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Influence of matrigel on biodistribution studies in cancer research

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Matrigel matrix is widely used for supporting tumour implantation. However, some tumour cells are unable to degrade matrigel matrix resulting in residual matrigel at the time point of the biodistribution study or therapy experiment. *In vitro* cell uptake of tumour affine compounds into tumour cells embedded in matrigel was compared with matrigel free tumour cells. Matrigel accumulation exceeded cellular uptake of the tumour affine peptides. This suggests that matrigel might have an influence on the acquired biodistribution data when it is still present at the time point of the study. A quantitation of residual matrigel in tumour explants fourteen days after tumour implantation with a matrigel-tumour cell mixture showed that the overall matrigel content in the case of MCF-7 and AR42J tumours was about 23%. In order to evaluate the extent of accumulation of compounds in matrigel, nude mice bearing either tumours, tumour-matrigel-mixtures or matrigel alone received intravenous injections of fluorophor tagged tumour specific peptides. Fluorescence microscopy of cryosectioned matrigel, matrigel-tumour mixtures and tumour explants showed that the labelled compounds were matrigel associated and tumour cell associated with a higher fluorescence intensity in matrigel. In summary, matrigel matrix can influence biodistribution studies. It leads to believe in a higher tumour accumulation. Therefore, either a number of control experiments or the separation of matrigel from the tumour is necessary in order to obtain correct biodistribution data.

1. Introduction

Animal tumour models are frequently used in cancer research. Many tumours (especially human tumours) do not grow in animal models without matrigel support, such as prostate tumours, breast tumours, small lung cell carcinomas and epidermoid tumours (Basler and Shapiro 1982; Noel et al. 1995; Yue and Brodie 1993). For supporting the implantation of various tumour cells into mice that otherwise cannot be inoculated in animals matrigel is widely used (Basler and Shapiro 1982; Noel et al. 1995). Matrigel matrix is a mixture of basement membrane proteins; its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin 1 derived from Engelbreth Holm Swar mouse sarcomas (Kleinmann and Martin 2005). At temperatures below 4 °C matrigel matrix is liquid, whereas at temperatures above 20 °C it solidifies and forms a gel. For matrigel supported tumour implantation cultured tumour cells are suspended in matrigel directly prior to the subcutaneous injection of the matrigel cell suspension. By time, matrigel is enzymatically degraded (Dolo et al. 1999; Wong et al. 1992). However, the amount of matrigel degrading enzymes varies on the type of cell line used, on the amount of cells mixed with matrigel and on the time period after the subcutaneous matrigel injection. In some cases cells do not express matrigel degrading enzymes. Balduyck et al. (2000) have shown that highly invasive MDA-MB-231 cells differentiate from the slightly invasive T47D, MCF-7 and BT-20 cells by the expression of MMP-1, MMP-3, MMP-9, and MMP-13. The proteolytic activity of MMP-9 plays an important role in the invasiveness of MDA-MB-231 cells through matrigel (Balduyck et al. 2000). Cells not expressing MMP-9 – such as MCF-7 cells – are unable to degrade the matrigel matrix (Balduyck et al. 2000). Matrigel degradation was reported to be supressed *in vivo* by recruitment of macrophages (Shen et al. 2006). Matrigel increased the initial rate of tumour growth, increased cell yield and produced tumours with a centralized area of residual matrigel and necrotic cells, with viable cells on the periphery of the mass (Wyrick et al. 1997). Even 30 days after meningioma inoculation residual matrigel can be found when tumours were inoculated by matrigel assistance, indicating incomplete matrigel degradation (Jensen 1998). Even in the absence of tumour cells, the formation of new blood vessels occurs (these new blood vessels can be determined as early as 7 days after matrigel implantation) which facilitate the accumulation of compounds in matrigel matrix (Shen et al. 2006; Kano et al. 2005). Kano et al. (2005) have shown that fluorescein dextran accumulates in matrigel plaques seven days after matrigel implantation. In order to clarify the role of matrigel matrix in biodistribution studies we investigated how long matrigel resides in animals after the subcutaneous injection of matrigel tumour

mixtures and of matrigel alone. In addition we evaluated the accumulation of two different peptides in matrigel in order to clarify whether compounds can show substantial matrigel accumulation that falsify biodistribution studies.

2. Investigations, results and discussion

2.1. In vitro affinity of compounds to matrigel

The uptake characteristics of tumour affine compounds into tumour cells, into matrigel and into tumour cells embedded in matrigel were determined by confocal laser scanning microscopy and additionally by fluorescence reader analysis in order to obtain quantitative uptake data. For the evaluation of the influence of matrigel on biodistribution studies we have chosen a wide variety of compounds that are known to show affinity to cells. Compounds with affinity to cells were chosen in order to allow uptake competition between the cells and matrigel. Octreotate, a cyclic somatostatine analogue, has affinity to somatostatine receptor expressing cells (MCF-7, AR42J) and is internalized by receptor mediated endocytosis (Ginj et al. 2006; Hofsli et al. 2002; Mier et al. 2002). FROP-1 and p160 were reported to be taken up by MCF-7 cells (Askolysis et al. 2005; Zhang et al. 2001; Mier et al. 2006). *N*-(2-Diethylaminoethyl)benzamides – such as procainamide – are known to show affinity to melanoma cells and other cell lines with great amounts of acidic cell organelles – such as HeLa (Wolf et al. 2007). Tat is a HIV derived cell penetrating peptide that was shown to enter a wide variety of cells (Chauhan et al. 2007). The *in vitro* uptake characteristics of the fluorescent-labelled compounds into tumour cells and matrigel could be directly followed by confocal laser scanning microscopy (a tunable ZeissLSM 510 UV). The micrographs along with quantitative uptake data are shown in Figs. 1 and 2.

In all cases with the exception of procainamide, matrigel showed a much greater uptake versus the cells embedded

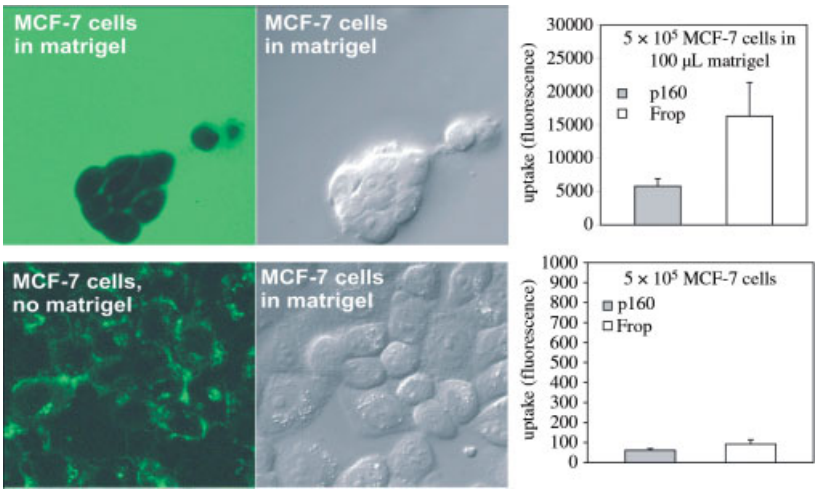

Fig. 1:
Confocal laser scanning micrographs of either MCF-7 cells embedded in matrigel or without matrigel following 1 h incubation with FITC labelled p160. In addition the quantitative uptake of FITC labelled p160 and Frop into MCF-7 cells embedded in matrigel or without matrigel following 1 h incubations

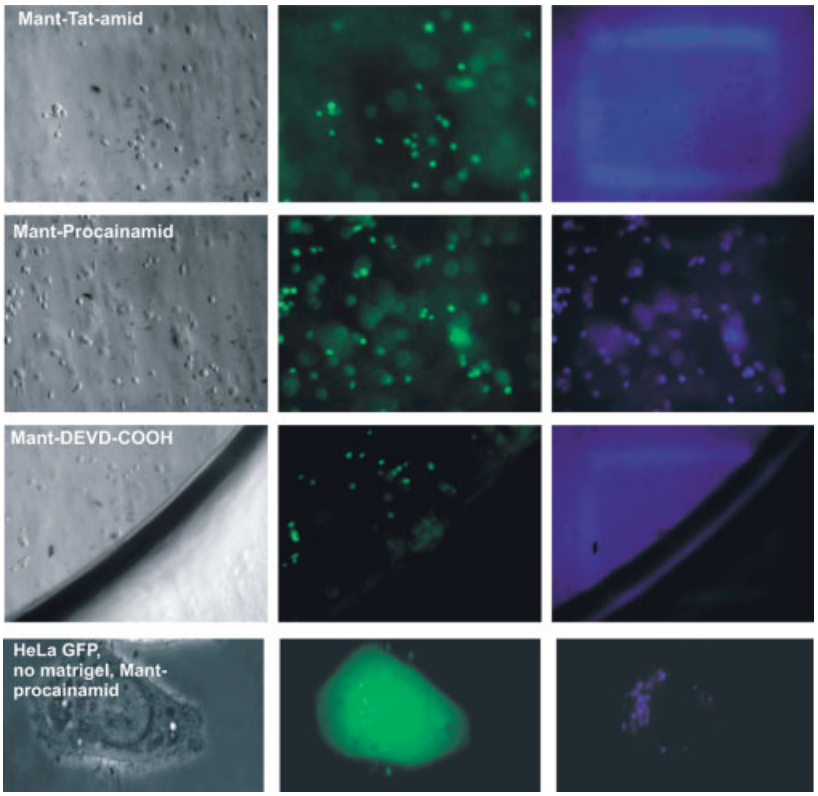

Fig. 2:
In vitro uptake study of Mant labelled Tat-peptide, Mant labelled procainamide and Mant-DEVD-COOH into HeLa GFP cells embedded in matrigel and of Mant labelled procainamide into Hela GFP cells in the absence of matrigel following 1 h incubation

in matrigel – as shown in Fig. 1 and Fig. 2. The MCF-7 affine peptides p160 and Frop-1 were taken up into the MCF-7 cells to a minor extend compared to matrigel as shown in Fig. 1. More than 95% of the fluorescence labelled peptides p160 and Frop-1 were matrigel associated. The same results (data not shown) were obtained in the case of rhodamine labelled octreotate.

In the case of procainamide the compound could only be detected within the cells, but not in the matrigel when HeLa cells were embedded in matrigel. However, intracellular uptake was much greater versus the uptake into matrigel free cells (Fig. 2). Matrigel seems to increase the extracellular concentration into the direct environment of the cells resulting in a much higher uptake by passive diffusion. In all cases about 8% of the incubation solutions were matrigel associated in the case of all tested compounds following 1 h incubations. These results indicate that in the presence of matrigel, not only the cells but the matrigel matrix as well can accumulate tumour affine compounds or alter the cellular accumulation.

2.2. Comparative quantitative determination of cellular uptake in the presence and absence of matrigel

In order to evaluate how the real cellular uptake could be determined in the presence of matrigel the following experiment was made. Cells embedded in matrigel and matrigel free cells were incubated with FITC labelled peptides p160 or Frop-1. Following 1 h incubation at 37 °C, the medium was removed and cells were washed. Matrigel embedded cells were treated with Matrisperse cell recovery solution and centrifuged in order to remove the liquefied matrigel from the cells (Kirshner et al. 2003; Rusus et al. 2005). The matrigel free cells were obtained by trypsination. In both cases equal cell counts were replaced into cell titer dishes and the fluorescence was determined. In both cases the same amount of cell associated fluorescence was measured. The results were comparable with those shown in Fig. 1 for cells in the absence of matrigel. This suggests that the separation of the cells form the matrigel seems to be a sufficient method to obtain he true cellular uptake.

2.3. Viability of matrigel embedded cells

The cell viability of cells embedded in matrigel was compared with the cell viability of cells not embedded in matrigel. Following a four day growth period cells were recovered from matrigel using Matrisperse. The matrigel free cells were harvested by trypsination. In both cases equal cells counts were examined by the tryptan exclusion assay showing that in both cases viability was about 93% $\pm$ 2.5%. Matrigel did not influence cell viability.

2.4. Residual matrigel

Matrigel residence time was determined by histological analysis of mice which had obtained fluorescent tagged matrigel matrix, non labeled matrigel or matrigel cell mixtures (MCF-7 or AR42J). As control tumor cells without matrigel were inoculated in mice as well. Thirteen days after the subcutaneous implantation of either fluorescence labelled matrigel alone or matrigel cell mixtures (MCF-7/AR42J) or tumours cells (MCF-7/AR42J) without matrigel, explants were screened for residual matrigel. In three different sections morphometric investigations showed that explants obtained from animals that had received matrigel tumour cell mixtures consisted of about 23 $\pm$ 3% matrigel. A selection of micrographs form the sections are shown in Fig. 3.

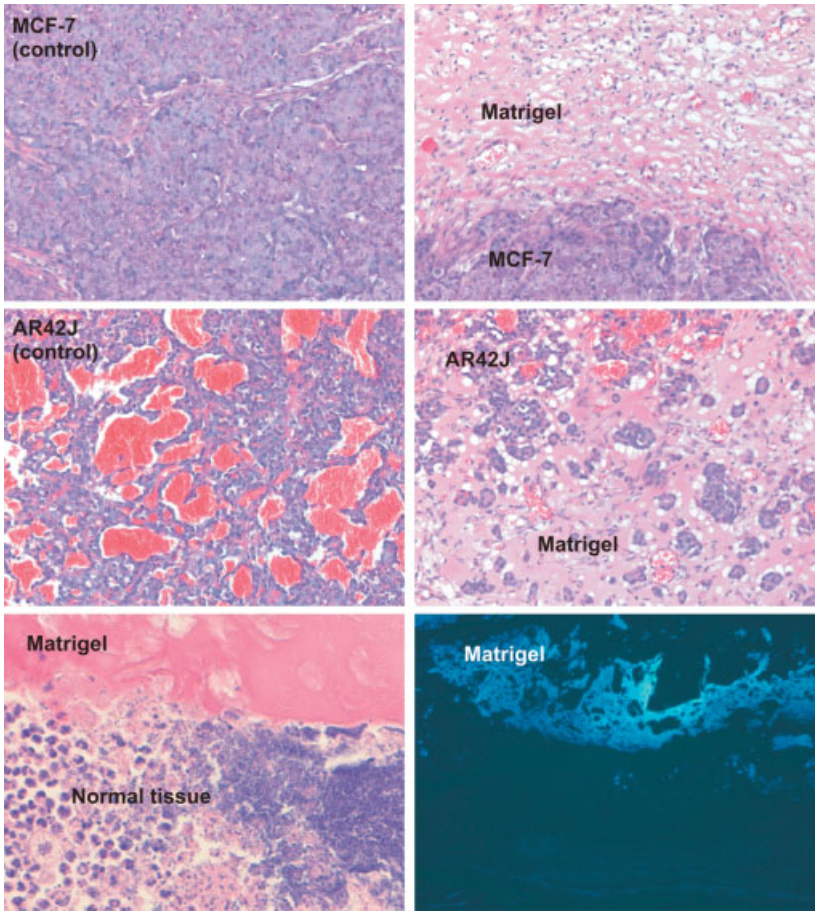

Fig. 3:
Matrigel residence time. 14 days after the inoculation explants were sectioned and examined. The tissue sections show MCF-7 and AR42J tumours in the absence of matrigel, mixtures consisting of matrigel and one of the cell lines as well as fluorophor tagged matrigel alone

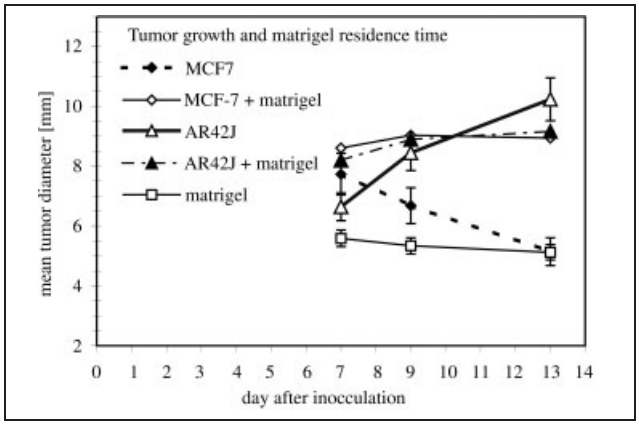

Fig. 4: Time depended tumour growth and matrigel residence time

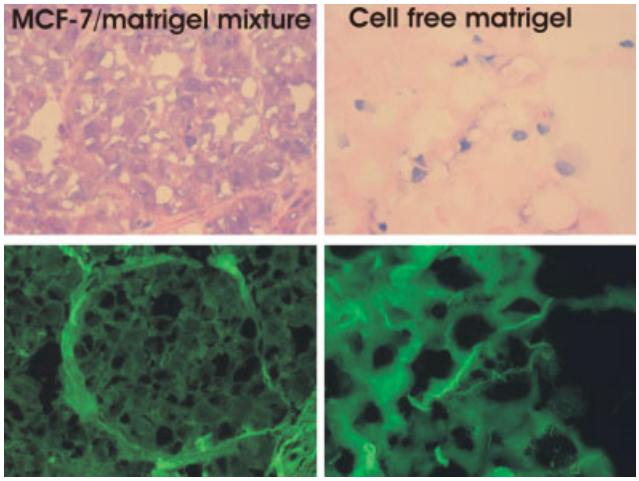

Fig. 5: *In vivo* uptake of FITC labelled p160 into MCF-7 matrigel mixtures and into matrigel 14 days after the implantation of either matrigel MCF-7 cells or matrigel alone. The explants were obtained 1 h after the i.p. injection of 10 mg/mL of FITC labelled p160, they were shock frozen and cryosectioned. The cryosectioned explants were examined by fluorescence microscopy

These results give proof that even in the presence of tumour cells matrigel is not degraded completely. Further proof that matrigel resides at the place of injection was obtained with *N*-methylanthraniloyl labelled (a fluorophor)matrigel matrix. Following a method of Hiratsuka matrigel was fluorophor labelled by reacting it with *N*-methylanthranoylic anhydride (Hiratsuka 1983). In the presence and the absence of tumour cells fluorophor tagged matrigel was subcutaneously injected into the left flank of six week old nude mice Balb C nu/nu. As shown in Fig. 3 fluorescent matrigel could be detected. In addition the influence of matrigel on tumour growth was investigated. When 1×10^7 cells were injected into mice, AR42J were progressively growing, whereas MCF-7 showed a decrease in tumour volume during the observation period. MCF-7 cells that were inoculated in the presence of matrigel and AR42J cells showed a constant diameter. Matrigel implants showed a constant diameter during the observation period. The results of the time dependent tumour growth are shown in Fig. 4.

As there is residual matrigel and matrigel can accumulate tumour affine compounds *in vitro*, *in vivo* uptake experiments were done in order to clarify the role of matrigel in biodistribution studies.

2.5. In vivo uptake experiment

Female nude mice with subcutaneous matrigel or matrigel cell mixtures received 12 days after the implantation of matrigel or the matrigel tumour mixtures intraperitoneal injections of 10 mg/kg bodyweight of FITC labelled p160 or FITC labelled Frop-1. One hour after the injection matrigel and the matrigel tumour mixtures were explanted and shock frozen. Cryosections of the explants were viewed by fluorescence microscopy. Matrigel and the tumour cells showed green fluorescence, indicating uptake of the FITC labelled peptides into matrigel and the cells with a higher fluorescence intensity in the matrigel (Fig. 5). These results show that in the presence of matrigel a too high tumour uptake can be measured.

2.6. Troubleshooting – prevention of matrigel associated artefacts

We advise one of the following procedures to avoid these artefacts. As it is difficult to separate matrigel from the tumour tissue mechanically, we propose the following methods to avoid matrigel associated artefacts in biodistribution studies and to measure the true tumour accumulation. In the case of intracellular compounds, after tumour explanation recovery of cells from matrigel matrix is accomplished by MatrisSperse solution that depolymerises the matrigel matrix within 7 h on ice or with Dispase, a metalloenzyme which gently releases the cells allowing for continuous culture. The cells can be separated from matrigel by centrifugation. As extracellular compounds might be washed away by the matrigel removal procedure another method must be used. The uptake of the compounds in control animals which received matrigel alone should be measured together with the quantity of matrigel. The amount of matrigel in explants consisting of tumour cells and matrigel should be determined. Then the amount of matrigel associated compound can be subtracted from the measured value for the matrigel tumour mixture in order to obtain the real tumour value.

In summary, this study indicates that care should be taken in biodistribution studies when tumour implantation was matrigel supported as residual matrigel can accumulate compounds or enhance the uptake of compounds into cells leading to believe in a higher tumour uptake.

3. Experimental

3.1. Chemicals

Sulforhodamine tagged octreoate (sulforhodamine B-(D)Phe-cyclo[Cys-Tyr-(D)Trp-Lys-Thr-Cys]-Thr(ol)) was purchased from Peptide Specialty Laboratories GmbH (Heidelberg, Germany). MANT-Tat_{49-57}-amid (*N*-methylanthranoyl(RKKRRQRRR)), 5-carboxyfluores-cein-Lys-p160 (FITC-KVPWMEPAYQRFL) and 5-carboxyfluorescein-Lys-FROP-1 (FITC-KEDYELMDLLAYLK) were obtained from Biosynthan GmbH (Berlin, Germany). Procainamide, FITC-dextran and acridine orange were obtained from Sigma-Aldrich (Taufkirchen, Germany). Procainamide was labelled as previously described (Wolf et al. 2007). Matrigel matrix phenolred free and Matrisperse Cell Recovery Solution (a mixture of dispase and collagenase) were obtained from BD Biosciences (Bedford, USA).

3.2. Cell culture

SkMel28 (human melanoma), MCF-7 cells (human mamma carcinoma) and AR42J cells (rat pancreas carcinoma) were obtained from the German Cancer Research Centre (DKFZ) tumour bank and grown at 37 °C as stock cultures in RPMI 1640 supplemented with 10% fetal calf serum and 1% glutamine as standard medium under a 5% CO_2 atmosphere (all components: Pan Biotech GmbH, Aidenbach, Germany).

HeLa cells expressing GFP were obtained from Biocat (Heidelberg, Germany) and grown in DMEM (Dulbeccos's Modified Eagles Medium) medium (Sigma Aldrich), supplemented with 10% FCS (fetal calf serum), 2% glutamine and 1% nonessential amino acids.

3.3. Fluorescence microscopy

5×10^4 cells were mixed with 100 µL liquid matrigel at 4 °C. The mixture was placed on glas coverslips and grown for 24 h at 37 °C using the culture conditions mentioned above. In addition 5×10^4 cells (without matrigel) were grown on glas coverslips for 24 h at 37 °C. Then the matrigel cell mixtures, cell free matrigel and matrigel free cells were incubated with the above mentioned compounds at indicated concentrations for 1 h. The cells were then washed three times with PBS and recovered with medium prior to confocal laser scanning microscopy. Confocal laser scanning microscopy was done on a tunable Zeiss LSM 510 UV instrument (Carl Zeiss, Jena, Germany) equipped with an Argon Laser (458, 477, 488, 514 nm and 30 mW) and an UV Argon laser (excitation 351–364 nm and 80 mW). For fluorescein labelled compounds and acridine orange the excitation wavelength was 488. Wavelengths between $505 < \lambda < 520$ nm were detected. The *N*-methylanthranoyl tagged compounds were excited at 359 nm, and wavelengths between $430 < \lambda < 450$ nm were detected. Images were processed with the LSM imaging browser software (http://www.zeiss.com) and exported as TIFF files.

3.4. Viability of cells embedded in matrigel

5×10^5 cells were embedded in 500 µL matrigel or were grown in the absence of matrigel for 5 days. Then matrigel was liquefied using Matrisperse following the suppliers instructions. Cells viability was determined using the tryptan exclusion assay.

3.5. Quantitative uptake studies

5×10^5 AR42J or MCF-7 cells embedded in matrigel or matrigel free were seeded in 48 well microtiter plates and grown for 24 h at 37 °C in RPMI 1640 supplemented with 10% fetal calf serum and 1% glutamine as standard medium. Cell free matrigel served as control. The cells were then incubated for 1 h in the presence of 10 µM of the above mentioned compounds. Medium was removed, cells washed 3 times and recovered with 100 µM PBS buffer. Cell associated fluorescence was measured with the Cytofluor microplate fluorescence reader device (Molecular Devices GmbH, Ismaning, Germany), at the following wavelengths: For fluorescein labelled compounds and acridine orange the excitation wavelength was 488. Wavelengths between $505 < \lambda < 520$ nm were detected. The *N*-methylanthranoyl tagged compounds were excited at 359 nm, and wavelengths between $430 < \lambda < 450$ nm were detected. Results were expressed as fluorescence intensities. Each determination was made in quadruplicate.

3.6. Comparative, quantitative uptake experiment

1×10^6 AR42J or MCF-7 cells either embedded in 100 µL matrigel or in the absence of matrigel were incubated with the above mentioned compounds for 1 h. Cells were released from matrigel by Matrisperse following the suppliers instructions. Equal cell counts of the incubated and washed cells were taken for fluorescence reader analysis. Cell associated fluorescence was measured and compared by the procedure described above.

Table 1: Time dependent tumour growth and matrigel residence time

Group	Number of animals	Type of tumour	Cell count for inoculation
1	5	matrigel	100 µL
2	5	AR42J	1×10^7
3	5	MCF-7	1×10^7
4	5	matrigel + AR42J	100 µL + 5×10^6
5	5	matrigel + MCF7	100 µL + 5×10^6

3.7. Animal experiments

One day before inoculation, the tumour cells were split 1 : 4 to achieve logarithmic-phase growth. For *in vivo* tumour studies either 1×10^7 MCF or 1×10^7 AR42J tumour cells suspended in a volume of 100 µL phosphate-buffered saline buffer, 100 µL of matrigel matrix lacking cells or mixtures of 5×10^6 cells in 100 µL matrigel were injected subcutaneously into the left flank of each of the female nude mice Balb C nu/nu (Charles River, Sulzfeld, Germany) weighing approximately 20 g. All animal experiments were performed in compliance with the German Animal Protection Laws (Permit 35-9185.81/G-128-06, Reg.-Präsidium, Karlsruhe, Germany).

3.8. Matrigel residence time and tumour growth in the presence and absence of matrigel

After the inoculation of the tumours, the injection of cell free matrigel or matrigel tumour cell mixtures, tumour growth and matrigel residence were determined by measuring two perpendicular diameters starting directly after tumour/matrigel inoculation. The inoculation schedule is given in Table 1.

These measurements were continued on every second or third day until the end of the experiment (two–three weeks after inoculation). At the end of the experiment animals were killed by CO_2. Tumours, matrigel and matrigel-tumour cell-mixtures were dissected and either fixed with a 10% formalin solution or shock-frozen in nitrogen. The shock-frozen explants were stored at −70 °C before histochemistry. Matrigel explants and tumours were sent to University Professor Dr. Achim Gruber, Institute for Animal Pathology of the Freie Universität Berlin in whose lab the samples were sectioned (6 µm) or cryosectioned (6 µm) with subsequent immunohistochemical analysis. Additionally matrigel explants of animals that had received fluorophor tagged matrigel were examined by fluorescence microscopy.

Formula for the calculation of the mean tumour diameter:

$$\text{mean diameter} = (\text{product of the two perpendicular diameters})^{1/2}.$$

3.9. Biodistribution studies

Fourteen days after tumour inoculation, injection of matrigel-tumour cell mixtures or the implantation of cell free matrigel the mice were divided into eleven groups and the accumulation of the compounds in matrigel-tumour cell mixtures, cell free matrigel or matrigel free tumours was examined. Division into different groups was performed. The division into groups, doses of the agents, dose schedules and route of injection of the treatment are summarized in Table 2.

The injection volume was 100 µL of one of the fluorophor tagged peptides. One hour after the injection, animals were killed with CO_2. Tumours, matrigel and matrigel-tumour cell-mixtures were dissected and shock-frozen in nitrogen. The shock-frozen explants were stored at −70 °C before cryosectioning. Matrigel explants and tumours were sent to University Professor Dr. Achim Gruber, Institute for Animal Pathology of the Freie Universität Berlin in whose lab the samples were sectioned (6 µm) or cryosectioned (6 µm) with subsequent immunohistochemical analysis. The explants were examined by fluorescence microscopy. Matrigel explants were compared with matrigel free tumours and matrigel tumour mixtures.

3.10. Statistical analysis

All measurements were expressed as mean ± SD (standard deviation). Statistical significance was evaluated by a two-sided Student's t-test assuming unequal variances followed by a one-way analysis of variance. The analy-

Table 2: Arrangement groups and dose schedule for mice bearing a subcutaneous tumour, subcutaneous matrigel or matrigel-tumour cell mixture for the determination of the influence of matrigel on biodistribution studies

Group	Number of animals	Type of tumour	Cell count for inoculation	Agent (10 mg/mL)[c]	Route of injection
1	5	matrigel[a]	100 µL	FITC-K-p160	i.p.
2	5	matrigel[a]	100 µL	FITC-K-FROP-1	i.p.
3	5	matrigel[a]	100 µL	Sulforhod-TOC	i.p.
4	5	AR42J[b]	1×10^7	Sulforhod-TOC	i.p.
5	5	MCF-7[b]	1×10^7	Sulforhod-TOC	i.p.
6	5	MCF-7[b]	1×10^7	FITC-K-p160	i.p.
7	5	MCF-7[b]	1×10^7	FITC-K-FROP-1	i.p.
8	5	matrigel + AR42J	5×10^6 + 100 µL	Sulforhod-TOC	i.p.
9	5	matrigel + MCF7	5×10^6 + 100 µL	Sulforhod-TOC	i.p.
10	5	matrigel + MCF-7	5×10^6 + 100 µL	FITC-K-p160	i.p.
11	5	matrigel + MCF-7	5×10^6 + 100 µL	FITC-K-FROP-1	i.p.

[a] Cell free matrigel; [b] Matrigel free tumours; [c] Dose for all groups, each animal received 100 µL

sis was performed using Analyse-It Software (Analyse-It Software, Ltd, Leeds, England Letchworth Hearts, UK). A P-value less than 0.05 was considered to be significant.

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