Matrix metalloproteinase inhibitor Ro 28-2653 in combination with estramustine: tumor-reducing effects on hormone-sensitive prostate cancer in rats

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Therapeutic efficacy of the novel matrix metalloproteinase (MMP) inhibitor, Ro 28-2653 (5-biphenyl-4-yl-5-[4-(-nitro-phenyl)-piperazin-1-yl]-pyrimidine-2,4,6-trione), has been shown in various models of different tumor entities. The tumor growth-reducing effect has been demonstrated in the orthotopic rat prostate Dunning model (subline MatLyLu). Based on these results we investigated Ro 28-2653 in combination with estramustine on the G subline of the Dunning tumor. This subline is characterized by a low metastatic ability and androgen sensitivity. Efficacy was determined by recording tumor growth *in vivo* by magnetic resonance imaging (MRI). Tumor cells were injected into the prostates of 81 Copenhagen rats. MRI was performed at day 100 and at day 126 after tumor cell injection. The duration of therapy was 17 days with daily oral application of Ro 28-2653 (100 mg/kg) and four i.p. injections of estramustine (7.5 mg/kg). Histological evaluations were conducted to provide further information about the effects on tumor morphology. Orthotopic tumor induction was successful in 100% of the animals. Tumor volume calculations with MRI showed a significant difference between the control groups, the animals treated with Ro 28-2653, and the animals treated with the combination of Ro 28-2653 and estramustine. The new MMP inhibitor Ro 28-2653 reduces tumor growth and provides a compatible therapeutic alternative for patients with prostate cancer. *Anti-Cancer Drugs* 16:855–861 © 2005 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2005, **16**:855–861

Keywords: Dunning tumor, magnetic resonance imaging, matrix metalloproteinase inhibitor, prostate cancer, rat

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Sponsorship: Ro 28-2653 was provided by Roche Diagnostics GmbH, Pharma Research (Penzberg, Germany). This study was supported by Roche Diagnostics GmbH.

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Received 26 April 2005 **Accepted** 4 June 2005

Introduction

Prostate cancer (PCa) is the most frequent cancer in men and the second leading cause of cancer death in the USA. In 2005, approximately 232 090 new cases of PCa will be diagnosed and 30 350 men will die of the disease [1].

For PCa patients with localized disease, the standard treatment procedure is radical prostatectomy or radiation therapy [2,3]. The standard therapy for advanced PCa is anti-androgen hormone therapy. After a limited effective time of anti-androgen therapy [3], the cancer will grow hormone independently [4]. In this phase of hormone-refractory PCa, there is no curative therapy, and the main objective of treatment must be to palliate symptoms and improve or stabilize quality of life.

Although those standard therapies are possible for the majority of the patients suffering from advanced PCa, little progress has been made in recent years toward improving survival [5]. Thus, it is of an enormous interest to develop new therapeutic strategies for the treatment of advanced PCa.

The evaluation of an acceptable animal model is necessary to improve new modalities of treatment. In the case of PCa, the Dunning R-3327 rat prostate adenocarcinoma is one such experimental tumor model of its human counterpart used to study tumor progression [6]. The sublines (H, G, HI-F, AT, MatLu and MatLyLu) differ in growth rate, differentiation, hormone responsiveness and metastatic ability, and reflect the variability of human PCa [7].

The tumor-reducing effect and a prolonged survival due to the synthetic matrix metalloproteinase (MMP) inhibitor Ro 28-2653 (5-biphenyl-4-yl-5-[4-(-nitro-phenyl)-piperazin-1-yl]-pyrimidine-2,4,6-trione) has been shown in the hormone-insensitive MatLyLu subline [8]. This compound has a high selectivity for MMP2, MMP9 and membrane type 1 MMP, and has previously demonstrated its inhibitory activity against MMPs expressed by tumor

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and/or stromal cells as a potent anti-tumor and anti-angiogenic agent [8–11]. MMPs, endogenous proteases responsible for the maintenance of homeostasis in the extracellular matrix, are capable of degrading components of the extracellular matrix like collagen, fibronectin and elastin [12]. MMPs are thought to mediate cancer progression through a variety of mechanisms. These include limiting apoptosis, promoting angiogenesis and effecting local tissue invasion by degradation of type IV basement membrane collagen. These mechanisms taken together promote metastatic tumor formation [13,14]. On the contrary, the G subline is a slow-growing, hormone-sensitive PCa. The aim of this study was to evaluate the potential positive treatment effect with Ro 28-2653 alone and in combination with the standard chemotherapy drug estramustine on the growth on this hormone-sensitive subline.

Materials and methods

Cell culture

At the day of injection, tumor cells (Dunning tumor, subline G; European Collection of Cell Cultures, Salisbury, UK) were removed from the tissue culture flasks with trypsin/EDTA. After trypsination of the cells, 4 ml RPMI 1640 was added. The tumor cells were centrifuged, washed twice in PBS, counted and resuspended in RPMI 1640 medium without supplements at a final concentration of 1×10^7 viable tumor cells/ml. The viability of the cells was controlled by Trypan blue staining and counted in a Neubauer chamber.

Animal model and therapy

The care and treatment of the animals was in accordance with German requirements, and all experiments performed were approved by the responsible local authority (Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit, Berlin, Germany). Male Copenhagen rats ($n = 81$) with a starting body weight of 200–220 g (Harlan Winkelmann, Borchen, Germany) were used for orthotopic cell injection using a suprapubic transverse incision. Animals were anesthetized with an intramuscular injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Tumor cells (1×10^6 in 100 µl) were injected into the ventral lobe of the prostate gland.

Animals were fed and watered *ad libitum* with daily monitoring of body weights. The total time of the operation procedure was approximately 10 min. The therapy was started at day 101 after the cell injection. At day 101 all animals had a palpable prostate tumor. The groups were treated as follows:

- Group 1: 0.2% Na-CMC (sodium carboxymethylcellulose), vehicle for Ro 28-2653
- Group 2: 0.9% saline solution, vehicle for estramustine
- Group 3: 0.2% Na-CMC + 0.9% saline solution
- Group 4: Ro 28-2653 (100 mg/kg)
- Group 5: estramustine (7.5 mg/kg)
- Group 6: Ro 28-2653 (100 mg/kg) + estramustine (7.5 mg/kg)

Groups 1–3 were combined to one vehicle (control) group.

The duration of therapy was 17 days, and over that period the animals received the MMP inhibitor Ro 28-2653 or its vehicle daily by oral application with a gastric catheter and four i.p. injections of estramustine or its vehicle in equal intervals. The total doses were Ro 28-2653 1.7 g/kg/animal, estramustine 30 mg/kg/animal and the analogous amount in the combination group. The dose and duration of the therapy was taken from Lein *et al.* [8] and previously unpublished studies. After the second magnetic resonance imaging (MRI) investigation the animals were sacrificed 126 days after tumor cell injection by an i.v. injection of T 61 under deep anesthesia.

Drugs

The synthetic MMP inhibitor Ro 28-2653 was provided by Roche Diagnostics (Penzberg, Germany). The cytostatic drug estramustine was prepared in ready-for-use vials by the pharmacy of the University Hospital Charité.

MRI

MRI was performed of each animal at day 100 and 126 after cell injection to assess individual tumor volume before and at the end of therapy. For MRI, the rats were anesthetized with 0.13 ml of the narcotic solution/100 g body weight (1 U 20 mg/ml xylazine and 4 U 100 mg/ml ketamine) in the leg muscle. MRI was performed at 1.5 T (Magnetom Vision; Siemens, Erlangen, Germany) using an extremity coil in which the animals were placed in couples in a horizontal dorsal position. All images were acquired with a T_1-weighted high solution spin echo sequence with the following parameters: TR/TE 400/20 ms, axial orientation, field of view 180 mm, matrix 256 × 512, slab thickness 2 mm, total acquisition time was dependent of the tumor volume and thus needed slices. Images were acquired before and after administration of a conventional, low-molecular-weight gadolinium-containing contrast medium (Magnevist; Schering, Berlin, Germany), which is a T_1-shortening contrast agent, at a dosage of 30 µmol/kg. The contrast agent was injected via an indwelling cannula (24 gage, 0.75 inch, 0.7 × 19 mm; Neoflon; Becton Dickinson, Helsingborg, Sweden) placed in a lateral tail vein of the animal. The application of contrast media was necessary to distinguish the tumor from adjacent tissues.

Analysis of tumor volume

MRI

The tumor volumes were determined from the cross-sectional view MRI slices inside the tumor and calculated

by the NHI Image 1.6 program. To calculate the volume of one MRI slice, first the region of interest, i.e. the outline of the tumor dimension, was determined and then all pixels automatically calculated by the program. This amount of tumor pixels was multiplied by the extent of the pixels designed by the MRI sequence. Then this two-dimensional area was multiplied by the slice distance factor and all slice volumes were added to produce the total tumor volume.

Macroscopic tumor calculation

After harvesting from an animal the tumor was resected and a macroscopic calculation of the tumor volume was conducted with the formula $V = (\text{length} \times \text{height} \times \text{breadth})\ \pi/6$. This is a generally accepted standard method; however, in reality, tumor dimension can vary beyond the assumed ellipsoidal form.

Histological analysis

Immediately after the second MRI session all animals were harvested, and 10 of the tumors of the group treated with Ro 28-2653 were resected and fixed in 4% formaldehyde solution. They were compared with 14 control animals from the vehicle groups. The tissue samples were processed routinely into paraffin and 3-µm sections were stained with hematoxylin & eosin for histological evaluation. The examination parameters were cell and nuclear polymorphism, rate of proliferation (mitosis per field), necrosis total area, apoptosis (per field of view), invasive growth, capsule formation, intratumoral fibrosis (collagen content), inflammatory cell infiltration, and content of intratumoral blood vessels.

Statistical analysis

Statistical evaluations were performed using the statistical program GraphPad Prism (GraphPad, San Diego, California, USA). The Kruskall–Wallis test and Dunn's multiple comparison test were used to compare more than two independent random samples. The Wilcoxon matched-pairs test was used to compare two dependent random samples. Differences of $p < 0.05$ were considered statistically significant. The Spearman correlation was used for non-parametric data.

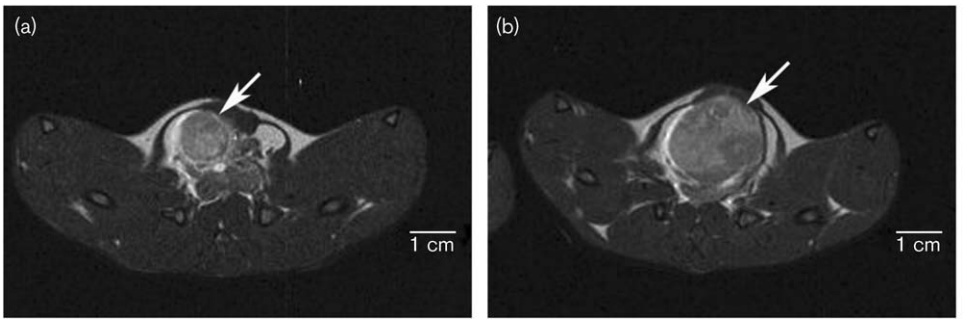
Results

The surgical procedure and tumor cell injection were well tolerated. In all rats, orthotopic tumors were successfully implanted by tumor cell injection. From the initial 81 rats in the first MRI session, eight rats died prior to the second MRI after 26 days due the large extent of the tumor volume. Therefore, 73 rats were examined at the second MRI. The total doses were as described in Materials and methods.

Tumor volumes

The first MRI was performed on day 100 after G tumor cell injection, when a local prostate tumor was palpable in all animals (Fig. 1a). After a therapy period of 17 days, tumor volumes were acquired with a second MRI on day 126 (Fig. 1b). Mean tumor volumes and SDs of all treated and untreated animals, as investigated by MRI, are given in Table 1. Since the three vehicle groups showed no significant differences they were combined into one single vehicle group and considered the control. The increase of tumor volume from the first to the second MRI is significant in all groups (Wilcoxon matched-pairs test, $p < 0.01$). Figure 2 shows the results as an individual tumor growth factor by calculating the ratio of tumor volume at the second MRI at day 126 to the first MRI at day 100 (volume 2/volume 1). The animals in Group 4 treated with Ro 28-2653 showed a significantly lower tumor growth than the animals in the combined vehicle group (Kruskall–Wallis, Dunn's post-test: $p < 0.001$). There is also a significantly lower tumor growth in Group 6 (Ro 28-2653 in combination with estramustine) versus the vehicle group (Kruskall–Wallis, Dunn's post-test: $p < 0.001$). There was no significant difference between

Fig. 1



MRI: *in vivo* axial section view of the prostate tumor (arrow). The first tumor volume was calculated at day 100 after G cell injection into the prostate (a). After 17 days of treatment a second MRI was performed at day 126 to identify the individual tumor growth of each animal (b, same animal).

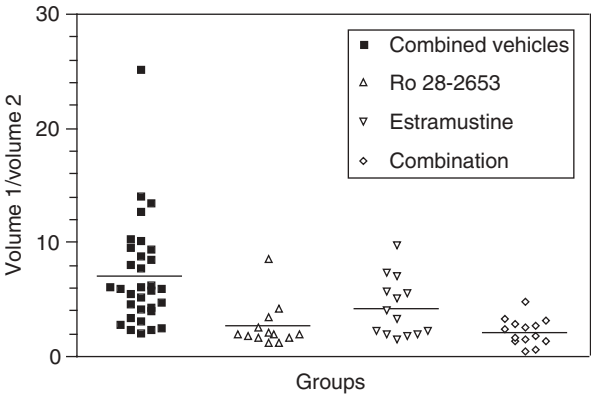
Group 4 (Ro 28-2653) and Group 6 (Ro 28-2653 in combination with estramustine). Group 5 (estramustine) showed no significant differences to the other groups.

The tumor volumes calculated at the second MRI were correlated to the macroscopic tumor volume calculated after tumor resection followed by the sacrifice of the animals (see Materials and methods) ($r_S = 0.46$, $p < 0.01$) and to the tumor weight ($r_S = 0.48$, $p < 0.01$ for macroscopic tumor volumes and tumor weights, and $r_S = 0.41$, $p < 0.01$ for MRI tumor volumes and tumor weights).

Histopathological evaluations

Due to the inhibitory effect of the MMP inhibitor Ro 28-2653 on the tumor growth, additional histological examinations were conducted to access further information about the morphologic structure of the tumor. First, three animals from each vehicle group were examined without any differences, so that they also were combined in one vehicle (control) group. Then a total of 14 control animals were investigated compared with 10 animals treated with Ro 28-2653.

Fig. 2



Scatter plot of the ratios of tumor volume 2 (MRI at day 126) to tumor volume 1 (MRI at day 100). It shows the growth of individual animal tumors. The animals treated with Ro 28-2653 alone and the animals treated with the combination of Ro 28-2653 with estramustine showed a significantly lower tumor growth than the animals in the combined vehicle group (Kruskall–Wallis $p < 0.0001$, Dunn's post-test is indicated as: $^{a}p < 0.001$ and $^{b}p < 0.001$). There were no significant differences between the other groups.

Vehicle groups (controls)

The tumors of the vehicle group (control) (Fig. 3a) represented moderate to less differentiated malignant tumors of the Dunning tumor cell line (G line) after orthotopic growth in the prostate. Most of the examined tumors showed a lobular structure with parts of solid adenocarcinoma as well as 'fibrosarcoma-like' structures. The tumors revealed a slight cellular and nuclear polymorphism. The capsule formation around the tumors and the intratumoral collagen content was slight. The tumors showed a moderate to severe content of intratumoral blood vessels. Inflammatory cell infiltration was seen in moderate to severe amounts. The tumors showed a locally invasive growth characteristic; however, invasion of blood and lymph vessels was rare. The percent of total intratumoral necrosis was low (5–20%). The rate of proliferation was low (two to four mitosis per field of view by × 200 magnification). The rate of apoptosis was moderate (four to six apoptotic cells per field of view by × 200 magnification).

Treated animals (Ro 28-2653)

Ten animals treated with Ro 28-2653 (Fig. 3b) were examined and seven of them had tumors that showed a clear increase in intratumoral necrosis compared with the control group. Three animals revealed slight differences regarding the histological tumor parameters. There was a slight increase in intratumoral collagen content and capsule formation. There was a distinctly lower content of intratumoral blood vessels observed in these animals. There were no differences with regard to the other examined histological tumor parameters.

Discussion

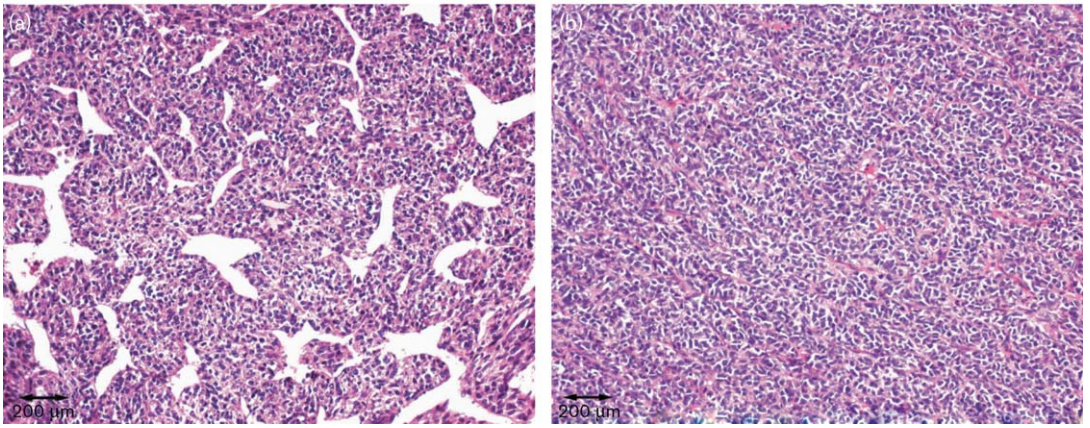
In this trial we evaluated the effect of the synthetic MMP inhibitor Ro 28-2653 alone and in combination with estramustine on the tumor growth of the G subline in the

Table 1 **Tumor volumes (cm^3) of all groups at the two MRI sessions**

Group	17 days of treatment	Tumor volume at day 100			Tumor volume at day 126		
		n	Mean	SD	*n*	Mean	SD
1	0.2% Na-CMC	11	1.81	2.76	11	8.13	5.35
2	0.9% saline solution	11	1.60	0.98	11	11.00	9.85
3	Na-CMC + saline solution	11	1.71	1.50	10	12.39	11.89
4	Ro 28-2653	14	2.05	1.82	13	5.39	6.82
5	Estramustine	15	2.32	2.70	14	8.34	10.61
6	Ro 28-2653 + estramustine	19	1.64	1.74	14	3.93	3.70

Data represent the tumor volumes before and after the 17 days of treatment. Groups 1–3 are control groups with the drug vehicles, Group 4 received Ro 28-2653 daily at 100 mg/kg. Group 5 received estramustine 4 times at 7.5 mg/kg. Group 6 was treated with the combination of Ro 28-2653 and estramustine.

Fig. 3



Histopathological findings (hematoxylin & eosin) of orthotopic tumors with × 200 magnification of a vehicle animal (a) and a rat treated with 100 mg/kg/day Ro 28-2653 (b). In vehicle animals, the tumors showed a moderate to severe content of intratumoral blood vessels and the percentage of total intratumoral necrosis was low. Some of the treated animals showed a clear increase in intratumoral necrosis and a distinctly lower content of intratumoral blood vessels.

orthotopic Dunning prostate tumor model of the rat. The aim of the study was to investigate the specific MMP inhibitor as a possible novel anti-cancer therapy.

MMPs play a key role in tumor invasion and the formation of tumor metastasis by degrading the extracellular matrix [15–19]. Ro 28-2653 is a member of a new generation of specific MMP inhibitors with high biocompatibility and inhibitory activity against MMPs expressed by tumor and/or stromal cells [11]. These anti-tumor and anti-angiogenic properties were confirmed in an earlier study on the tumor growth of the Dunning MatLyLu tumor in the same animal model [8,20]. This is an androgen-independent, highly metastatic and anaplastic tumor. The results of the MatLyLu model correspond to other cancer studies, but the effectiveness in this specific aggressive tumor was remarkable. In contrast, the Dunning G subline is an androgen-sensitive cell line with low metastatic ability and longer growth rate [21]. Therefore, confirmation in the G subline was of interest and an assumption for further clinical studies.

To our knowledge this is the first study of this new MMP inhibitor on the G subline of the Dunning model that is characterized by its similarities to the androgen-dependent state in the PCa growth. The G cells generated a slowly and homogeneously growing orthotopic tumor with high interindividual differences in the first detection of the tumor. To avoid this huge range of tumor volumes, the starting point of treatment ought to be fixed depending on a defined tumor volume in each animal. We started the treatment when all animals had a palpable tumor detectable, limited by the time schedule and complexity of MRI investigations. Due to the two MRI investigations, it was possible to obtain the individual progression of every tumor volume. Despite the correlation between the macroscopic and the tumor volume achieved by MRI, the latter method is a more accurate strategy due to detailed imaging with high-resolution sequences. Additionally, it allows us to pursue the individual development of the tumor of every single animal *in vivo*.

Our results show that the daily oral application of the MMP-inhibitor Ro 28-2653 is able to reduce the tumor growth of the G cell Dunning PCa of the Copenhagen rat in a significant manner in comparison to the control groups. In clinical studies, a particular therapeutic effect of estramustine has been previously demonstrated [22,23]. Therefore, we chose estramustine to evaluate a possible further effect of Ro 28-2653. However, there was no additional or synergistic effect on the reduction of tumor growth between the combined therapy group (Ro 28-2653 and estramustine) and the group treated only with Ro 28-2653. Thus, the reducing effect of tumor growth is exclusively generated by the MMP inhibitor. Eight animals died prior the second MRI investigation. In the combined therapy group, the highest loss of five animals was observed. Biocompatibility is the limiting factor of this therapy pattern, notably due to estramustine. Toxicity and side-effects are known for the application of estramustine [23]. In contrast, the daily applied Ro 28-2653 was well tolerated.

The anti-angiogenic effect of MMP inhibitors, in general, and the novel synthetic Ro 28-2653 with high selectivity for MMP2 and MMP9, in particular, has been demonstrated in several studies [11,24–26]. This property of

this new MMP inhibitor could be one explanation for the reduction of tumor growth. This is also reflected in the results of the histological investigations. Ten animals treated with Ro 28-2653 were evaluated to determine whether possible anti-angiogenic effectiveness is reflected in an objective morphological reduced level of vessels in the PCa. A higher total area as a consequence of reduced tumor vessels in these animals, compared with the control animals, could support this hypothesis. This descriptive analysis revealed that some different pathological pathways must occur during the growing process of the tumor. Although there was no statistically significant lower content in intratumoral blood vessels, there is an anti-proliferative 'hint' in the increase in intratumoral necrosis that could lead to a reduced tumor volume.

This insight about the characteristics and role in physiological and pathological processes of MMPs led to the conclusion that the development of synthetic MMP inhibitors could be a promising anti-cancer therapy. Despite the promising ability of broad-spectrum MMP inhibitors, such as batimastat or marimastat, to delay primary tumor growth and to block metastasis [27,28], clinical trials unfortunately revealed disappointing results [29–31]. Recent investigations revealed that that certain MMPs can have dual effects on cancer development [32] and broad-spectrum MMP inhibitors may affect the natural host defense mechanism against tumors [33]. In addition, it has been shown that there exists a diversity of substrates targeted by MMPs, e.g. growth factor receptors, cell adhesion molecules, chemokines, cytokines, apoptotic ligands and angiogenic factors [34]. Thus, the direct degradative action on extracellular matrix components is not the only basic mechanism in cancer progression [13,35,36]. MMPs seem to play essential roles in the early stages of cancer [34]. In a recent study, Ro 28-2653 demonstrated its potent anti-invasive, antitumoral and anti-angiogenic properties, in contrast to the use of broad-spectrum MMP inhibitors, which showed no benefit [11]. In our study, the tumor-reducing effect and the biocompatibility of Ro 28-2653 could be confirmed in the G cell PCa tumor model. Since the therapy was started at the point of established tumors, it is possible that this treatment employed in earlier stages, analogous to localized disease, could yield better results. Beyond this, continuous application of the MMP inhibitor during the whole period of study could also provide better effects. In summary, our study confirms the ability of MMP inhibitor Ro 28-2653 to have a tumor growth-reducing effect in orthotopic PCa in Copenhagen rats. These results should encourage further studies and the application in humans.

Acknowledgements

The authors thank Sabine Becker, Silke Klotzek and Ines Baumert for valuable technical assistance.

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