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ORIGINAL ARTICLE

Enhanced inhibitory effect of the matrix metalloproteinase inhibitor Ro 28-2653 in combination with estramustine and etoposide on the prostate carcinoma in the rat Dunning orthotopic tumor model

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Abstract *Purpose*: Therapeutic efficacy of the novel matrix metalloproteinase (MMP) inhibitor Ro 28-2653 has been shown in various models of different tumor entities. We hypothesized that the inhibitor effect of Ro 28-2653 on the tumor growth could be improved by combination with chemotherapeutic drugs and examined therefore the effect of Ro 28-2653 alone and in combination with etoposide or estramustine in the MatLyLu Dunning R-3327 rat tumor model characteristic for the androgen-independent prostate cancer (PCa). *Methods*: In vitro effects were estimated measuring the proliferation of MatLyLu cells incubated with the three agents alone or in combination using the XTT test. The in vivo effects of the agents alone or in combination were examined by measuring the tumor weight 18 days after tumor cell injection. *Results*: The proliferation rate was only inhibited by etoposide while that effect was increased in combination with Ro 28-2653 and estramustine. Ro 28-2653 reduced the tumor weight by 86%. That effect was significantly increased in combination with etoposide to 92%. *Conclusions*: The inhibitory effect of the MMP inhibitor Ro 28-2653 on the tumor growth in the Dunning PCa model is enhanced by the standard chemotherapeutic drug etoposide. A combined application of both agents could be considered as potential tool to improve the therapy of patients with advanced PCa after failure of hormonal treatment.

Keywords Prostate cancer · Matrix metalloproteinase inhibitors · Combined chemotherapy · MatLyLu cells · In vitro and in vivo inhibitor effect

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Introduction

The antiandrogen hormone therapy is the standard therapy for advanced prostate cancer (PCa) but is effective for only a limited period of time. Followed by a progressive hormone-refractory phase the cancer will grow hormone-independent with no currently available curative therapy. The survival of these patients in general does not exceed 1–2 years [31]. Therefore, new therapeutic strategies for the treatment of the hormone-refractory PCa are urgently required.

In men suffering from hormone-refractory PCa, estramustine and etoposide are standard chemotherapeutic drugs. Estramustine phosphate, an oral microtubule active agent, is a nitrogen mustard derivative of estradiol-17-beta-phosphate. The combination of the hormonal effect of estrogen and the cytotoxic action causes the mechanism of action in PCa [7]. Because of its significant gastrointestinal toxicity used as a single agent, many efforts have been made to decrease the side effects by joining the efficacy on PCa with other agents [17, 21, 37]. Numerous studies revealed that estramustine as an option for combination therapy, for example with mitoxantrone and taxanes, has shown promising effects on hormone-refractory PCa [10, 12,

20, 29, 35, 36]. Although the docetaxel-based therapy is now suggested the standard of care for hormone-refractory PCa, the significance of combinations with other drugs remain to be investigated [32]. In addition, etoposide, an antineoplastic agent by inhibiting topoisomerase II, in combination with estramustine may offer alternatives in PCa treatment [34, 38].

Recently, inhibitors of matrix metalloproteinases (MMPs) have been evaluated for tumor inhibiting properties. MMPs are implicated in invasion and metastasis of tumors by degrading the extracellular matrix and basement membrane in physiological and pathological conditions [19, 39]. Ro 28-2653, a new synthetic specific MMP inhibitor, has shown its inhibitory effect against MMPs expressed in tumor cells as potent antitumor and antiangiogenic agent with promising results [1, 25, 28].

The aim of this study was to evaluate the efficacy of Ro 28-2653 in combination with estramustine and etoposide as a potential new therapeutic strategy in patients with hormone-refractory PCa. We conducted in vitro assays and in vivo studies in Copenhagen rats with the MatLyLu Dunning R-3327 prostate adenocarcinoma accepted as model for androgen-independent, highly metastatic, and anaplastic growing prostate tumors [26].

Materials and methods

Preparation of drugs

The cytostatic drugs estramustine and etoposide were prepared as stock solutions in ready to use vials by the pharmacy office of the University Hospital Charité, Berlin, Germany and then diluted with 0.9% NaCl solution for corresponding concentrations. The synthetic MMP inhibitor Ro 28-2653 (5-biphenyl-4-yl-5-[4-(-nitro-phenyl)-piperazin-1-yl]-pyrimidine-2,4,6-trione) was provided by Roche Diagnostics GmbH, Pharma Research (Penzberg, Germany). For culture experiments, it was dissolved in dimethyl sulfoxide (3 mg + 40 µl dimethyl sulfoxide, then addition of 19.96 ml RPMI medium 1640) and then diluted with RPMI medium 1640 to the corresponding concentrations. For animal experiments, Ro 28-2653 was dissolved in 0.2% sodium carboxymethylcellulose.

Cell line and cell culture

The androgen-independent rat prostate R-3327 MatLyLu Dunning tumor cell line was maintained in RPMI 1640 (Bio Whitaker, Verviers, Belgium) with 2 mmol/l L-glutamine supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 as described previously [24].

Cell proliferation assay

Dunning cells were harvested at 60–70% confluence. The proportion of living and dead cells was estimated by the trypan blue exclusion assay and only cell preparations with living cells > 95% were used for the experiments. Then 500 cells/well in 0.1 ml were seeded onto 96-well microtiter plates and allowed to adhere during overnight incubation. Twenty-four hours (day 0) after seeding the medium was removed and renewed with medium supplemented with estramustine (final concentration 10 and 100 nmol/l), etoposide (100 and 1,000 nmol/l), Ro 28-2653 (0.4 and 2.0 µmol/l), or the combinations, each with corresponding control wells and incubated for 48–96 h. Proliferation was measured afterward using the standard XTT assay (Roche, Mannheim, Germany). That assay is based on the cleavage of the tetrazolium salt XTT (sodium 3′-[1-(phenylaminocarbony)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene sulfonic hydrate) by viable cells to form soluble formazan dye that can be directly quantified by photometry. Briefly, to each well with cells grown as described 50 µl of the XXT labeling reagent were added, incubated for 2–6 h at 37°C, and the formazan absorbance was read on a microplate reader (Anthos HT3, Anthos Labtec Instruments, Salzburg, Austria) using 540 or 620 nm band pass filters. The different incubation time for the final reading of the absorbances was selected to obtain absorbances of > 1.0 OD for the controls to perform experiments with comparable analytical sensitivities.

Tumor cell implantation

The performance of the animal study was in accordance with German requirements and was approved by the responsible local authority (Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit, Berlin, Germany). Male Copenhagen rats with a starting body weight between 180 and 220 g (Charles River, Sulzfeld, Germany) were used for orthotopic cell implantation as described [24]. Briefly, at the day of injection, cells were removed from the tissue culture flasks with trypsin/EDTA. After trypsination of the cells, 4 ml RPMI 1640 were added and the cells were centrifuged, washed twice in PBS, counted, and resuspended in RPMI 1640 medium without supplements at a final concentration of

1×10^6 viable tumor cells/ml. Then 1×10^5 MatLyLu cells were injected into the ventral prostatic lobe of the animals anesthetized with an intramuscular injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Animals were fed and watered ad libitum with daily monitoring of body weights.

Experimental treatment

For the estramustine and etoposide combination study ($n = 5$ per group) the animals were treated as following by daily intraperitoneal injection of the drugs: group 1 received 0.9% NaCl solution as the vehicle of estramustine and etoposide, group 2 estramustine (7.5 mg/kg), group 3 etoposide (25 mg/m^2), and group 4 estramustine (7.5 mg/kg) and etoposide (25 mg/m^2). For the etoposide and Ro 28-2653 combination study ($n = 8$ per group) the animals were treated as following by daily intraperitoneal injection regarding etoposide and its corresponding vehicle of 0.9% saline solution and daily oral gastric catheter application regarding Ro 28-2653 and its corresponding vehicle of 0.2% sodium carboxymethylcellulose: group 1 received intraperitoneally 0.9% NaCl solution, group 2 0.2% sodium carboxymethylcellulose, group 3 etoposide (25 mg/m^2) and the vehicle of Ro 28-2653, group 4 Ro 28-2653 (100 mg/kg) and the vehicle of etoposide, and group 5 etoposide (25 mg/m^2) and Ro 28-2653 (100 mg/kg) as combination treatment. The animals were weighed daily during the treatment period. Before, every day after tumor cell implantation and at the day of sacrifice the animals were weighed. Treatment was initiated 6 days after tumor cell injection; the duration of therapy was 12 days until day 17 after cell injection. On day 18 all animals were sacrificed and the tumors were weighed.

The doses for estramustine and etoposide were selected as the halves of the doses previously applied in the subcutaneous MatLyLu model [30, 33], since we observed severe side effects (increased loss of body weight, dermal irritation, edema) in pilot biocompatibility studies of both drugs using the original doses. The doses for Ro 28-2653 were used as described in detail previously [1, 25].

Statistical analyses

The software GraphPadPrism for Windows, version 4.03 (GraphPad, San Diego, CA, USA) was used to perform statistical analyses. Student's *t*-test and ANOVA analysis with Dunnett's multiple comparison test were used. Differences of $P < 0.05$ were considered statistically significant.

Results

In vitro effects

Figure 1 shows the effects of estramustine, etoposide, and Ro 28-2653 as single agents and in various combinations on the proliferation of the MatLyLu cells after 72 h incubation. Although we also made proliferation studies at other time points, the demonstration of data at the time point of 72 h was chosen because these results showed a distinct tendency and facilitates the clarity of results. The results can be summarized as follows:

- Etoposide was the only single agent that reduced cell proliferation significantly compared to controls and the other single agents. That effect was concentration-dependent ($P = 0.0059$), as shown by comparison of the inhibition at 100 and 1,000 nmol/l.
- Neither Ro 28-2653 or estramustine as single agents nor their combined use inhibited the proliferation of the MatLyLu cells.
- Ro 28-2653 significantly increased ($P = 0.0013$) the inhibitory effect of etoposide by about 20% if the combination Ro 28-2653 (2 µmol/l) and etoposide (1,000 nmol/l) was used. The tendency of that strengthening effect became already obvious with the lower concentration of Ro 28-2653 ($P = 0.061$).
- The combination of etoposide and estramustine showed a similar inhibitory effect as the combination Ro 28-2653 and etoposide. Estramustine (100 nmol/l) also significantly enhanced the inhibitory effect of etoposide (1,000 nmol/l) by about 20% ($P = 0.0008$).

The effect of some combinations on the proliferation of cells was also measured after a time-dependent incubation between 48 and 96 h (Fig. 2). The effect of etoposide or the combination of etoposide with Ro 28-2653 was different to the controls already after 48 h incubation. However, the difference between etoposide and the combination of etoposide and Ro 2.0 became not significant before 72 h incubation (at 72 h: $P = 0.0013$; at 96 h: $P = 0.0449$).

In vivo effects

The applications of the drugs were well tolerated although a loss of body weight was observed both in the controls and treatment groups. The loss of the body weight started at the days 12–15 after tumor cell implantation. Referring to the body weight at the beginning until the end of the treatment, there was a reduction in body weights by 5.2% in the control group, 12.9% in the estramustine group, 8% in the

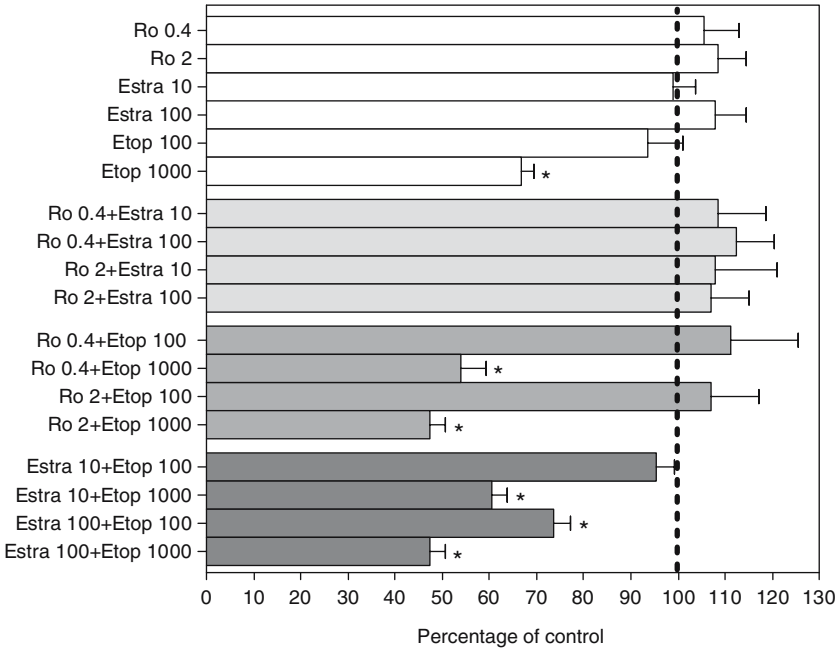

Fig. 1 Effect of etoposide, estramustine, and Ro 28-2653 as single agents and in combination on Dunning tumor cell proliferation. Data are given as arithmetic means and standard error of 4–6 experiments. To facilitate the direct comparison between the various treatment groups and the controls, the inhibitory effect was related to the controls without drug, shown as *dashed* 100% *line*. The absorbances measured in controls ranged between 1.250 and 1.690 OD. Significant decreases (at least $P < 0.05$) from the control were indicated as *asterisk* at the *error bar*. The agents and their concentrations are indicated by the following abbreviations: *Estra* estramustine, *Etop* etoposide, *Ro* Ro 28-2653 following the final concentrations used in the experiments (nmol/l for estramustine and etoposide; µmol/l for Ro 28-2653)

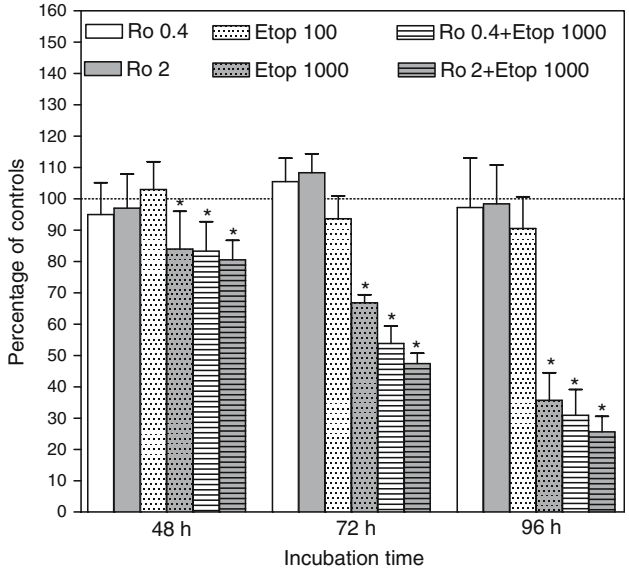

Fig. 2 Time-dependent effect of etoposide and Ro 28-2653 as single agents and in combination on Dunning tumor cell proliferation. Data are given as arithmetic means and standard error of 4–6 experiments. Further details are given in the legend of Fig. 1

etoposide group, 17.1% in the estramustine and etoposide combination group, 3.7% in the Ro 28-2653 group, and 17.6% in the Ro 28-2653 and etoposide combination group. The loss of body weight showed a significant difference between the control group and the estramustine and etoposide combination group ($P < 0.05$) and the Ro 28-2653 and etoposide combination group ($P < 0.05$). There was also a significant difference between the Ro 28-2653 group and the Ro 28-2653 and etoposide combination group ($P < 0.01$).

Figure 3 summarizes the tumor growth in the experimental groups as orthotopic mean tumor weights 18 days after the cell implantation. Since there were no significant differences of the tumor weights between the vehicle groups, they were combined to one group indicated as control group. After MatLyLu cell inoculation the tumors in the control group grew to a mean weight of 13.9 g after 18 days. ANOVA analysis proved that all treatment groups ($P < 0.01$) except the group treated with estramustine ($P > 0.05$) showed a significantly lower tumor growth than the control group. Comparing the effects of the single agents estramustine, etoposide, and Ro 28-2653, the MMP inhibitor was most effective and surpassed the inhibitory effect of the combination of estramustine and etoposide. The tumor weight reduction of etoposide was 37%, of the combination of etoposide with estramustine 62%, and of Ro 28-2653 86%. The inhibitory effect of Ro 28-2653 was significantly enhanced to 92% when

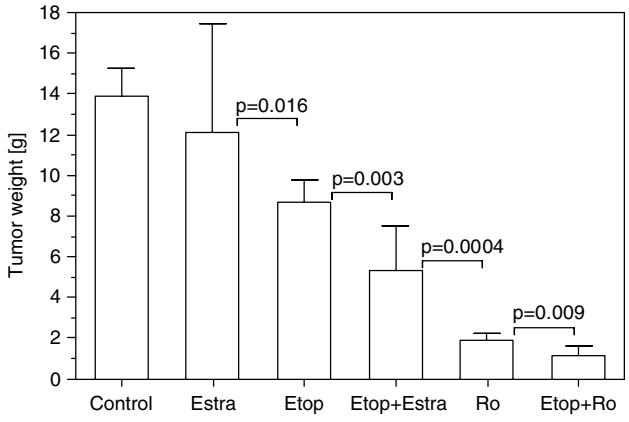

Fig. 3 Effect of etoposide, estramustine, and Ro 28-2653 as single agents and in combination on the tumor weights measured 18 days after tumor cell injection. Data are given as arithmetic means and 95% confidence intervals. The treatment of all animals was initiated 6 days after tumor cell injection with vehicles applied intraperitoneally or orally (controls), with estramustine (Estra, 7.5 mg/kg), etoposide (Etop, 25 mg/m^2), Ro 28-2653 (Ro, 100 mg/kg), or the combinations of etoposide with estramustine or Ro 28-2653. The tumor weights of the controls significantly differed from those of all other groups except the group treated with estramustine (ANOVA with Dunnett's post-test; $P < 0.01$). In addition, significant differences (Student's *t*-test) between various groups are indicated

it was combined with etoposide (Fig. 3). Consequently, the percentage tumor weight difference between that group and the combined etoposide and Ro 28-2653 groups was 30%. This result showed that the combination of Ro 28-2653 with etoposide was more effective to reduce the tumor growth than etoposide or Ro 28-2653 alone. Thus, a synergistic inhibitory effect between Ro 28-2653 and etoposide can be assumed following a comparable approach by evaluating data obtained with high-density focused ultrasound combined with chemotherapy of paclitaxel plus estramustine on Dunning tumors [30].

Discussion

We studied the effect of estramustine, etoposide, and the synthetic MMP inhibitor Ro 28-2653 as single agents and combined with each other to evaluate the specific MMP inhibitor as a possible novel anticancer combination therapy. As briefly pointed out in the introduction, etoposide and estramustine are standard drugs used in the chemotherapy of the hormone-refractory PCa, while the inhibitory efficacy of the MMP inhibitor Ro 28-2653 on the PCa progression was previously shown in the orthotopic MatLyLu Dunning prostate tumor model of the rat [24, 25]. We chose this model because it is an accepted standard PCa model for evaluating the effects of PCa therapy [26]. The effects of estramustine and etoposide in the subcutaneous variant of the model were also comprehensively described [33].

When Ro 28-2653 or estramustine were used as single agents or in combination in the MatLyLu cell culture, no effect on the proliferation could be observed. These in vitro data confirmed the effect of estramustine on MatLyLu cells [33] and also our results with non-selective and selective MMP inhibitors [24, 25]. Only etoposide was able to inhibit the cell proliferation rate. This might be explained by its direct intracellular activity by inducing a premitotic blockage in the cell cycle process either in late S or early G2 phase by binding to the topoisomerase II [5]. It is remarkable that Ro 28-2653 enhanced the inhibitory effect of etoposide although that agent alone did not influence the cell proliferation rate (Figs. 1, 2). The reason for that phenomenon cannot been explained until now since dose–response curves under the aspect of isobologram analyses were not performed. In spite of the limitation of this study, already cell culture experiments let us assume that both agents could be more effective when applied in combination. A similar phenomenon was observed for the combination of etoposide and estramustine [33].

So we evaluated the possible therapeutic effect of these drugs as a combination therapy in the MatLyLu Dunning rat model (Fig. 3). Despite the loss of body weights as shown in the results, the single drugs and drug combinations were relatively well tolerated. The combination groups with etoposide resulted in a higher loss of body weight than the other groups despite the lower tumor weights. It can be concluded that etoposide turns out adverse effects in an unknown manner, shown by affecting the body weight if it is used in a combination treatment. That effect was obvious although we used only the half of the etoposide dose used in the subcutaneous MatLyLu model [30, 33]. The macroscopic examination of the tumors corresponded to our previous observations and further histological examinations were not performed since the typical histological findings were described previously [1, 24, 25]. The investigation on tumor growth in an intact host demonstrated that the combination treatment with estramustine and etoposide significantly reduced the orthotopic tumor weight after 18 days. A similar effect was previously shown in the traditional subcutaneous MatLyLu model [33]. Since estramustine, in contrast to etoposide, resulted in no effect on tumor growth, we decided to evaluate further single and combination

study of etoposide with Ro 28-2653 (Fig. 3). In contrast to the in vitro studies, Ro 28-2653 significantly influenced tumor progression as shown by reduction of tumor weight. Ro 28-2653 as single agent as well as in combination with etoposide could inhibit the tumor growth drastically.

Since Ro 28-2653 alone does not show an antiproliferative effect in cell culture but exposes its antitumoral growth inhibition in the animal organism, it can be concluded that other, e.g., extracellular factors, might play a crucial role. These data support the results already demonstrated in other experiments that metalloproteinase inhibitors only minimally influence the proliferation rate in cell culture but has an effect in tissue by means of the inhibition of MMPs [24, 40].

The complex interplay between tumor progression and the surrounding cellular matrix components within the body might be a possible reason for these observations. MMPs are known to play a critical role in physiologic and pathologic events, such as wound healing, tissue remodeling, tumor growth, vascularization, and metastasis [8, 11]. They are proteolytic enzymes able to promote tumor progression by degrading extracellular matrix and thus facilitating cell migration, invasion, and neovascularization [16]. Especially the synthesis of MMP-9 by tumor cells and/or host stromal cells plays a crucial role for local promotion of tumor progression and invasion [15], with a subsequent increase in distant metastasis [27]. It contributes to tumor angiogenesis by promoting blood vessel morphogenesis and pericyte recruitment [13]. These facts result in the consideration that the inhibition of MMP-9 might be beneficial to the hosts by inhibiting tumor progression and angiogenesis.

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 [28]. It is characterized by a high selectivity for MMP-2, MMP-9, and membrane type 1-MMP which are most consistently detected in malignant tissues and are associated with tumor aggressiveness, metastatic potential, and a poor prognosis. The anti-invasive, antitumor, and antiangiogenic characteristics of MMP inhibitors in general has been demonstrated in several studies [4, 18, 23]. In particular, Ro 28-2653 demonstrated these properties in contrast to the use of broad-spectrum MMP inhibitors, which showed no benefit [28]. These qualities of Ro 28-2653 could be one explanation for the reduction of tumor growth. Own studies verified these effects in the same animal model on the hormone-insensitive R-3327 subline but also on the hormone-sensitive G subline [1, 25]. The combination treatment with Ro 28-2653 and etoposide showed a significantly higher reduction of tumor weight than Ro 28-2653 as a single agent. As shown in Sect. "Results" we assume a synergistic inhibitory effect between Ro 28-2653 and etoposide due to the two different mechanisms of action of etoposide and Ro 28-2653. A similar conclusion was drawn when the effect of high-density focused ultrasound combined with chemotherapy was studied on the Dunning tumor model [30]. However, it should be pointed out that an isobologram analysis in that and also in our study could not be performed to prove that assumption because of the limited number of data.

The characteristics of MMPs and their role in physiological and pathological processes prove that the development of synthetic MMP inhibitors could be a promising tool in anticancer therapy. Unfortunately, clinical trials revealed disappointing results despite the promising ability of broad-spectrum MMP inhibitors to delay primary tumor growth and to block metastasis [6, 9, 14]. Recent investigations revealed that MMP inhibitors may affect the natural host defense mechanism against tumors [3] and that certain MMPs and thus also the inhibitors can have dual effects on cancer development [2, 22]. Several MMPs are involved in the modulation of angiogenic factors and the synthesis of endogenous angiogenic inhibitors [13]. Our previous experiments showed a lower content of intratumoral blood vessels after the treatment of the tumor bearing rats with Ro 28-2653 which could be explained by the antiangiogenic effect of this MMP inhibitor [1]. Consequently, the direct degradation effect on extracellular matrix components is not the only basic mechanism of MMPs in cancer progression [8].

In summary, the synthetic MMP inhibitor Ro 28-2653 in combination with conventional anticancer chemotherapeutic drugs significantly intensified the efficiency of both single therapies to reduce the tumor growth in the rat Dunning tumor model. We believe that Ro 28-2653 or other MMP inhibitors should be considered as potential tools to improve the therapy of patients with advanced PCa after failure of hormonal therapy.

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