Short Communication

PAR2 Knock-Out C57Bl6 Mice as a Model for Evaluating Metastases of Cancer Cells: Pilot *in Vivo* Study of the Metastatic Potential of B16 Melanoma in Knock-Out (*PAR2*-/-) Animals

(PAR-2 / trypsin / melanoma / metastasis / knock-out)

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Abstract. Proteinase-activated receptor 2 (PAR-2) is a ubiquitous surface molecule. It belongs to the family of G protein-coupled receptors activated by site-specific proteolysis by trypsin. Altered function of PAR-2 has been described in different malignant tumours, both *in vivo* and *in vitro*. In the present study, we investigated differences of metastatic spread of B16 melanoma in knock-out animals compared with C57Bl6 mice. Knock-out mice B6.Cg-F2rl1^{tm1Mslb}/J (*PAR2*-/-) and C57Bl6 controls were subcutaneously inoculated with the B16 melanoma tissue cell line. Fourteen days after inoculation, all primary tumours were removed and histopathologically analysed. After one month, animals in both group started to die. Autopsy showed metastatic spread of the melanoma to various organs in both groups. Our experiment confirmed growth and metastatic spread in both groups of mice. Excised tumours differed in volume and weight; average weight (0.62 g in *PAR2*-/- and 0.4 g in control animals). Metastatic spread was observed in both groups and reached 80 % in *PAR2*-/- and 50 % in control animals. While in control mice only lung metastases were observed, local tumour recurrence, renal and lung metastases were observed in *PAR2*-/- mice. The absence of functional PAR-2 could be an important factor influencing the growth and spread of melanoma *in vivo,* probably associated with tumour cell migration, invasiveness and metastasis formation.

Introduction

The role of various proteinases and their inhibitors in cancer development and malignant behaviour and the relationship of these enzymes to the prognosis of cancer is a constant topic of discussion in professional literature. The role of tissue matrix metalloproteinases, cathepsins and other enzymes produced by cancer cells has been extensively investigated. However, there are many other enzymes produced by malignant cells and/or tumour-associated cells such as fibroblasts and immunocompetent cells. One of these enzymes, trypsin, is a proteinase that is produced by many tissues including exocrine pancreatic and intestinal Paneth cells; additionally, enhanced production of trypsin has been reported in malignant cells. Trypsin and similar enzymes act on the cell surface via specific receptors – proteinase-activated receptors (PARs).

Proteinase-activated receptor 2 (PAR-2) is a ubiquitous surface molecule participating in many biological processes. It belongs to the family of G protein-coupled receptors activated by tethered ligand sequences within the amino-terminal part of the molecule that is made accessible by site-specific proteolysis. PAR-2 activation, after site-specific proteolysis of the N-terminus by trypsin and presentation of the tethered ligand sequence

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Abbreviations: FCS – foetal calf serum, HE – haematoxylin-eosin, PAR – proteinase-activated receptor, *PAR2*-/- – knock-out mice B6.Cg-F2rl1^{tm1Mslb}/J, WT – wild type.

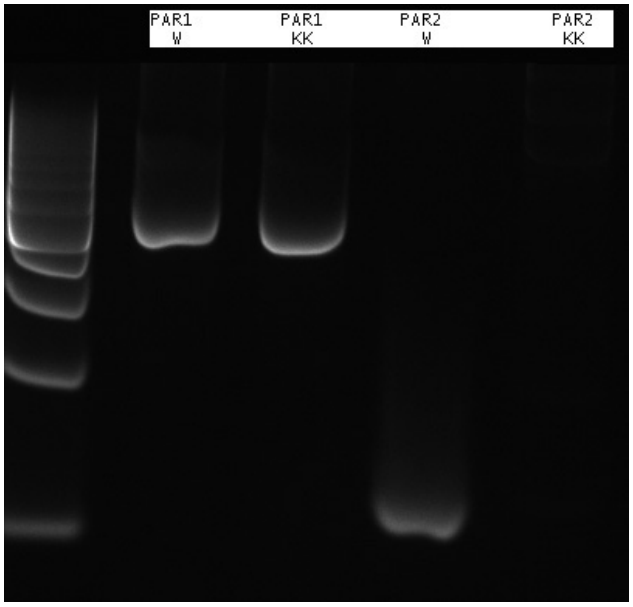

Fig. 1. RT PCR demonstrated the presence of PAR-1 in both groups of mice and lack of PAR-2 in knock-out animals and its expression in controls, W - wild type and KK – knock-out

(SLIGRL in mice) to extracellular domains of the receptor, participates in tissue growth and differentiation, regeneration and repair, inflammatory response regulation and also in malignant transformation (Adams et al., 2011; Hansen et al., 2011). The role of environmental body rate of proteinases (including trypsin and trypsin-like ones) and anti-proteinases, resulting in a "certain" level of proteolytic activity influencing PAR-2 relative to tumour cells, has already been investigated in an *in vitro* model of breast cancer (Matej et al., 2007). Addition of trypsin to medium lacking foetal calf serum (FCS) demonstrated poor cellular growth, most probably caused by attenuated surface adhesion, since the metabolic activity of breast cancer cells was higher in trypsin-treated groups in the presence or absence of FCS. Taken together, the action of trypsin via PAR-2 has been clearly demonstrated; however, this action was limited by the presence of anti-proteinases in the plasma.

On the other hand, despite its ubiquitous body distribution, the role of PAR-2 in the stromal, non-tumourous tissues, in cancer growth and metastasis formation is still poorly understood, while the altered function of PAR-2 has been described in a variety of malignant tumours (Nakanuma et al., 2010; Suen et al., 2010; Wang et al., 2010; reviewed by Elste and Petersen, 2010). However, only a few of the current studies suggest an important role for PAR-2 in cancer tissue development and local progression (Zhang et al., 2009; Mannowetz et al., 2010; Yang et al., 2010), and very little is known about the role of PAR-2 in development of distant metastases.

For these reasons, we sought to evaluate the systemic behaviour of cancer in a metastatic model in which knock-out animals lacking *PAR2* genes were inoculated with different malignant cell cultures. As a pilot study, we decided to evaluate the role of PAR-2 in the generalized spread of melanoma B16 in *PAR2* knock-out mice compared to our previous observations using wild-type (WT), C57Bl6, mice (Wald et al., 2001).

Material and Methods

Animals

The study was performed in accordance with guidelines on animal experimentation of the First Faculty of Medicine, Charles University in Prague and all procedures were approved by the animal experimentation review committee. In the experiment, seven male inbred PAR-2-deficient B6.Cg-*F2rl1*^{tm1Mslb}/J mice (Jackson Laboratories, Bar Harbor, ME) and wild-type inbred C57Bl6 mice (body weight = 18–20 g – AnLab s.r.o., Charles River, Prague, Czech Republic) were used. Mice were kept in a barrier facility for animals, provided with radiation-sterilized bedding (SAWI Research Bedding, JELU-WERK, Germany), fed with radiation-sterilized ST-1 diet (Bergmann, Prague, Czech Republic), and received autoclaved water *ad libitum*.

RT PCR

Total RNA was used for evaluation of the PAR-2 status in both groups of animals (TRIzol®Reagent, Invitrogen, Grand Island, NY). Primers for *PAR2* were 5' - T G G C C A T T G G A G T C T T C C T G T T - 3' , 5'-TAGCCCTCTGCCTTTTCTTCTC-3', and for *PAR1* 5'-TCCTTTCTCACACTTCCACC-3' and 5-GTTCAGGGCTAAACTCTACC-3'. DNA polymerase was used for reverse transcription and PCR (SuperScript® III One-Step RT-PCR System with Platinum®*Taq;* Invitrogen, Grand Island, NY). Amplification cycles consisted of 45 s at 93 °C, 45 s at 55 °C and 1 min at 72 °C for 30 cycles. The PCR reaction was performed in a PCR thermo-cycler (MJ Research, Bio-Rad Laboratories, Inc., Hercules, CA). Products were analysed electrophoretically in 1% agarose gel with ethidium bromide.

Tumour cells

B16 melanoma cells were diluted to a concentration of 4.0×10^6/0.2 ml in suspension. Under thiopental anaesthesia, B16 cells, at the above-mentioned dose, were subcutaneously inoculated into the *PAR2*-/- and WT C57Bl6 mice, on the left side of the trunk.

Tumour growth

Fourteen days after the B16 melanoma cell subcutaneous transplantation, the primary tumours were removed and measured. The volume was calculated from the formula {V = 1/2.A.B2} where A = the largest dimension of the tumour, B = the smallest dimension of the tumour. The malignant aetiology of the process was checked histologically.

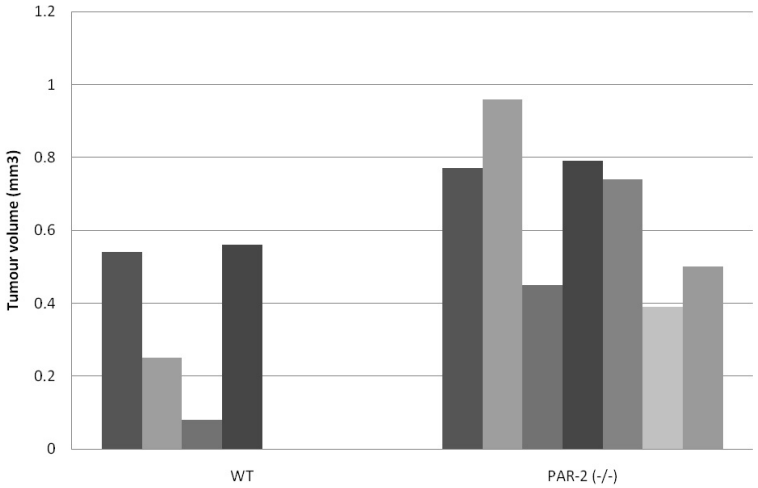

Fig. 2. Volume of explanted tumours was higher in knock-out animals than in controls

Observation, autopsy and histology

Deterioration of animals was followed in both groups every day for a total of 100 days. All surviving animals were sacrificed after this time period. Dissection and histological verification was carried out in all animals, those that died of the disease and those that were sacrificed after 100 days. The lung, liver and local recurrences of primary tumours and metastases into the axillary lymph nodes, as far as they were macroscopically distinct, were subjected to histopathological examinations. Additionally, they were fixed in 10% buffered formalin and embedded in paraffin blocks. Five μm serial slices were subsequently prepared and stained with haematoxylin-eosin (HE). The lungs were embedded *in toto* according to the above procedure and transversally cut at the level of the heart ventricles, and the prepared paraffin blocks were cut serially. After HE staining, metastatic processes were either verified or excluded.

Statistics

The standard two-sampled *t*-test was used for testing tumour weight comparisons between WT and *PAR2*-/- groups. The same test was used for comparing the tumour volume between WT and *PAR2*-/- groups. Survival curves of WT and *PAR2*-/- groups were tested as statistical distributions using the two-sampled Kolmogorov-Smirnov test of distribution equality. All the statistical calculations were performed using the MATLAB Statistical Toolbox (MathWorks, Natick, MA), with a critical probability of 0.1 in response to small sample sizes.

Results and Discussion

Using RT PCR we confirmed that the knock-out animals were lacking PAR-2 RNA (Fig. 1). Our experiment confirmed growth and metastatic spread in both groups of mice and met the primary objective of this pilot study, i.e. verification of metastatic tumour dissemination in *PAR2*-/- mice.

The excised tumours differed in volume (Fig. 2); the tumours also differed in average weight; 0.62 g in *PAR2*-/- and 0.4 g in the control animals (Fig. 3). The tumour weight equality hypothesis for WT vs. *PAR2*-/- groups was not rejected (P value = 0.186). The tumour volume

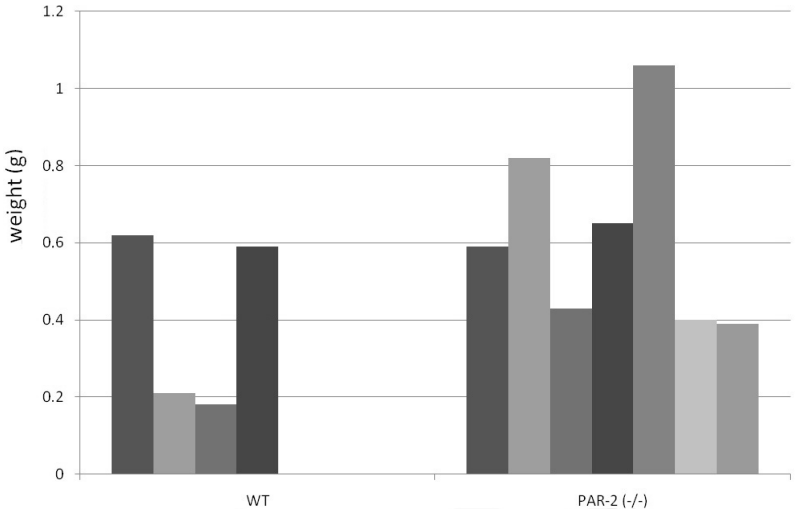

Fig. 3. Weight of explanted tumours was higher in knock-out animals than in controls

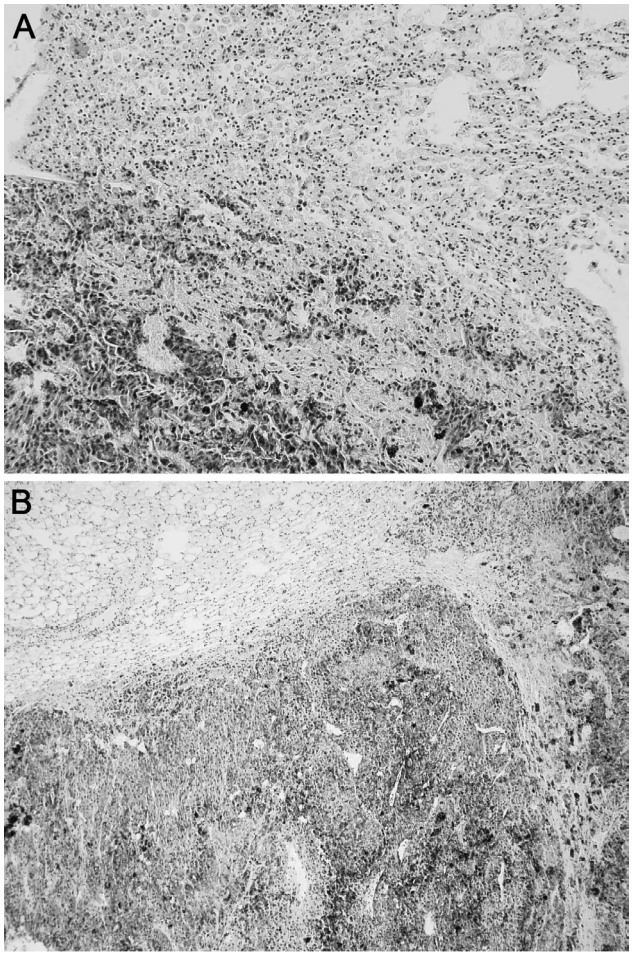

Fig. 4. Metastasis of the tumours in the lungs in control animal (A) and in knock-out mouse (B). Pigmented highly malignant tumour is compatible with melanoma. There are slightly more prominent regressive changes in the tumour of the wild-type animal.

differences between groups were significant (P value = 0.057). Microscopically, we observed regressive changes in primary tumours (pigmented melanoma) in both groups of investigated animals. Distant metastases were recorded in 2/3 (0.667) of WT animals and in 5/7 (0.714) of *PAR2* -/- animals without any statistically significant difference. Metastatic spread was observed in both groups and reached 80 % in *PAR2*-/- and 50 % in control animals. In control mice, only lung metastases were observed (Fig. 4); however, local tumour recurrence, renal and lung metastases were observed in *PAR2*-/- mice (Table 1). Only mice with recorded metastatic spread contributed data to survival analyses. A difference in survival times between animal groups was observed; knock-out animals had shorter average survival (47.2 days) compared to the controls (57.5 days). Statistically, there were no significant differences between survival curves (P value = 0.8271) of WT and *PAR2*-/- groups. However, it is worth mentioning that these data are only preliminary and cannot be statistically evaluated any further because of the small numbers of investigated animals (Fig. 5).

Despite the fact that our study was not designed to evaluate differences between the groups, one interesting observation jumps to centre-stage and fairly seeks scholarly attention. Tumours in the *PAR2*-/- mice were larger and heavier; additionally, the metastatic spread of these tumours involved other organs (kidney) and local recurrences were observed in this group of mice as well. These findings may raise important questions about the role of PAR-2 in the environment for tumour growth and dissemination despite its presence or absence on tumour cells themselves. PAR-2 acts as a pro-inflammatory agent inducing production of different mediators (Al-Ani et al., 2010; Yoshida et al., 2011), vasodilatation (Bhatt et al., 2010) and increased vascular permeability (Kawabata et al., 1998). Links between cancer spread, inflammation, vascular growth and coagulation/fibrinolysis are obvious, and the direct action of PAR-2 has already been partly elucidated (McEachron et al., 2010). PAR-2, in general, appears to be involved in many processes related to membrane internalization and intracellular sorting (Déry et al., 1999; Soh et al., 2010); however, it also seems to play an important role in the immune system. PAR-2 directly triggers development of dendritic cells (Fields et al., 2003; Jiang et al., 2010) and promotes dendritic cell antigen transport and T-cell activation *in vivo* (Ramelli et al., 2010). It is obvious that the absence of PAR-2 can lead to attenuation of the specific cellular anti-tumour activity, although further investigations are needed to verify this hypothesis.

Conclusion

The absence of functional PAR-2 could be an important factor influencing the growth and spread of mela-

Table 1. Evaluation of the metastatic spread in both groups of animals

WT	***PAR2*-/-**
Lung	Local forelimb and hindlimb lymph node, lung
Lung	Local forelimb and lung
No metastasis recorded	Local hindlimb lymph node, kidney, lung
No metastasis recorded (died only one day after tumour extirpation, not entered into the survival analysis)	Lung
	Lung
	No metastasis recorded
	No metastasis recorded

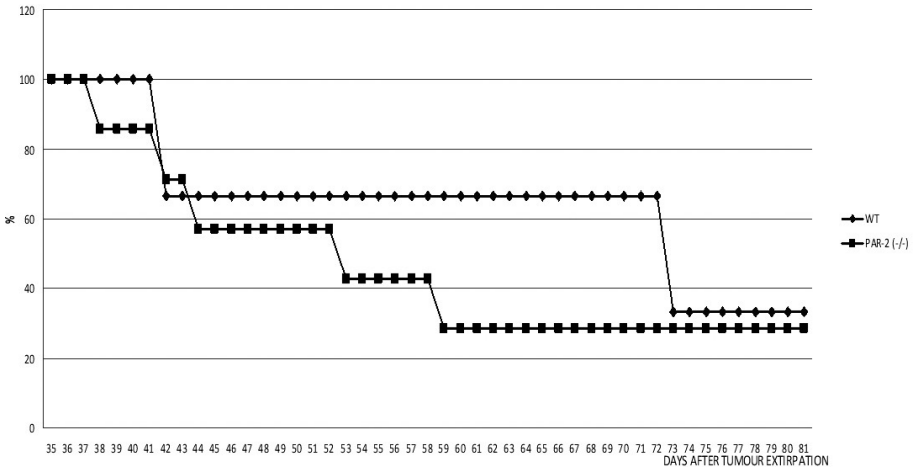

Fig. 5. Survival curves of investigated groups of mice (WT – 3 mice, *PAR2*-/- – 7 mice). There is no statistically significant difference between the two groups, namely due to small numbers of investigated animals.

noma *in vivo,* probably through the process of tumour cell migration, invasiveness and metastasis formation. However, further studies involving larger samples sizes will be needed to confirm our results.

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References

Adams, M. N., Ramachandran, R., Yau, M. K., Suen, J. Y., Fairlie, D. P., Hollenberg, M. D., Hooper, J. D. (2011) Structure, function and pathophysiology of protease activated receptors. *Pharmacol. Ther*. **130,** 248-282.

Al-Ani, B., Hewett, P. W., Cudmore, M. J., Fujisawa, T., Saifeddine, M., Williams, H., Ramma, W., Sissaoui, S., Jayaraman, P. S., Ohba, M., Ahmad, S., Hollenberg, M. D., Ahmed, A. (2010) Activation of proteinase-activated receptor 2 stimulates soluble vascular endothelial growth factor receptor 1 release via epidermal growth factor receptor transactivation in endothelial cells. *Hypertension* **55,** 689-697.

Bhatt, D. K., Ploug, K. B., Ramachandran, R., Olesen, J., Gupta, S. (2010) Activation of PAR-2 elicits NO-dependent and CGRP-independent dilation of the dural artery. *Headache* **50,** 1017-1030.

Déry, O., Thoma, M. S., Wong, H., Grady, E. F., Bunnett, N. W. (1999) Trafficking of proteinase-activated receptor-2 and β-arrestin-1 tagged with green fluorescent protein. β-Arrestin-dependent endocytosis of a proteinase receptor. *J. Biol. Chem.* **274,** 18524-18535.

Elste, A. P., Petersen, I. (2010) Expression of proteinase-activated receptor 1-4 (PAR 1-4) in human cancer. *J. Mol. Histol.* **41,** 89-99.

Fields, R. C., Schoenecker, J. G., Hart, J. P., Hoffman, M. R., Pizzo, S. V., Lawson, J. H. (2003) Protease-activated receptor-2 signaling triggers dendritic cell development. *Am. J. Pathol.* **162,** 1817-1822.

Hansen, K. K., Oikonomopoulou, K., Baruch, A., Ramachandran, R., Beck, P., Diamandis, E. P., Hollenberg, M. D. (2008) Proteinases as hormones: targets and mechanisms for proteolytic signaling. *Biol. Chem.* **389,** 971-982.

Jiang, B., Grage-Griebenow, E., Csernok, E., Butherus, K., Ehlers, S., Gross, W. L., Holle, J. U. (2010) The role of proteinase 3 (PR3) and the protease-activated receptor-2 (PAR-2) pathway in dendritic cell (DC) maturation of human-DC-like monocytes and murine DC. *Clin. Exp. Rheumatol.* **28 (Suppl 57),** 56-61.

Kawabata, A., Kuroda, R., Minami, T., Kataoka, K., Taneda, M. (1998) Increased vascular permeability by a specific agonist of protease-activated receptor-2 in rat hindpaw. *Br. J. Pharmacol.* **125**, 419-422.

Mannowetz, N., Würdinger, R., Zippel, A., Aumüller, G., Wennemuth, G. (2010) Expression of proteinase-activated receptor-2 (PAR2) is androgen-dependent in stromal cell line (hPCPs) from benign prostatic hyperplasia. *Prostate* **70,** 1350-1358.

Matej, R., Mandáková, P., Netíková, I., Poucková, P., Olejár, T. (2007) Proteinase-activated receptor-2 expression in breast cancer and the role of trypsin on growth and metabolism of breast cancer cell line MDA MB-231. *Physiol. Res.* **56,** 475-484.

McEachron, T. A., Pawlinski, R., Richards, K. L., Church, F. C., Mackman, N. (2010) Protease-activated receptors mediate crosstalk between coagulation and fibrinolysis. *Blood* **116,** 5037-5044.

Nakanuma, S., Tajima, H., Okamoto, K., Hayashi, H., Nakagawara, H., Onishi, I., Takamura, H., Kitagawa, H., Fushida, S., Tani, T., Fujimura, T., Kayahara, M., Ohta, T., Wakayama, T., Iseki, S., Harada, S. (2010) Tumour-derived trypsin enhances proliferation of intrahepatic cholangiocarcinoma cells by activating protease-activated receptor-2. *Int. J. Oncol.* **36,** 793-800.

Ramelli, G., Fuertes, S., Narayan, S., Busso, N., Acha-Orbea, H., So, A. (2010) Protease-activated receptor 2 signalling promotes dendritic cell antigen transport and T-cell activation in vivo. *Immunology* **129,** 20-27.

Soh, U. J., Dores, M. R., Chen, B., Trejo, J. (2010) Signal transduction by protease-activated receptors. *Br. J. Pharmacol.* **160,** 191-203.

Suen, J.Y., Gardiner, B., Grimmond, S., Fairlie, D. P. (2010) Profiling gene expression induced by protease-activated

receptor 2 (PAR2) activation in human kidney cells. *PLoS One* **5,** e13809

Wald, M., Olejár, T., Sebková, V., Zadinová, M., Boubelík, M., Poucková, P. (2001) Mixture of trypsin, chymotrypsin and papain reduces formation of metastases and extends survival time of C57Bl6 mice with syngeneic melanoma B16. *Cancer Chemother. Pharmacol.* **47 (Suppl),** S16-22.

Wang, X., Liu, H. T., Li, S., Li, K., Lin, N., Fan, Q. X., Zheng, Y. L. (2010) Prognostic value of protease-activated receptor 2 expression in oesophageal squamous cell carcinoma. *J. Int. Med. Res.* **38,** 1381-1388.

Yang, X. P., Li, Y., Wang, Y., Wang, Y., Wang, P. (2010) β-Tryptase up-regulates vascular endothelial growth factor expression via proteinase-activated receptor-2 and mitogen-activated protein kinase pathways in bone marrow stromal cells in acute myeloid leukemia. *Leuk. Lymphoma* **51,** 1550-1558.

Yoshida, N., Takagi, T., Isozaki, Y., Suzuki, T., Ichikawa, H., Yoshikawa, T. (2011) Proinflammatory role of protease-activated receptor-2 in intestinal ischemia/reperfusion injury in rats. *Mol. Med. Report.* **4,** 81-86.

Zhang, X., Wang, W., True, L. D., Vessella, R. L., Takayama, T. K. (2009) Protease-activated receptor-1 is upregulated in reactive stroma of primary prostate cancer and bone metastasis. *Prostate* **69,** 727-736.