

Dose dependent role of Emodin and BTB14431 in suspension colon cancer model in rats

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Abbreviations: intravenously (iv), intraperitoneally (ip)

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Abstract

Background. An “In Silico 2D/3D Conformer Screening” for structural similar antitumor substances to Curcumin was carried out and the novel anthraquinone BTB14431 was found. Emodin, contained in several Chinese medical plants and BTB14431 are known to be potential inhibitors of the COP9-signalosome - stabilizing the tumor suppressor protein p53. The aim of this study was to analyze the suppressing effects on colorectal cancer in a standardized rat model (WAG/Rij).

Methods. A suspension of CC531 colon cancer cells was applied to the cecum after laparotomy and, additionally, at the back of animals. Therapy was conducted twice daily for 7 days, with increasing doses of BTB14431, Emodin and with isotone sodium chloride solution (control) intravenously (iv) or intraperitoneally (ip). Therapy was initiated the day of tumor cell application. Peripheral blood samples were taken before surgery and on day 7. 21 days after the end of therapy, the animals were euthanized and tumor growth was evaluated.

Results. Data showed a downward trend of the total tumor growth after iv and ip treatment with low doses of BTB14431 and Emodin. Differential blood analysis showed apoptosis, but no major changes in hemogram. Increasing doses of Emodin elevated total mortality rate exponentially.

Conclusions. Although apoptosis was verified, no significant tumor suppressing effects could be observed for iv and ip treatment of both agents in our model. This stays in contrast to former in vitro studies. Agents remain viable novel substances. They will be the subject of upcoming studies. Additional data is needed to evaluate the significance of the “In Silico Screening” to identify potential in vivo anti-tumor drugs.

Keywords. Angiogenesis; tumor; Emodin; BTB14431; rats

Introduction

In recent years, the number of novel anti-tumorigenic agents, which are effective in vitro, increased dramatically. Several different substances out of that group have been studied in vivo. However, high amount of side effects, low efficacy or high costs might impede their clinical use.

On the other hand, a number of preclinical and clinical studies revealed that Taurolidine (TRD), a novel compound derived from the biogenic amino acid Taurine, possesses anti-angiogenic and cancer chemopreventive properties [Daigeler, 2008; Walters, 2007; Opitz, 2007; Chromik, 2007]. Besides other antitumorigenic mechanisms, TRD blocks protein biosynthesis as we have described before [Braumann, 2004]. The development and molecular investigation of new drugs with potential antineoplastic effects, namely drugs, which are at least as safe and effective as TRD, are expensive and time-consuming. Therefore, we were interested to detect structural homologues [Lipinski, 2000] of antineoplastic molecules using the “In-Silico-Screening” method [Fullbeck, 2005]. Several substances were perceived, which have been tested in different concentrations in molecular experiments [Braumann, 2008]. Among other agents, two of them were found to block tubulogenesis, an analogy of angiogenesis, in vitro. Because of their almost optimal chemical qualities, Emodin and BTB14431 were chosen to be examined in vivo.

Emodin is a structural homologue of Curcumin, the yellow pigment of curry, which has anti-oxidative, anti-inflammatory as well as anti-septic properties. Moreover, it is an in vivo inhibitor of angiogenesis [Arbiser, 1998]. The special characteristics of the substance has been published previously [Fullbeck, 2005]. Curcumin and Emodin were described to inhibit the COP9 signalosome-associated kinases [Berse, 2004; Uhle, 2003].

BTB14431 also inhibits the mentioned kinases in different cell lines (human cervix carcinoma-HeLa, B8 mice fibroblasts). It has been shown to increase the intracellular level of p53 (a cell cycle protein, which correlates with apoptosis) and to decrease the intracellular level of the transcription factor c-Jun [Fullbeck, 2005]. Both are known to induce apoptosis. Cell viability was previously assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method as recommended by the manufacturer (Sigma-Aldrich, Germany). An 80% reduction of cell viability was identified. Our own experiments demonstrated that the inhibition constant ($IC_{50} = 50\%$ inhibition in vitro) of BTB14431 was very low ($17\ \mu\text{M}$). That indicates a high receptor affinity. Unfortunately, there was no information in the literature concerning the doses needed for Emodin and BTB14431 in mammals. Molecular calculations and expected intracellular concentrations led to our

decisions made in the performed rat model. Therefore, we were interested in the safety

toxicity and the antitumorigenic in vivo effects of Emodin and BTB14431.

Methods and materials

Animals

All procedures were performed under protocols approved by the Local Committee for Animal Use and Care (Approval number G0192/06, LaGeSo Berlin, Postfach 31 09 29, 10639 Berlin, Germany) and following UKCCCR-guidelines for animal welfare [Workman, 1988]. Originally, the preliminary aim of the study was to determine the proper drug dose in terms of related safety and toxicity. However, the Local Committee for Animal Use and Care only approved the antitumorigenic element.

120 male WAG/Rij rats (Charles River, Sulzfeld, Germany) were acclimated to a 12 – hour light cycle controlled environment for 7 days before investigations (humidity 50 – 60%, temperature 24 – 26 °C). The animals were allowed standard laboratory food and water ad libitum. Animal weight was in median 22 grams (SD 20-25 g) at arrival.

Tumor cell line

CC-531 (donation from Medical School University Frankfurt/Main, Germany) is a chemically induced colon adenocarcinoma cell line derived from the WAG-Rij rat [Gutt, 2001]. The cells were maintained under standardized and sterile conditions (37°C, 5.2% carbon dioxide, and 94.8% oxygen [O₂]) in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mmol/l of glutamine, 50 lg/ml of streptomycin, and 50 U/ml of penicillin (all reagents from Gibco BRL, Karlsruhe, Germany). Before injection, the cells were harvested with EDTA and then washed three times in PBS (both from Gibco BRL, Karlsruhe, Germany) by centrifugation (Hereaus-Sepatech Varifuge 3.0 R; Kendro Hereaus, Burladingen, Germany) at 1,000 rpm at a temperature of 4°C for 5 minutes. Vitality was determined by the trypan blue method using a Neubauer counting chamber [Braumann, 2000]. Cells were resuspended in a serum-free medium to the desired concentration on prompt application.

Therapy agents

BTB14431 and Emodin were applied in specific doses (see table 1); BTB14431 was applied with 0.3, 1.7, and 3.3 milligram (mg) per kilogram (kg) body weight (bw). The agent Emodin was applied with 5.0 and 10 mg per kg bw.

Comment: Decisions made for the used doses will be described in the discussion.

The pure powder of both agents Emodin (ACROS Organics, Geel, Belgium) and BTB14431 (Sigma-Aldrich, Steinheim, Germany) was dissolved in a 5.0% solution of polyvinylpyrrolidone (PVP, Sigma-Aldrich, Steinheim, Germany) and isotone sodium chloride solution (0.9%). After 3 minutes at 2,000 revolutions per minute (rpm) in a shaker (MS1 minishaker, IKA Works Inc., Staufen, Germany), the solution was placed in an ultrasonic bath (Sonorex RK255H, Bandelin electronics, Berlin, Germany) at a temperature of 40 °C (degrees of Celsius) for 20 minutes until it was completely dissolved. Sterility was achieved using a bacterial filter (0.22 μm) (Braun, Melsungen, Germany) before administration.

Study design

The influence of an iv versus an ip application of BTB14431 or Emodin on ip and subcutaneous (sc) tumor growth in WAG/Rij rats was tested. A dose response study was performed.

The surgical interventions were performed undergoing general anesthesia using weight depended ip injections of xylazine (12 mg/kg bw) and ketamine (75 mg/kg bw). A median laparotomy of 4 cm was performed. The cecum and the small intestine were exenterated out of the abdominal cavity for 20 minutes to simulate operative stress. After the ip administration of 10^5 colon adenocarcinoma cells (CC-531) in 1 ml serum-free medium, the abdomen was closed using a 4-0 vicryl running suture technique. To simulate distal metastasis 10^5 tumor cells in 1 ml serum-free medium were sc implanted at the back of the animals. All

rats (n=120) were randomized into 12 groups (n=10, table 1). To reduce sample or procedure bias, groups were evenly divided either in iv or ip treatment per day.

For the iv treatment a jugularis vein port catheter system (Access technologies, Skokie, Illinois, USA) was implanted for 7 days. The first treatment was performed at the end of surgery. Irrelevant differences in body weight and standardized general conditions, such as temperature, time schedule and blinded investigators led to a comparable treatment of the animals. Rats were treated twice a day (12-hourly, 1 ml each, 14 injections in total). They were allowed standard laboratory food and water ad libitum. To avoid negative side effects the agents were carefully applied iv with a minimum injection time of 1.0 minute. After 7 days of treatment the vein port catheter system was removed under general anesthesia.

Peripheral blood samples were taken from the tail vein just before surgery and one day after the last injection under general anesthesia. Next to a hemogram the number of leukocytes and thrombocytes as well as the hematocrit was determined.

To examine the effect of treatment, the body weight was recorded 7 days and 1 hour prior to laparotomy, as well as subsequently, 7 and 28 days, respectively. Four weeks after the tumor cell implantation, all rats were euthanized with carbon dioxide poisoning following general anesthesia. Two blinded assessors performed the autopsy whereas documentation was transcribed by a third person. The number of ip and sc tumor formations as well as the total tumor weight was recorded.

Complete blood count

Peripheral blood samples were taken from the tail vein under general anesthesia just before intervention and one day after the last treatment to examine the influence of BTB14431 and Emodin on systemic hematopoiesis reactions. The number of leukocytes, erythrocytes, and thrombocytes as well as the hematocrit value was calculated (using a Neubauer's counting chamber).

Hemogram: The effects of the antitumorigenic agents on the different blood count were

automatically and manually analyzed by a master practitioner “blinded” for the treatment. To determine the different types of leukocytes (neutrophil, eosinophil, and basophil granulocytes, lymphocytes, and monocytes) and changes in cell morphology, cells were stained with haematoxylin-eosin (HE). The quantitative amounts were manually determined and checked for aberrations as well as apoptosis characters.

Wound healing

Operative wounds and scars were examined macroscopically daily by an independent physician. At the beginning of the autopsy the scar tissue was resected for histological examination. Three sections were evaluated: upper, middle, and lower third. Sections were stained for HE to determine cell dissemination as well as cell- and nucleus morphology (circular, ovoid, spindle-shaped). Sections were evaluated using a light microscope and were documented with a digital camera (Olympus C2020Z, Japan).

Statistics

For evaluation purposes, the Kruskal-Wallis-test was used for unequal distribution. Single comparisons were performed by the Mann-Whitney-test and for categorical data Fisher’s exact test was used. Results were considered significant if the P values were less than 0.05. Tests were performed with statistical software SPSS 18.0 for Windows (USA).

Results

The study was carried out with 120 rats separated into 12 different treatment groups according to the applied drug, dose, and way of application (see Table 1). 105 animals lived throughout the entire treatment program. Most of the other animals died during or immediately after the iv treatment (n= 14). One animal died before treatment without any intervention. The fatality is detailed in table 2. All animals in group VI died. Therefore, no data could have been analyzed.

Total tumor weight

tumor growth after treatment with the lowest BTB14431-dosis (group II; median 0,17 mg; range 0.02-0.8 mg) and Emodin (group V; median 0.08 mg; range <0.01-3.78 mg), a statistical significance in comparison to group I (control; median 1,2 mg; range 0-3.35 mg) could not be shown (group II $p=0.08$; group V $p=0.3$). The therapy with BTB14431 in higher doses like in group III (median 0.66 mg; range 0.01-3.4 mg; $p=1,0$) and IV (median 1.99 mg; range 0.14-4.72 mg; $p=0.24$) did not lead to a decrease of tumor growth.

IPT: The tumor load among the ip treated animals including the control group was much lower than among the iv treated (see fig.1b). But neither the BTB14431 treatment (group VII: median 0.07 mg; range 0-1.65 mg; $p=0.77$ / group IX: median 0.07 mg; range 0-1.67 mg; $p=0.60$ / group X: median 0.02 mg; range 0-0.41 mg; $p=0.30$) nor the Emodin (group XI: median 0.03 mg; range 0-0.53 mg; $p=0.73$ / group XII: median 0.05 mg; range <0.01-0.91 mg; $p=0.45$) treatment resulted in different tumor growth compared to the control group VII (median 0.1 mg; range <0.01-1.31 mg).

Intraperitoneal tumor weight (table 3)

IVT: The tumor growth was reduced only after treatment with BTB14431 at the lowest dose (group II, $p= 0.05$) in comparison to group I (control group). The other treatments did not alter the tumor load: group V ($p=.2$), group IV ($p=.2$), and group III ($p=.9$) to group I.

IPT: None of the treatments led to a changed tumor growth in comparison to group VII (control group): group XII ($p=.8$), group XI ($p=.9$), group X ($p=.2$), group IX ($p=.5$), group VIII ($p=.7$).

Subcutaneous tumor weight (table 3)

IVT: Neither the tumor load of group V ($p=.3$), nor of group IV ($p=1.0$), nor of group III ($p=.5$) or of group II ($p=.2$) differed to group I (control group). Although the box-whisker-plots show a clear reduction of tumor burden in the groups II and V, no statistical significance was proved. This seems to be attributed to a massive tumor load either of one animal in group II (extreme value 400 mg) and in group V (282 mg).

IPT: The tumor loads did not differ: group XII ($p=.9$), group XI ($p=.8$), group X ($.7$), group IX ($p=.8$), group VIII ($p=.8$) in comparison to the control group VII.

Body weight (bw, table 3)

IVT: The bw did not vary during the perioperative course between group I and V ($p>.05$).

IPT: Neither treatment location (ip vs. iv) nor dose applied in groups VIII to XII led to a significant change of bw compared to the control group (VII, $p>.05$).

However, comparison of the body weight changes during the perioperative course after IVT and IPT demonstrated a significant reduction of bw after intravenous medication: group I (-6.25%) versus group VII (-2.1%, $p=.001$), group II (-4.4%) versus group VIII (-.8, $p=.01$), group III (-3.9%) versus group IX (2.6%, $p=.06$), group IV (-5.2%) versus group X (-1.2%, $p=.01$), and group V (-6.25%) versus group XI (-3.5%, $p=.04$). Group VI versus group XII could not be analyzed due to missed values.

Peripheral complete blood count

There were no differences statable in leukocyte, thrombocyte, and erythrocyte count preoperatively (immediately before treatment at T1, see table 2) in comparison to the end of treatment (T7) after IVT and IPT ($p>.05$, data not shown).

Differential blood count (hemogram)

A slight reduction of lymphocyte and monocyte count and an insignificant increase of neutrophil and eosinophil granulocyte count were analyzed ($p =$ not significant, $p=ns$, figure 2a and 2b). This effect was independent from application form (IVT or IPT). Both agents lead to characteristic morphologic cell changes (e.g. blebbing, cell shrinkage, and nuclear fragmentation. Intravenous application of Emodin and BTB14431 lead to apoptosis. A sign of apoptosis can be seen (fig.3A and 3B) in differential blood sample nuclear fragmentation and blebbing and irregular bulge in plasma membrane.

Wound healing

Since there are no data in the literature about the effects of Emodin and BTB14431 on wound

healing, this influence was observed as well.

Three sections of each scar were analyzed at day 28. No macroscopic and microscopic skin lesions were detected. We were interested in the microscopic configuration of the fascia site tissue. A primary wound healing was observed in all cases. All evaluated staining sections presented themselves in a typical phase III of wound healing. Next to state of the order of the collagen fibers (van Gieson's stain, figures not shown) was still a high density of fibroblasts and capillary vessels independent of the treatment strategy.

Discussion

Here we describe the potential of BTB14431 and Emodin to inhibit the growth of tumor cell lines in vivo. Emodin is an active component of a traditional Chinese and Japanese medicine isolated from the root and rhizomes of *Rheum palmatum* L.

Our results showed a slight downward trend of the total tumor growth after iv treatment with the lowest BTB14431 (in group II) and Emodin dose (in group V). We did not achieve a statistical significance in comparison to the control (group I). Tumor load among the ip treated animals including the control group was much lower than compared to the iv treated. This could be attributed to the additional interventional protocol of the iv group. Here surgery was needed to implant iv catheter systems resulting in a significant higher operative stress. Furthermore, ip treatment may interfere with tumor cell adhesion in the intraperitoneal cavity through a diluting effect. However, neither the BTB14431 treatment nor the Emodin treatment resulted in a different tumor growth compared to the control group VII. Recently it was shown that BTB14431 showed to be more efficient in inducing apoptosis or necrosis in HeLa cell lines than Emodin itself [Fullbeck, 2005]. In our in vivo experiments with colon adenocarcinoma cells the effects seen were comparable to Emodin.

Several authors have assessed the antineoplastic role of Emodin, mostly in vitro. Evidence for the anti-cancer activity of Emodin especially in colon cancer is limited. Kamei et al. studied

the inhibitory effects of chinones and Emodin in tumor growth on the HCT-15 cells of human colon carcinoma [Kamei, 1998]. Following an incubation with various doses authors reported a 50 % suppression of in vitro tumor growth for an Emodin concentration of von 12,5 $\mu\text{g/ml}$. Antineoplastic effects were found only for anthracinones with 2 or more hydroxyl groups.

We decided to examine the antineoplastic effects with comparable doses in this current in vivo experiment. Doses used (6.0 $\mu\text{g/ml}$, 34 $\mu\text{g/ml}$, and 66 $\mu\text{g/ml}$) did not lead to a suppression of tumor growths in our animal model but increased toxicity. Other results were discussed contrary in the literature. A “successful” in vitro experiment observed increased apoptosis and interruption of cell cycle in the G2/M-phase in HCT-116 colonic tumor cells after administration of a 40 μM Emodin solution [Lu, Zhang & Qian, 2008]. On the contrary, Zhang et al. found that administration of Emodin 40 μM significantly inhibited tumor growth and prolonged survival in mice bearing HER-2/neu overexpressing human breast cancer cells (MDA-MB453, BT-483, AU-565, MDA-MB-361), though no cell death was observed. Furthermore, the combination of Emodin and Paclitaxel synergistically inhibited the anchorage-dependant and -independent growth of HER-2/neu overexpressing human breast cancer cells in vitro and synergistically inhibited tumor growth and prolonged survival in athymic mice bearing sc xenografts of human tumor cells, which expresses high levels of p185. The authors concluded that their results could have important implications in chemotherapy for HER-2/neu-overexpressing breast tumors [Zhang, 1995]. Chan et al. selectively blocked the growth of v-ras-transformed human bronchial epithelial cells. Half-maximal inhibition of cell growth occurred at a concentration of 4 micrograms/ml ($\mu\text{g/ml}$). In contrast, Emodin at a concentration of 100 $\mu\text{g/ml}$ had little effect on the growth of normal human bronchial epithelial cells. Cell cycle analyses indicated that treatment with Emodin arrested the v-ras-transformed cells in the G2/M phase of their cell cycle [Chan, 1993].

Shieh et al. investigated the cytotoxic effect of Emodin on various human hepatoma cell lines.

Results demonstrated that Emodin exhibited strongly suppressing effects on HepG2/C3A,

PLC/PRF/5, and SK-HEP-1 cells, with the IC₅₀ value of 42.5, 46.6, and 53.1 μ M, respectively. Furthermore, Emodin induced apoptosis in HepG2/C3A cells was clearly verified by the appearance of DNA fragmentation and sub-G(1) accumulation [Shieh, 2004]. HepG2/C3A cells were found to be arrested in G2/M phase after the cells were treated with 60 μ M Emodin for 48 h. Furthermore, significant increase in the levels of apoptosis-related signals such as p53 (419.3 pg/ml), p21 (437.4 units/ml), Fas (6.6 units/ml), and caspase-3 (35.4 pmol/min) were observed in Emodin treated HepG2/C3A cells. Authors concluded that Emodin displays strong inhibitory effects on the growth of various human hepatoma cell lines and stimulates the expression of p53 and p21 that resulted in the cell cycle arrest of HepG2/C3A cells at G(2)/M phase. The prominent finding shows that Emodin could be a useful chemotherapeutical agent for the treatment of hepatocellular carcinoma (HCC). Emodin was shown to induce apoptotic responses in the human hepatocellular carcinoma cell lines (HCC) Mahlavu, PLC/PRF/5 and HepG2 [Jing, 2002]. The addition of Emodin to these three cell lines led to inhibition of growth in a time and dose-dependent manner. Moreover, Emodin generated reactive oxygen species (ROS) in these cells. This led to a reduction of the intracellular mitochondrial trans-membrane potential (Δ Psi_m), followed by the activation of caspase-9 and caspase-3, leading to DNA fragmentation and apoptosis. The authors concluded that enhancement of generation of ROS, Δ Psi_m disruption and caspase activation may be involved in the apoptotic pathway induced by Emodin.

In our experiments we observed signs of apoptosis in differential blood analysis. It has been shown that Emodin possesses anti-inflammatory effects with suppression of lymphocyte proliferation and cytokine production [Liu, 2009]. The immunosuppressive effect of Emodin and BTB14431, respectively, may account for the lack of tumor growth suppression in vivo, especially at high doses. Furthermore, some of the results may be explained by a direct cytotoxic effect of Emodin. Consequently local tumor treatment might be more effective by avoiding systemic toxicity and immune-suppression. This has to be evaluated in further

studies especially in light of Emodin-enhanced sensitivity of cancer cells to chemotherapeutic drugs.

In summary, the actual literature and our preliminary in vitro results: significant antineoplastic effects were only described in vitro, but complex tumor biology and mechanism as well as dose dependent toxicity did not lead to discover the adequate dose or the sufficient method to verify the data in animal models. Growth of metastasis include specific procedures such as adhesion, cell migration and invasion [Woodhouse, Chuaqui & Liotta, 1997]. Newer studies show that Emodin might have an inhibitory role in these events and might interact with Matrix-Metalloproteinases (MMP). For human colorectal cancer, an overexpression of MMP-2 was described [Grigioni, 1984].

As most previous studies applied Emodin ip or orally, we cannot directly compare assessment of our iv dose in comparison to previous studies. Serum drug level and dose is a well-established problem when assessing a new antineoplastic agent. Thus, the limited scope of therapeutic drug level monitoring in cancer chemotherapy results from the complex biochemical mechanisms that contribute to antineoplastic activity and obscure the relationships among drug serum levels and therapeutic benefits. Moreover, new agents for cancer chemotherapy are being introduced at a more rapid rate than for the treatment of other diseases. The successful application of therapeutic drug level monitoring may require several years of intensive study of the significance of serum drug levels. However, this monitoring can be of considerable value during phase I clinical trials of new antineoplastic agents in order to assess drug metabolism, bioavailability, and inter-subject variability; these are important parameters in the interpretation of clinical studies, but have no immediate benefit to the patient.

Conclusion

Although representative apoptotic effects were observed in pathohistochemical staining of

blood cells, no significant tumor suppressing effects could be observed for iv and ip treatment of both agents in our model. Emodin is difficult to dissolve completely and to apply in relevant dose for iv injection in animals. However, agents tested remain interesting new substances that will be subject for further studies in different doses and in modified animal models in our clinic. In Silico Screening might be helpful for identifying potential antitumor drugs – but failed in this in vivo model.

Acknowledgements

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Tables and figures

Table 1

Group	Intravenous treatment (IVT)	Group	Intraperitoneal treatment (IPT)
I	Isotone sodium chloride iv (control group)	VII	Isotone sodium chloride ip (control group)
II	BTB14431 iv 0.3 mg/kg	VIII	BTB14431 ip 0.3 mg/kg
III	BTB14431 iv 1.7 mg/kg	IX	BTB14431 ip 1.7 mg/kg
IV	BTB14431 iv 3.3 mg/kg	X	BTB14431 ip 3.3 mg/kg
V	Emodin iv 5 mg/kg	XI	Emodin ip 5 mg/kg
VI	Emodin iv 10 mg/kg	XII	Emodin ip 10 mg/kg

Doses are given in mg per kg of the body weight (bw)

Table 2

Group	D1	D2	D3	D4	D5	D6	D7	Removal of port catheter	Total
VII						1		1	2
VIII						1			1
IX									
X									
XI	1			1	1		2		5
XII	1	1		1	1		2		6

D1 to D7 – treatment at day 1 to 7, removal of port catheter system was performed at day 7; Reasons for death: Control group: port catheter related sepsis at day 6; bleeding after port removal; Group VII: during injection - cardiac and circulation arrest (CCA); Group XI: during injection – CCA; Group XII: during injection – CCA

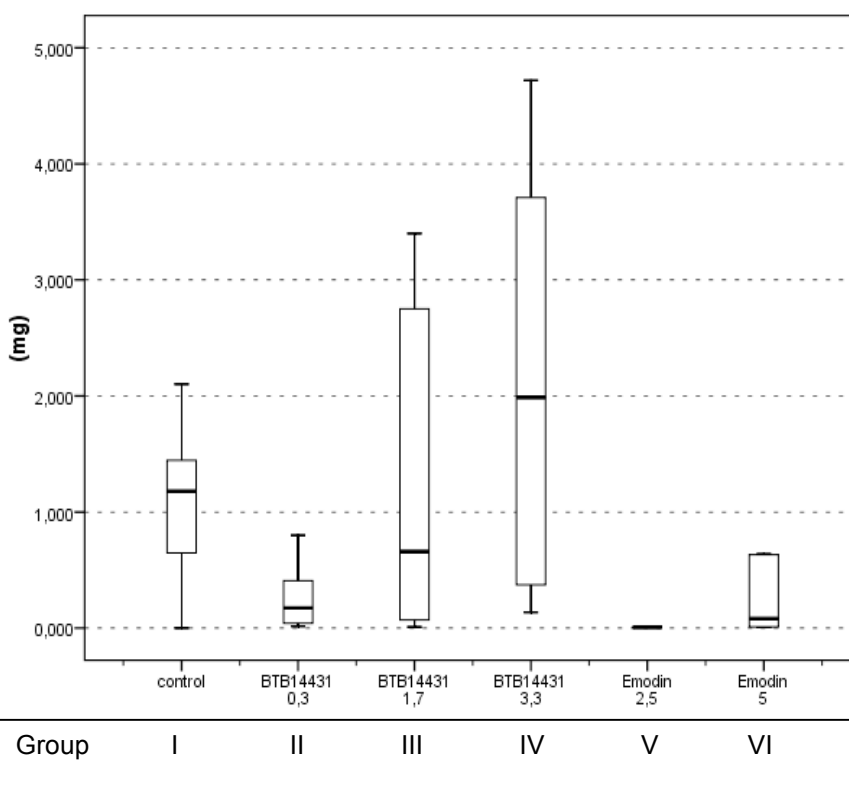
Table 3

Group	Intravenous treatment				Group	Intraperitoneal treatment			
	ip-TM-weight	sc-TM-weight	bw day 1	bw day 28		ip-TM-weight	sc-TM-weight	bw day 1	bw day 28
I	.80 (0-3.0)	.13 (0-.53)	242 (230-299)	271 (257-302)	VII	.02 (<.01-1.2)	≤.01 (0-.3)	242 (206-262)	270 (246-281)
II	.07 (<.01-0.8)	≤.01 (0-.4)	257 (228-356)	281 (250-350)	VIII	.01 (0-1.6)	≤.01 (0-0.19)	235 (219-266)	261 (245-294)
III	.59 (.01-3.3)	.01 (0-.4)	240 (223-374)	275 (249-364)	IX	.01 (0-1.6)	.02 (0-.16)	232 (223-272)	261 (250-298)
IV	1.85 (.13-4.3)	.1 (<.01-.42)	242 (219-262)	264 (244-284)	X	.01(0-.22)	≤.01 (0-.19)	239 (209-258)	270 (240-290)
V	.08 (<.01-3.5)	.01 (0-0.28)	241 (224-256)	266 (253-276)	XI	.03 (0-.51)	≤.01 (0-.02)	238 (206-251)	259 (235-272)
VI	-----	-----	-----	-----	XII	.05 (<.01-.69)	≤.01 (0-.44)	238 (216-256)	257 (238-280)

For assignment of the groups see table 1. ip-TM-weight: intraperitoneal tumor weight, sc-TM-weight: subcutaneous tumor weight; tumor load is given in gram (g) – median and range; bw: body weight; day 1: beginning of treatment; day 28: weight at autopsy; group V: only one animal evaluated, group VI all animals died

1 Figure 1a Tumor weight after intravenous treatment

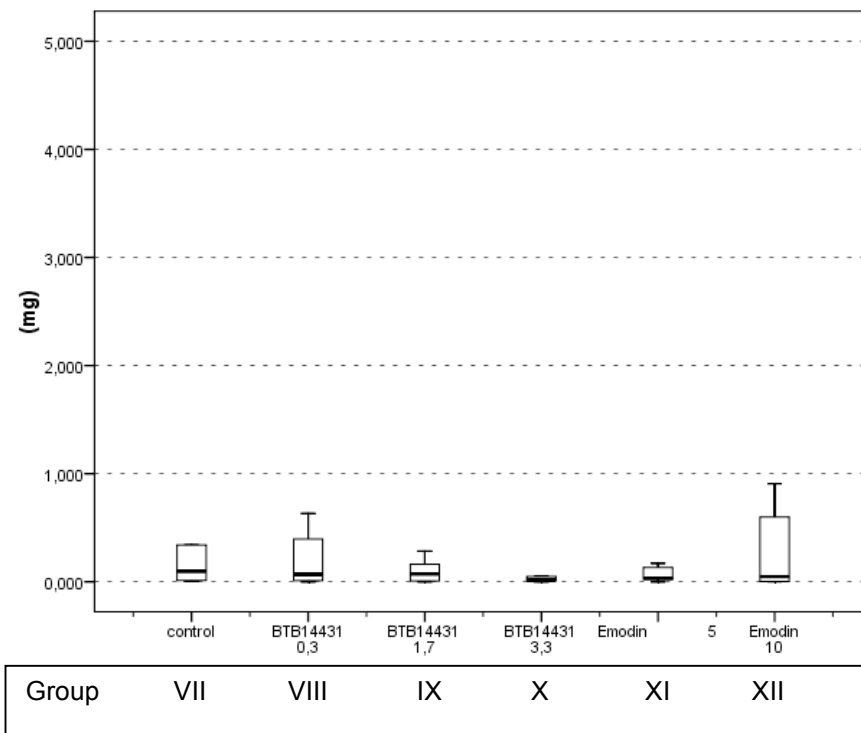
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Complete tumor weight (in mg) after intravenous treatment (given in mg/kg bw) with BTB14431 and Emodin compared to the control group (isotone sodium chloride solution)

1 Figure 1b Tumor weight after intraperitoneal treatment

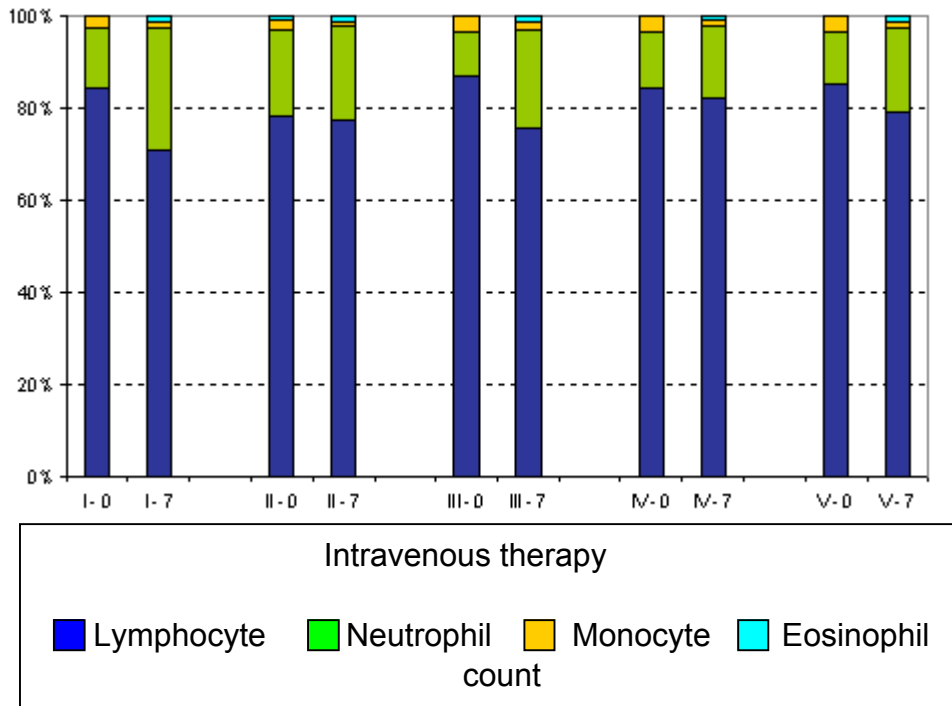
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Entire tumor weight (in mg) following intraperitoneal treatment with BTB14431 and Emodin (given in mg/kg bw) compared to the control group (isotone sodium chloride solution)

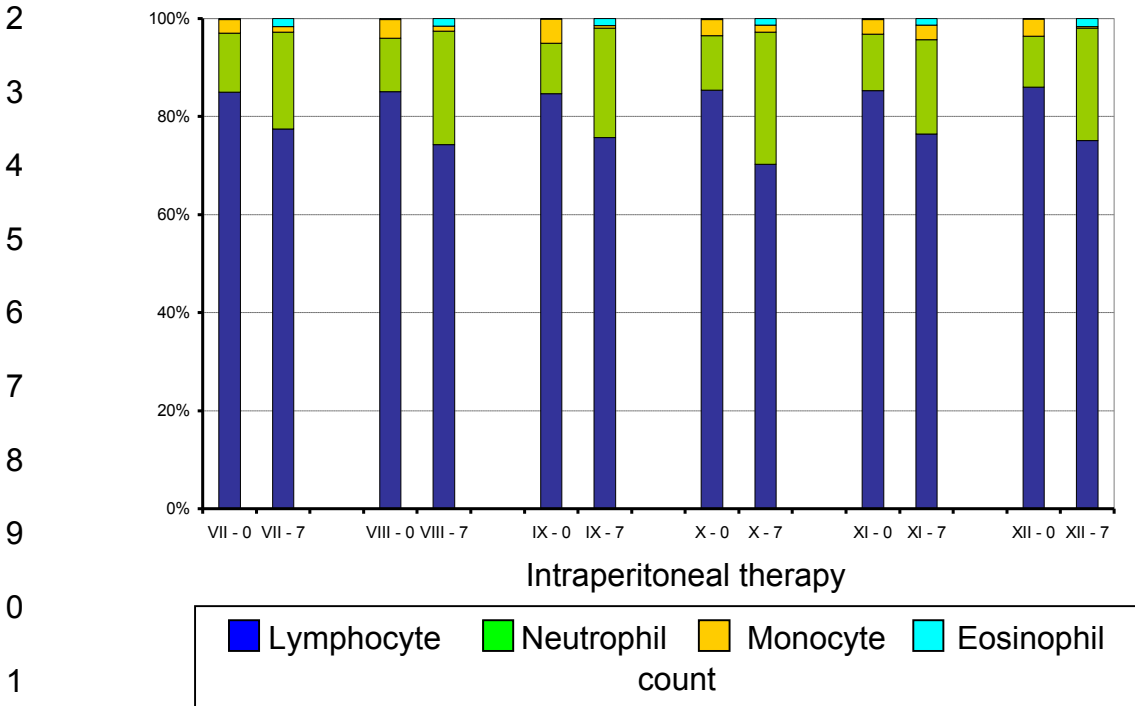
1 Figure 2a

2



For identification of the groups see table 1 (Roman numerals, e.g. group one left two bars). Figure shows relative rate of leukocyte count (mean) in percent of 100%: pre op (0, left bar) and after last treatment (e.g. second bar). Neutrophil count increases whereas monocyte and lymphocyte count decreases during treatment. Eosinophil count increases in a lower rate.

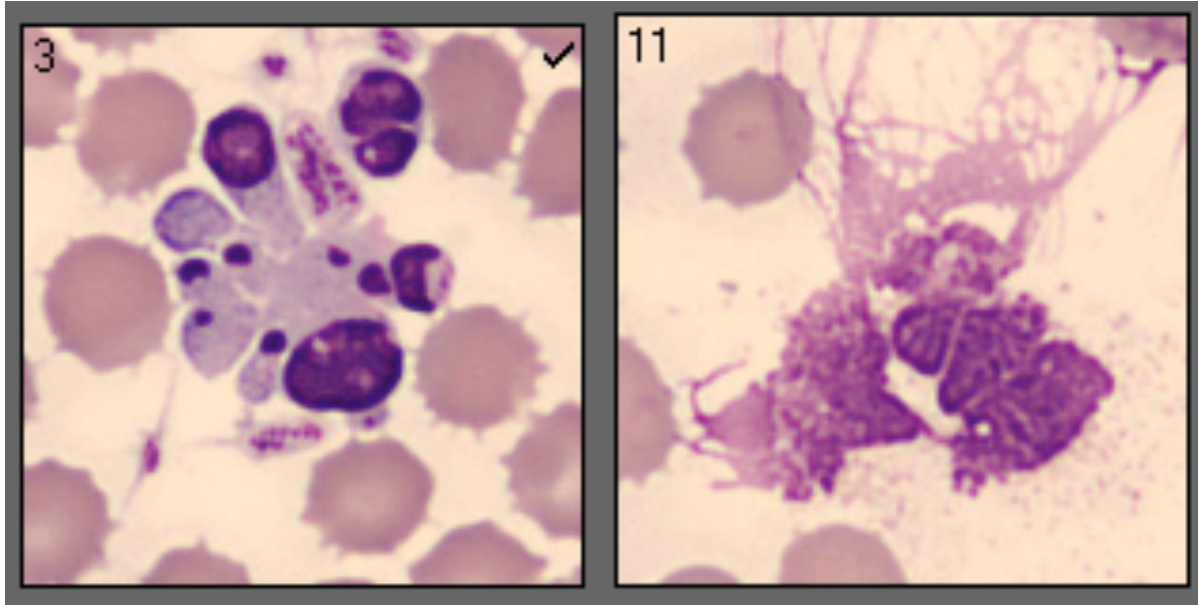
1 Figure 2b



12 Bar chart illustrates mean of leukocyte count in percent (compared to 100%).

13 Neutrophil count increases while reduction of monocyte and lymphocyte count was
14 observed at the end of treatment. Eosinophil count increases.

1 Figure 3A



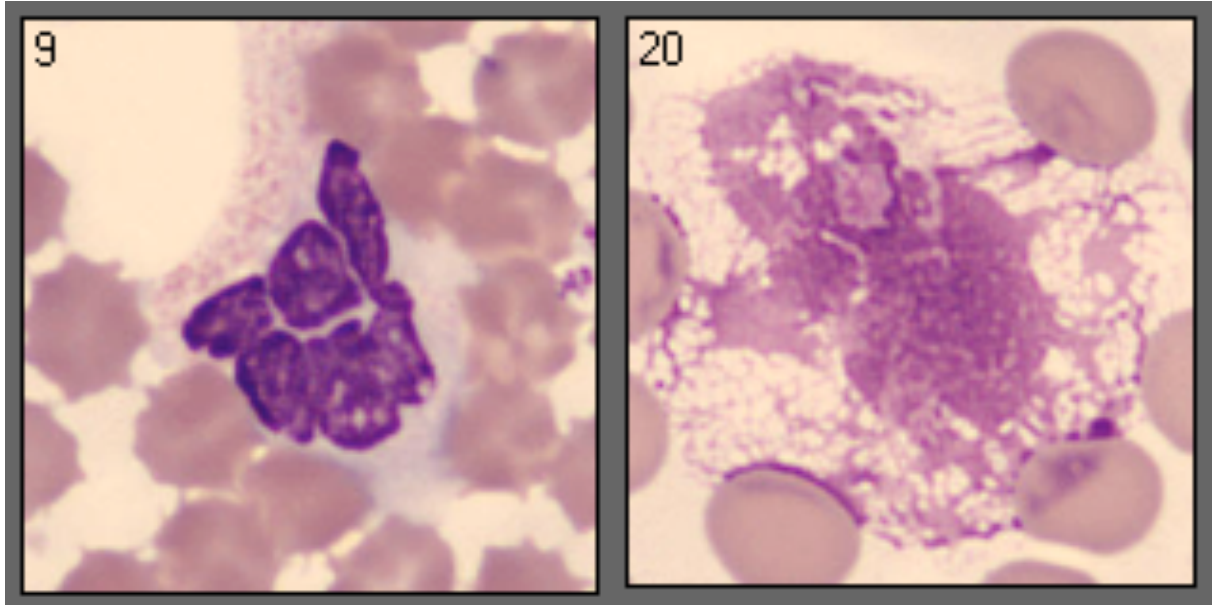
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3

4 Intravenous application of Emodin is shown to induce apoptosis in differential blood
5 sample (e.g. in pictogram 3 'nuclear fragmentation' and in pictogram 11 'blebbing' are
6 seen; Magnitude x 10 for both)

7

1 Figure 3B



2

3 Intravenous application of BTB14431 caused apoptosis (effects seen are comparable

4 to Emodin – see fig. 3A; e.g. in pictogram no. 9 ‘nuclear fragmentation’ and in

5 pictogram no. 20 ‘blebbing’ are seen)

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