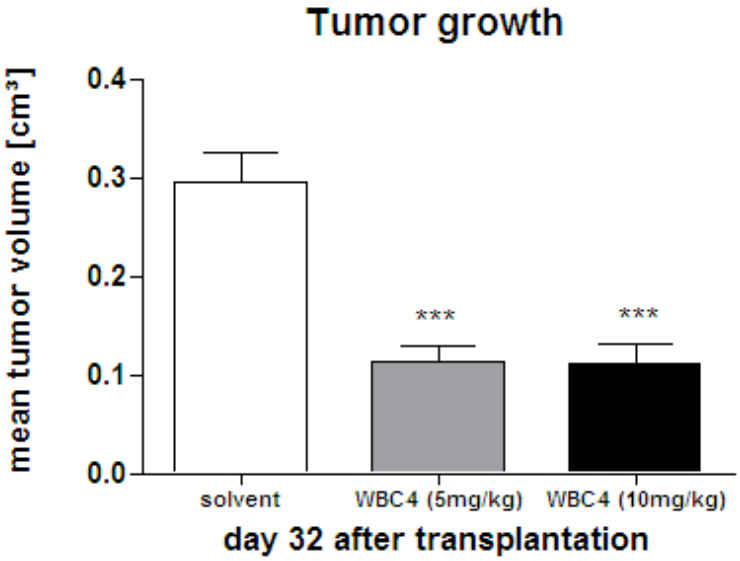
CONCLUSION

The experimental anticancer drug WBC4 shows cytotoxic activity against a wide range of cancer cell lines mostly at nanomolar concentrations. None of the NCI 60 cancer cell lines showed GI50 values above 1.5 μM, which is a good starting point for further preclinical development.

Due to a long-standing interest in renal cell cancer CAKI-1 cells were chosen for the *in vivo* investigations [23, 24]. From the first *in vivo* experiments using non-tumor bearing mice and a single injection a MTD value of 10 mg/kg was determined for WBC4. In the xenograft experiment using CAKI-1 tumor-bearing mice this MTD proved to be already quite high and 2 out of 6 mice died. When exposed to 5 dosages of 5 mg/kg of WBC4 none out of 6 mice died during the treatment. A good T/C value of 0.38 at the end of the experiment combined with reversible body weight loss and tolerable toxicity demonstrated that WBC4 has a useable therapeutic index in its given formulation. Particularly encouraging is the fact that copper can be used as a cytotoxic reagent [26]; copper is an essential element for humans and should not have a long-term toxicity associated with it, since the human body is able to manage copper levels with known transport mechanisms. Therefore, WBC4 is a promising anticancer drug candidate for clinical testing.

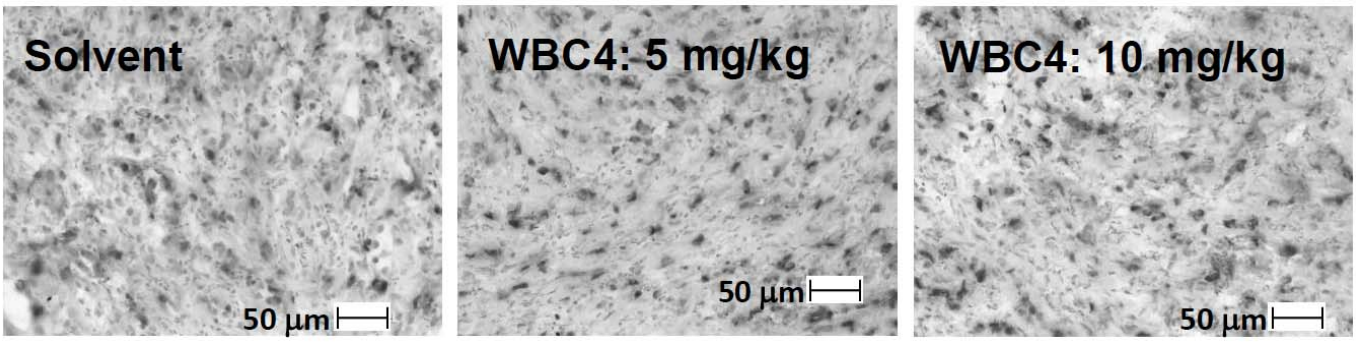


A



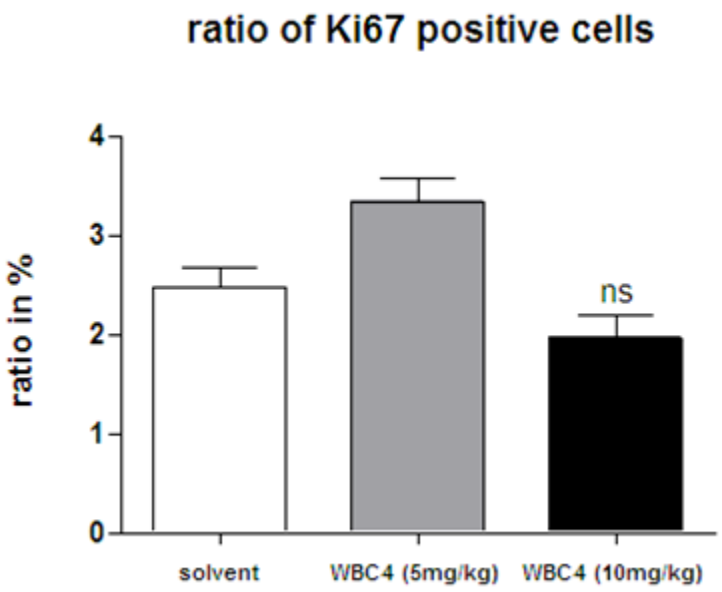
B

Fig. (2). Influence of WBC4 on growth of CAKI-1 xenotransplant tumors in NMRI nu/nu mice. Treatment of animals with WBC4 at doses of 5 and 10 mg/kg significantly reduces tumor growth (**A**). This is also reflected by comparison of the mean tumor volumes at day 32 (**B**). The asterisks (***) mark levels of significance: $p < 0.001$; the standard deviations are given as SEM.



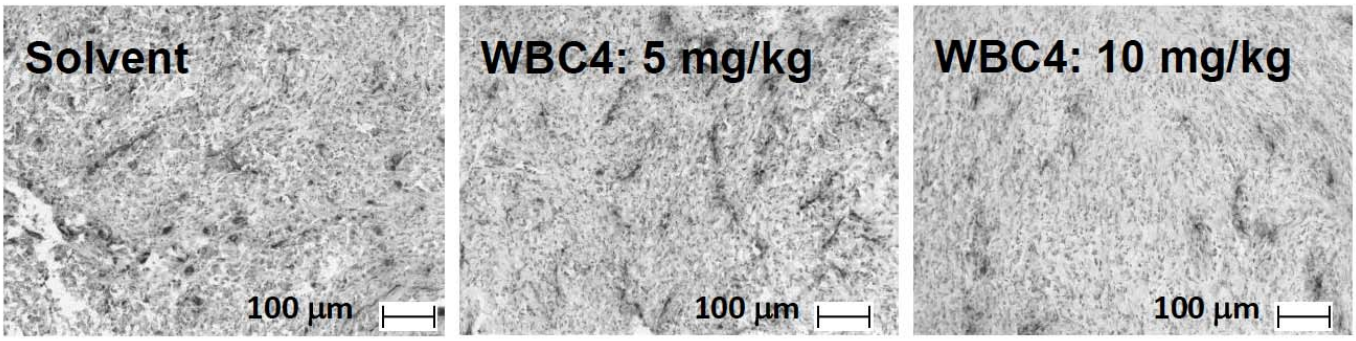
A

Fig. (**3**). Contd…



B

Fig. (3). Immunohistochemistry for Ki-67 (**A**) and ratio of Ki-67 positive cells in control and WBC4 treated tumors. (**A**) The dark staining in the tumor sections indicates Ki-67 positivity. (**B**) Quantitative analysis of ratios of Ki-67 positive cells revealed no significant ($p>0.05$) differences between treated and control animals. Standard deviations are given as SEM.



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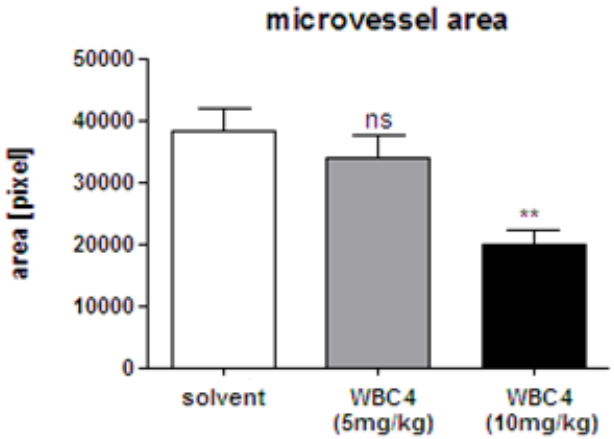


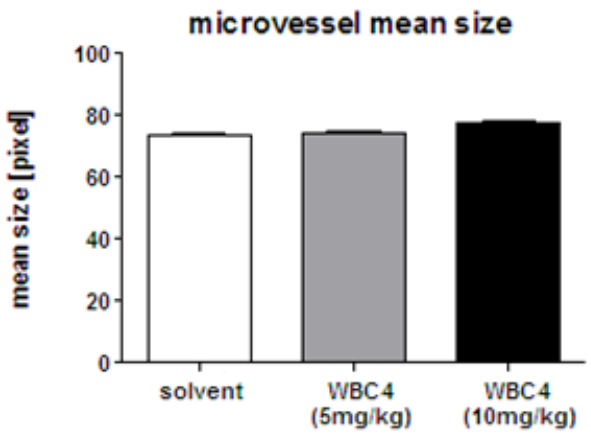
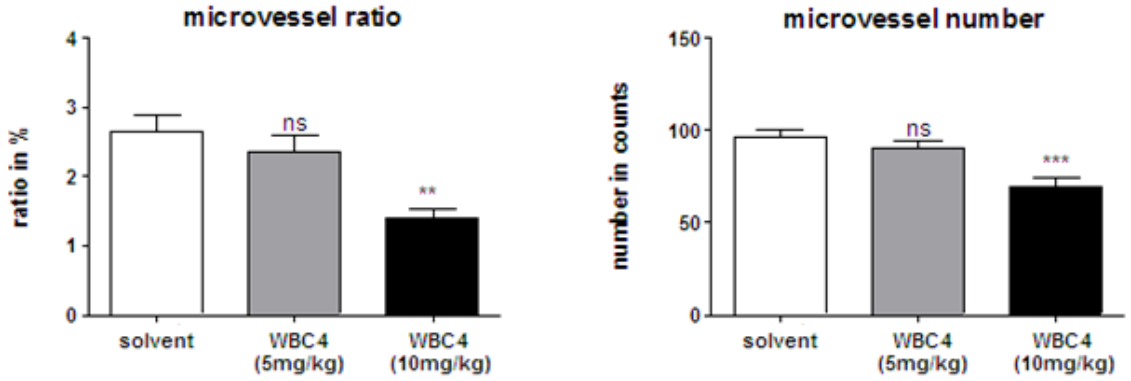


Fig. (**4**). Contd…



B

Fig. (4). Immunohistochemistry for CD31 (**A**) and quantitative analysis for microvessel area, mean size ratio and number (**B**). (**A**) The dark staining in the representative tumor sections indicates CD31 positivity from the microvessels. (**B**) Quantitative analysis for microvessel number shows significant reduction in the high dose WBC4 treated group. Similarly, high dose WBC4 treatment significantly reduces microvessel area and ratio. Levels of significance: "**", $p<0.01$; "***", $p<0.001$. Standard deviations are given as SEM.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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