

## Modelling of Tumour–Host Coexistence *In Vitro* in the Presence of Serine Protease Inhibitors

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**Abstract.** *The activities of cell surface serine proteases are markedly enhanced in malignant tumours. Proteolytic degradation of the extracellular matrix and basal membrane of normal cells is an important event for tumour cell growth and invasion. Two well-known broad-spectrum inhibitors of serine protease, Foy-305 and Ono-3403, were evaluated for their ability to affect the growth rate and survival of MCF7 breast cancer cells co-cultured with MRC5 lung fibroblasts as feeder cells in the absence of serum. Flow cytometry and differential staining demonstrated that in the mixed culture, the rate of tumor growth was dependent upon the presence of the feeder MRC5 lung fibroblasts and could be obviated by the additional presence of the inhibitors of serine proteases.*

Malignancy is a state that emerges from a tumour-host microenvironment in which the host participates in the induction, selection and expansion of the neoplastic cells. Malignant tumour cells recruit the vasculature and stroma through the production and secretion of stimulatory growth factors and cytokines (1). The locally activated host microenvironment (both cellular and extracellular elements) in turn modifies the proliferative and invasive behaviour of the tumour cells (2). The interactions of tumour cells with various host cells (platelets, lymphocytes and endothelial cells), the extracellular matrix (ECM) and the basement membrane (BM) are crucial parameters for tumour cell growth and invasion, as analysed in detail by Liotta and Kohn (3).

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It is well known that tumour cells secrete various proteolytic enzymes, such as matrix metalloproteinases and serine proteinases (tissue plasminogen activator, urokinase, thrombin, and plasmin). The activities of cell surface proteinases are enhanced in tumours and these enzymes contribute to the degradation of the BM and the ECM (4).

The direction of tumour cell invasion and migration can be influenced by fibroblast-produced chemoattractants for tumour cells, and by the construction of preferred adhesion pathways. Local attractants include scatter factor/hepatocyte growth factor, proteolysed matrix fragments or cytokines and growth factors, such as epidermal growth factor and transforming growth factor- $\beta$  released from the degraded matrix (5, 6), which promote further tumour cell growth. The ECM modifies the BM, resulting in decomposition of the boundaries in the normal tissues. During metastasis, tumour cells leave the stroma and enter nearby lymphatic and blood vascular channels (7). Intravascularly circulated tumour cells attach to endothelium in target organs and are stimulated to grow as colonies inside the vessel.

Stromal therapy could emerge as a viable approach to cancer prevention and intervention (8, 9). Tumour invasion and metastasis formation can be reduced by certain protease inhibitors at the stromal level (10).

In the present study, we investigated the growth of human breast cancer cells in mixed cultures with normal human fibroblast cells. This co-cultivation was performed in the presence and absence of serum. In order to investigate the nature of the coexistence and the therapeutic perspectives of intervention in tumour growth, we investigated the tumour growth-suppressing activities of two synthetic serine protease inhibitors in the presence of normal cells.

### Materials and Methods

*Compounds.* Synthetic serine protease inhibitors FOY 305 (Foypan<sup>®</sup>) and ONO 3403 were obtained from Ono Pharmaceutical Co. Ltd., Osaka, Japan (11). ONO 3403 is an analogue of FOY 305 (12). The cytotoxicity of these compounds against the cell lines

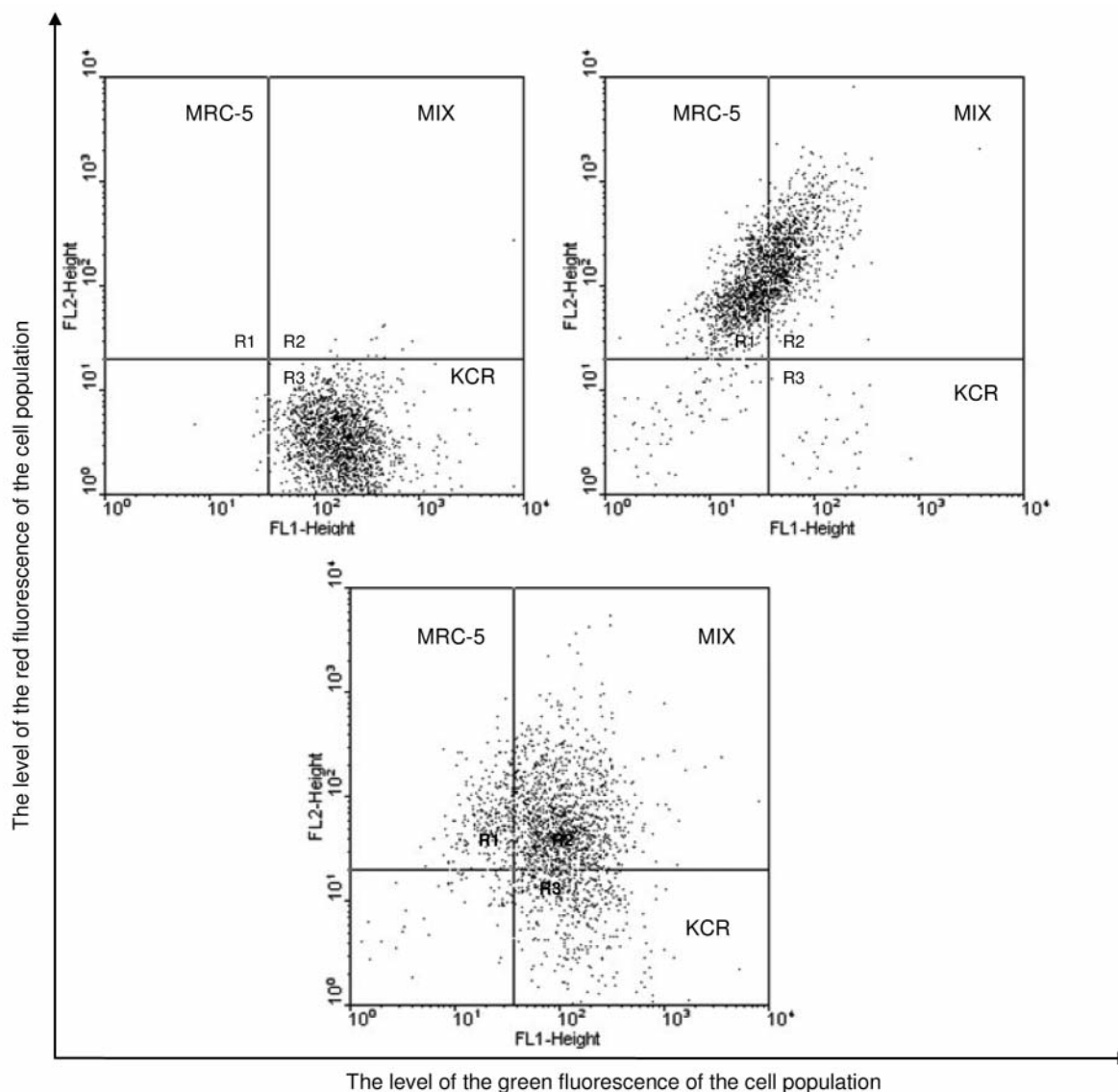


Figure 1. Examples of the results of the flow cytometry. The samples were analyzed for red fluorescence (quadrant R1) and green fluorescence (quadrant R3) by flow cytometry. The assay gives information about the number of the KCR (quadrant R3), MRC-5 (quadrant R1) and mixed (quadrant R2) cells.

employed in this study was determined and the inhibitory dose level of 50% ( $ID_{50}$ ) values of the compounds were as follows: FOY 305: >100  $\mu\text{g/ml}$ , ONO 3403: >120  $\mu\text{g/ml}$  on both cell lines incubated for 48 h. The inhibitors were dissolved in dimethyl sulfoxide to prepare stock solutions at a concentration of 1.0 mg/ml.

**Cell cultures.** Normal and tumour cell lines were obtained from the ATCC collection of LGC Promochem, Teddington, UK, and were used in the co-cultivation studies as follows.

The drug-resistant subline of breast cancer MCF7 cells (also known as KCR) (ATCC: HTB22) was grown in RPMI-1640 medium supplemented with 2 mM L-glutamine and heat-deactivated 10% foetal bovine serum (FBS) supplemented with penicillin, streptomycin and nystatin. Doxorubicin at 1  $\mu\text{M}$  was added to the

medium so as to maintain the overexpression of the ABC transporter P-glycoprotein (Pgp 1) of the breast cancer MCF7 cells. Pgp1 is the protein that extrudes anticancer agents prior to their reaching their intended targets and hence functions as an efflux pump. Overexpression of this transporter is responsible for the multidrug-resistant properties of cancer cells.

Normal human lung fibroblast MRC-5 cells (ATCC: CCL-171) were cultivated in Eagle's MEM (Sigma- Aldrich Ltd, Budapest, Hungary) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10% FBS and antibiotics.

**Cell membrane labelling.** The co-existence of normal MRC5 cells and MCF7/KCR breast cancer cells was modelled *in vitro* by double staining using different ratios of cells and measuring the fates of the

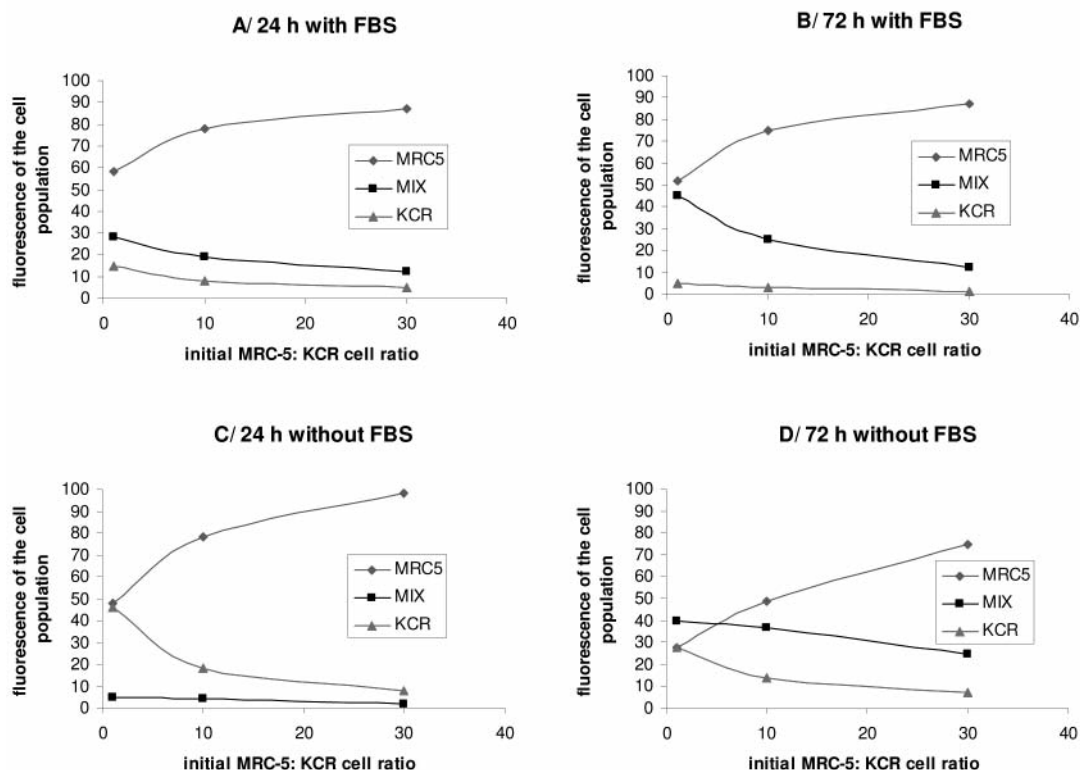


Figure 2. Percentage of different cell populations in co-cultivation under different conditions. Values are averages of four independent measurements. The x-axis presents the cell ratios in the experiments. One unit of KCR cells and 3, 10 and 30-fold higher numbers of MRC5 cells were used at 0 h (at the beginning of the experiment). The y-axis presents the fluorescence intensities of cell populations in co-cultures.

two individual cell lines. In order to clearly attribute the changes in the co-cultures to one or the other cell line, the cells were labelled with the PKH membrane linker dyes PKH67 Green and PKH26 Red Fluorescent Cell Linker Kits (Sigma-Aldrich Ltd, Budapest, Hungary). The PKH Fluorescent Cell Linker Kits allow the fluorescent labelling of live cells. The KCR cells were stained with PKH67 and the MRC5 fibroblast cells were stained with PKH26 and performed according to the manufacturer's instructions. Briefly, the cells were washed with medium without FBS. Ten million cells were added to a polypropylene tube, centrifuged at 2,000 rpm for 2 minutes and the supernatant carefully removed to minimize cell loss. Staining solutions of PKH67 and PKH26 were prepared in polypropylene tubes by dilution in diluent C (supplied with the kit) immediately prior to cell staining. Staining was initiated by rapidly adding a 2x concentrated cell suspension, prepared by re-suspending the cell pellet in 1 ml of diluent C, to the 2x dye solution. Staining was stopped after 3 min by adding an equal volume (2 ml) of FBS over a period of 1 min followed by the addition of an equal volume of complete medium containing 10% FBS. The cells were then centrifuged and washed three times with 10 ml of complete medium. All steps were performed at room temperature and under sterile conditions. The cells were cultured in FBS-free and in FBS-supplemented medium for 24 and 72 h before measurement of the distribution (MCF7/KCR:MRC5) of the mixed cell population. The KCR and MRC-5 cells were used in different proportions (1:3, 1:10, 1:30) in order to investigate whether the cell number and ratio influence the result in mixed cultures. Stained cells were suspended

with 0.25% trypsin-EDTA, incubated at 37°C for 2 minutes, and the suspension centrifuged and washed once in FBS-free medium. The levels of green and red fluorescence of the cell population were simultaneously measured (Figure 1) with the aid of a Becton Dickinson FACS scan flow cytometer (Oxford, UK). PKH67 is a green fluorochrome with excitation at 490 nm and emission at 504 nm, while PKH26 is a red fluorochrome, with excitation at 551 nm and emission at 567 nm.

## Results

As is evident from Figure 2A and 2B, in the presence of FBS the difference in the proliferation of the two cell types was minimal. However, under FBS-free conditions (Figure 2C and 2D), the proportion of the tumour cells was found to be significantly higher than that of the co-cultured normal lung fibroblasts. In the absence of FBS, the ratio of the two cell types changed dramatically in favour of the KCR tumour cell line. Noteworthy changes were found in the samples grown at a tumour cell: fibroblast cell ratio of 1:3

The FBS components serve as growth promoters for normal and cancer cells. In the absence of FBS, cancer and normal cells cannot grow (data not shown). As the cancer cells continued to grow in the absence of FBS whereas the normal fibroblasts continued to decrease in number due to starvation and eventual

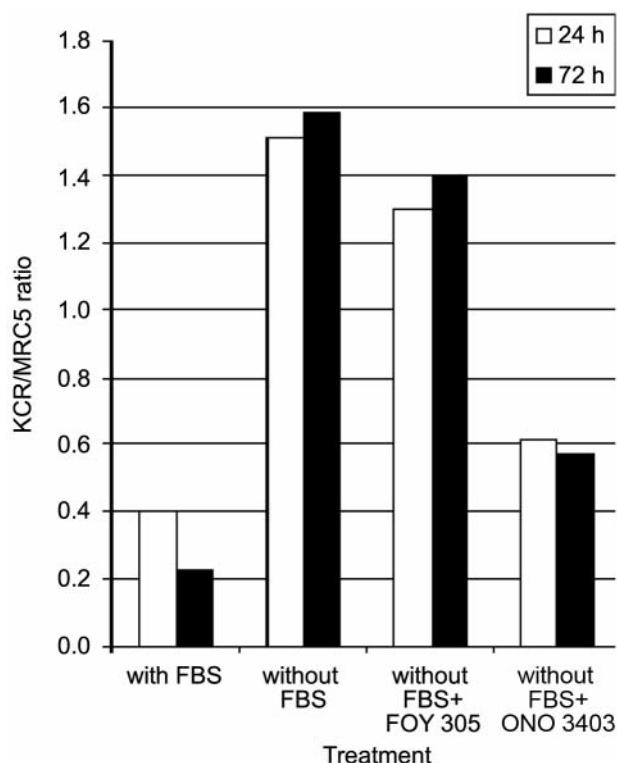


Figure 3. Ratio of KCR: MRC5 cell populations after co-cultivation for 24 or 72 h in the presence of different protease inhibitors: FOY 305 and ONO 3403 were applied at 20  $\mu\text{g/ml}$ .

cell death, the death of the latter cells may have yielded products that may serve as alternate sources of nutrients for the cancer cells provided that these products can be utilized by the membrane based proteases of the cancer cells.

To test this hypothesis, parallel FBS-free, mixed cultures containing the two protease inhibitors were used (Figure 3). The normal cells were more sensitive to (FBS) starvation than the cancer cells, as predicted. Without FBS addition to the culture medium, a tumour cell growth inhibitory effect was found after incubation of 24 h in the presence of ONO 3403 while FOY 305 moderately reduced the proportion of MCF7/KCR cells. The effects of the three compounds on the ratio of cancer cells MCF7/KCR to normal MRC5 cells were even more pronounced after an incubation period of 72. These inhibitors resulted in the reduction of cancer cells with time, suggesting that the membrane-bound proteases of the cancer cells were involved in the processing of products yielded from dead or dying normal cells

## Discussion

Cell-cell contact, cytophagocytosis or autophagocytosis may be responsible for the interaction between normal and cancer cell populations. Since the human cancer cells were able to

survive on normal cell cultures in the absence of serum (FBS), we conclude that cancer cells exist in a predator-prey relationship in which they live as parasites on normal cells (13). The occurrence of cytophagocytosis in tumorous tissue is known to be associated with different tumours. Autophagy is regulated by G proteins, which control the lysosomal-autophagic catabolism in cancer cells when the extracellular amino acid level reduces the formation of autophagic vacuoles containing cytoplasmic material destined for lysosomal degradation (14). When cancer cells survive or grow in FBS-free medium, they feed on various nutrients by ingesting different metabolites, cell debris, dead or living cells by autophagy or cannibalism-like mechanisms.

In our earlier reported model for study of the host-cancer relationship and their coexistence, the host cells were regarded as prey and the cancer cells as predators (15). Coexistence is a situation that could be regarded as a regulated mechanism in tumour growth. Mathematical models can significantly assist an understanding of malignant transformation, overgrowth and factors affecting the numbers of one of two competing populations and the relationship to the magnitude of entropy production (16).

In our study, we presumed that tumour growth and invasion can be modified by some compounds at a stromal level. Consequently, it was presumed that the interaction between tumour and normal cells in a FBS-free medium can be studied as model of propagation or invasion in the presence of protease inhibitors. The matrix metalloproteases and serine proteases are membrane-anchored enzymes that degrade the ECM under the control of an activation cascade (3). Through this function, malignant tumour cells can migrate and invade distant organs. FOY 305 is a broad-spectrum synthetic serine protease inhibitor of enzymatic activities, including trypsin, thrombin, kallikrein and plasmin (17). Previous studies have shown that FOY 305 inhibits carcinogenesis and delays tumour development in mouse skin, most likely *via* an inhibition of proteolytic enzymes, which are in turn induced by carcinogenesis and are involved in tumour development (18). A phase analysis by flow cytometry demonstrated that this serine protease inhibitor suppressed the cell cycle progression in the G1- or S-phase (19).

ONO 3403, an analogue of FOY 305, was recently found to exert a more potent inhibitory activity toward trypsin, plasmin, pancreatic kallikrein and thrombin than did FOY 305 (20). In our study, we too observed that ONO 3403 was more potent than FOY-305. The compounds additionally inhibited metastasis formation (8). ONO 3403 reportedly displays growth-inhibitory activity against transformed murine fibroblasts (20), the human pancreatic carcinoma cell line PANK-1, Mia Paka-2 and BxPC-3, the human bladder carcinoma cell line T24, human epidermoid carcinoma A431 (9) and also malignant human neuroblastoma cell lines *in*

*vitro* (21). This paper provides evidence that inhibitors of serine proteases were able to reduce the rate of growth of cancer cells co-cultivated with normal cells in the absence of serum as growth-promoting factor. It is concluded that tumour invasion and metastasis formation can be reduced by certain protease inhibitors at a stromal level.

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