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The impact of altered matriptase activity on cell viability and spheroid growth in 2 and 3D cell cultures

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Table of contents

1. List of abbreviations

AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
bFGF	basic fibroblast growth factor
C26	murine colorectal adenocarcinoma epithelial cell line
DMEM/F-12	Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture
ECM	extracellular matrix
EDG	endothelial differentiation gene
FBS	foetal bovine serum
HAI-1	hepatocyte growth factor activator inhibitor type 1
HGF/SF	hepatocyte growth factor/scatter factor
HT-29	human colon epithelial cells from a colorectal adenocarcinoma cell line
IPEC-J2	intestinal porcine epithelial cells from an unsuckled, neonatal piglet
KD	Kunitz domain
LDL	low density lipoprotein
MCTS	multicellular tumour spheroid
MI-432	matriptase inhibitor – 432
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, a water-soluble formazan dye compound
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAR-2	protease-activated receptor 2

TI	therapeutic index
TJ	tight junction
TTSP	type II transmembrane serine protease
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor

2. Introduction

The European Commission are aiming to phase out animal testing in medical research, although they are waiting for a suitable alternative to become available first. Therefore, finding alternatives to animal testing is a key strategy throughout Europe and around the world. One such alternative are the 3D cell culture systems, which provide a much more accurate representation of *in vivo* conditions than the traditionally used *in vitro* monolayer cell culture, which does not truly represent the physiological cell-cell connections in 3 dimensions, or the relationship between cells and the extracellular matrix (ECM) as would be found within the body.

An essential part of anti-cancer drug testing is the preclinical trial, during which the potential new drugs are tested in both *in vitro* conditions and *in vivo* laboratory animals. Prior to the *in vivo* animal testing, the drugs are first tested extensively *in vitro*, using cell cultures, to determine the cytotoxicity and effect of the drugs. With the monolayer cultures, factors such as the drug diffusion through the layers of cells and ECM cannot be determined, whereas with the 3D cultures, such parameters can be investigated in *in vitro* models.

One type of the 3D cell cultures is the ball-like culture, the spheroid. A potential problem with the development of the 3D spheroid cell cultures is the poor reproducibility of standard, identical spheroids for research purposes. Different methods have been developed to try to reproduce identical spheroids on a mass scale. A current method under investigation for this purpose is the use of non-adhesive agarose gel casts developed from silicone micro moulds, and the formation of 3D cell aggregates from cell lines in the identical wells of the micro mould. The standard mould cast produces standard size wells, with identical diameters, which allow the formation of identically sized spheroids. This gives the advantage of producing many spheroids of similar size with relative ease. As the cells form their intercellular adhesions, they produce ECM to support the 3D structure development.

Matriptase is a type II transmembrane protease which plays a physiological role in the growth and maturation of epithelial cells and in the maintenance of epithelial homeostasis. Under pathological conditions, the impact of matriptase activity in tumour growth and metastasis has been newly discovered and there is growing evidence which reveals that enhanced matriptase expression in tumours can be of diagnostic importance in effective cancer treatment.

In our experiment, 3 different cell lines were used for our *in vitro* studies- a non-carcinogenic cell line (IPEC-J2), and 2 tumorigenic cell lines (HT-29 and C26). The cells were also used to investigate the correlation between the impact of matriptase activation and inhibition on cell viability and tumour cell growth, which could be a potential target for anti-cancer drugs.

The main purpose of the spheroids is to mimic, as closely as possible, the *in vivo* cellular conditions in an *in vitro* environment, and enable the additional experimental parameters of surface area and spheroid diameter to be measured in response to drug applications, such as potential anti-cancer agents.

3. Scientific goals

The main aim of these experiments was to create viable 3D spheroid cell cultures from different epithelial cell lines including both cancerous (HT-29 and C26) and non-tumorigenic (IPEC-J2) cells. The ability of the different epithelial cell lines to form spheroids, as well as the dimensions, such as surface areas, of the formed cellular aggregates of different cell lines were monitored. The effects of the non-selective and selective matriptase inhibitors, as well as the effects of the chemical inducer of matriptase, suramin, at different concentrations on the growth and dimensions of the spheroids and on the cell viability using monolayers were also determined.

4. Literature overview

4.1. Application of cell monolayers and spheroids as an in vitro model system

The *in vitro* models should resemble, as closely as possible, the *in vivo* state. This enables the results to be as true a representation as possible of the *in vivo* condition, which allows a more accurate prediction of drug effects, such as alteration in cell function and toxicity on the host cells. The use of 3D cell cultures, known as spheroids or, when created from a tumour cell lines, multicellular tumour spheroids (MCTS), are a more precise representation of the *in vivo* structure (where cells can synthesise their own ECM, and so have both cell-cell and cell-ECM contacts) in contrast to the traditionally used 2D cell monolayer models, which only have cell-cell contacts (due the reduced capability of the 2D *in vitro* cells to generate an ECM). Additionally, the presence of an ECM in the 3D cell models causes an increase in interstitial pressure, creating an additional barrier to drug diffusion, potentially contributing to drug resistance, which is similar to the *in vivo* situation. As a result, spheroids could be a useful model for anticancer drug testing, as they are more precise in mimicking an avascular solid tumour nodule (Gong et al., 2015).

In vitro experimentation to produce reliable results with 3D cell models depends on the reproducibility of uniformly sized spheroids. Previously used methods to generate spheroids have had difficulty in creating easily reproducible, uniform 3D models. By using agarose gel templates containing uniformly sized micro-wells, formed from commercially available cell culture plates, large scale production of spheroids can be achieved with relative ease, and simple monitoring of the spheroid development using optical microscopy is also possible. By controlling the cell seeding volume, the size of the produced spheroids can also be controlled (Gong et al., 2015).

4.2. The importance of spheroid size

The size of the individual spheroid cultures is important, as the physiological and biological processes within the spheroids change with the spheroid size. Experiments showed that 3D spheroid cultures of a smaller size have a higher proliferation capacity, compared to spheroids of larger sizes. It was also noted that larger MCTS (>200 μm diameter) contained a central, hypoxic, necrotic core, then a layer of quiescent cells, and then a peripheral proliferating cell

layer, which is a structure similar to that of a solid tumour nodule. It is also reported that larger sized spheroids exhibited higher drug resistance in comparison to smaller spheroids, which correlates with their study regarding vascular endothelial growth factor (VEGF) (released in hypoxic conditions, such as in the central necrotic core) which has been shown to be connected to chemoresistance (Gong et al., 2015).

4.3. Characterisation of IPEC-J2, HT-29 and C26 cell lines

IPEC-J2 cell line is a non-transformed intestinal cell line originally derived from jejunal epithelial isolated from a neonatal, unsuckled piglet (Cencic and Langerholc, 2010). They have structures such as microvilli and tight junctions (TJ), like the *in vivo* cells, so can be applied as an *in vitro* simple epithelial monolayer model system of the porcine enterocytes (Schierack et al., 2006).

HT-29 cells- are human colon adenocarcinoma cells which have the morphology of epithelial cells. It was found that if glucose is replaced with galactose in the cell culture medium, the HT-29 cells will differentiate in to functional enterocytes, a type of epithelial cell (Brehier and Thomasset, 1988).

C26 cells are derived from a murine colorectal tumour, formed from epithelial cells. They are described as a having a low tendency to metastasise, although they have a high mortality rate (Aulino et al., 2010).

3D spheroid cell models can be grown from both non-tumorigenic and tumorigenic cell lines. Cancer cells have different properties, such as a different glycosylation pattern, and can also display different biological functions, such as increased cell proliferation, in comparison to healthy cells (Cencic and Langerholc, 2010).

4.4. The biological role of matriptase

The enzyme matriptase has become a source of interest for those researching in to cancer and the regulation of cell proliferation. Matriptase is a type II integral transmembrane serine protease (TTSP), which has been shown to have important roles in epithelial homeostasis. Matriptase seems to be essential, in animal models, for postnatal survival, epidermal barrier function, stratum corneum and hair follicle development and thymocyte survival and plays an important role during neural tube closure (Lee et al., 2005; Chen et al., 2012).

Matriptase is synthesised as a single chain zymogen which is converted, via cleavage at its intact active triad, in to the two-chain active form of the enzyme (Lee et al., 2005; Lee, 2006). Matriptase has a cognate inhibitor, called HAI-1, which is required for the regulation, both in terms of activation and inhibition of the enzyme. The requirement for HAI-1 in the activation of matriptase indicates a close temporospatial relationship between matriptase and its cognate inhibitor, suggesting that matriptase activation and inhibition is a very tightly controlled process (Lee et al., 2005; Lee, 2006).

It is reported that HAI-1 is a type I integral membrane protein which is synthesised already in its functional form, and contains 2 Kunitz-type domains (KD). KDs competitively inhibit their target serine proteases. Between the two KDs is a LDL receptor class A domain, which is required for matriptase activation. Whereas the bonds between latent matriptase and HAI-1 are very weak, HAI-1, almost immediately after matriptase activation, binds very strongly (although non-covalently) to activated matriptase. Other than binding with HAI-1, matriptase activation can be controlled by ectodomain shedding, a process which is thought to be activated at the same time as the activation of matriptase (Lee et al., 2005; Lee, 2006).

Many of the pathological roles of matriptase closely involve HAI-1 as well. Matriptase can result in malignant cancer formation. If the balance between the expression of matriptase and HAI-1 is lost, resulting in a relative increase of matriptase to HAI-1, it is linked to increased cancer development and metastasis, poor patient outcome, and correlates with different clinical tumour stages (Lee et al., 2005; Lee, 2006).

Even a small increase in matriptase activity in relation to HAI-1 activity results in the formation of cancers, and increases the risk of tumour metastasis. However, it was also reported that if matriptase increases in proportion with HAI-1, then spontaneous cancers did not develop. Whilst several substrates for matriptase have been identified, there are three protein substrates in particular which could contribute to the cancer-related roles of matriptase. These are: urokinase-type plasminogen activator (uPA), hepatocyte growth factor/scatter factor (HGF/SF) and protease-activated receptor 2 (PAR-2) (Uhland, 2006).

uPA has roles in tissue remodelling, invasiveness and angiogenesis. HGF/SF has many roles, including increased cell motility, and has mitogenic, morphogenic and motogenic properties which have been shown to have roles in tumorigenesis, tumour cell invasion and angiogenesis

(Uhland, 2006). PAR-2 potentially promotes tumour cell growth and vitality (Lee et al., 2005; Lee, 2006).

Additionally, a secreted form of matriptase has been shown to cleave elements of the ECM- laminin and fibronectin- which mediate cellular attachment and migration (Uhland, 2006). However, these roles may be important for physiological processes such as wound healing and growth (Benaud et al., 2001). Matriptase activity is also needed for proper epithelial barrier integrity, and modulation of matriptase activity could be useful in the therapeutic treatment of epithelial barrier dysfunctions (Pászti-Gere et al., 2015).

Whilst certain naturally occurring protease inhibitors, such as soybean trypsin inhibitor, can also inhibit matriptase, there is great interest in drug development of other matriptase inhibitors as potential anti-cancer treatments (Chen et al., 2012).

4.5. Substances regulating matriptase activity

4.5.1. Suramin as a physiological inducer of matriptase

Suramin is a symmetric polyanionic naphthlyurea which, although it has a very low therapeutic index (TI) due to its severe toxicity, could potentially be developed in to a new class of antineoplastic drugs, as it has been shown to have angiostatic properties, inhibiting bFGF-induced angiogenesis (Firsching et al., 1995). It is found to be a chemical inducer of matriptase because after suramin treatment, the matriptase activation foci were found to be relocated in the cytoplasm (Lee et al., 2005; Lee, 2006).

4.5.2. Non-selective and selective matriptase inhibitors

Some derivatives of sulfonylated 3-amidinophenylalanine are selective matriptase inhibitors. Of these derivatives, MI-432 was found to be one of the most potent matriptase inhibitors (Hammami et al., 2012; Meyer et al., 2013). CVS-3983, an arginal derivative, is a selective matriptase inhibitor, with the ability to suppress the growth of androgen independent prostate tumour xenografts (Galkin et al., 2004).

5. Materials and methods

5.1. Cell cultures and media

The IPEC-J2 cell line used in this study was derived from jejunal epithelia of an unsuckled, neonatal piglet and was a kind gift from Dr. Jody Gookin, North Carolina State University, USA. IPEC-J2 cells are a non-transformed cell line that in some respects mimics *in vivo* conditions when cultured on membrane inserts. The cells form a differentiated layer and are attached to each other via apical tight junctions. IPEC-J2 cells were seeded at a density of 1.5×10^5 per well on six-well plates with Transwell polyester membrane inserts (pore size 0.4 µm; surface area 4.67 cm^2; Sigma, Germany) coated with rat tail collagen (Sigma) in a 1.5 ml apical and 2.6 ml basolateral volume. The cells were maintained in a complete medium containing 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12) supplemented with 5% FBS, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 5 ng/ml epidermal growth factor and 1% penicillin-streptomycin (all from Fisher Scientific, USA). The cell cultures (***Fig. 1.***) were tested by PCR and were found to be free of mycoplasma contamination.

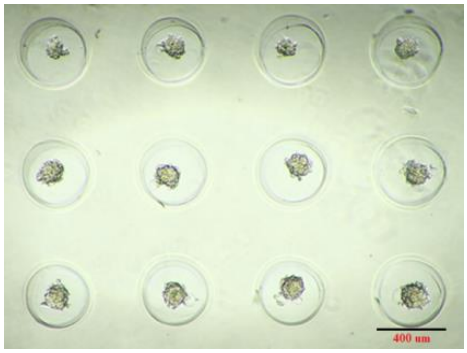


Fig. 1. *IPEC-J2 cells forming cell aggregates*

C26 cells were a kind gift from Semmelweis University. Their culturing medium consisted of: RPMI 1640 (Roswell Park Memorial Institute) (Sigma-Aldrich), fortified with 2mmol L-glutamine and 10% foetal calf serum. The conditions for the culture (***Fig. 2.***) were 37˚C and 5% CO_2. The incubation time was a three days.

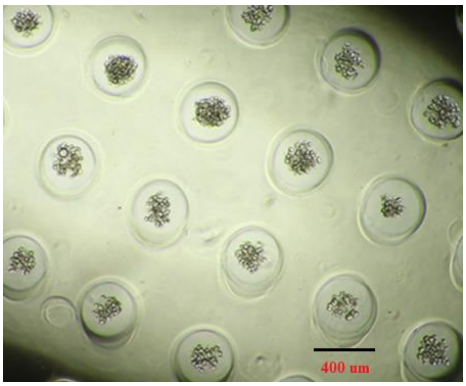


Fig. 2. *C26 tumour cells forming spheroids in an agarose gel micro mould cast*

HT-29 cells (***Fig. 3.***) are human colon epithelial cells from a colorectal adenocarcinoma, also a kind gift from Semmelweis University, Budapest. The culture medium consisted of: Eagles DMEM (Eagles Dulbecco's Modified Eagles Medium) (Sigma-Aldrich) and 10% foetal calf serum. The culturing conditions were also 37˚C and 5% CO_2, for three days.

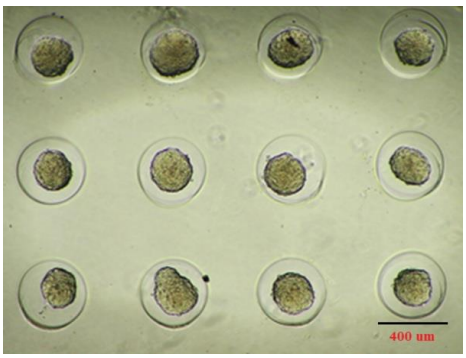


Fig. 3. *HT-29 cells forming spheroids in an agarose gel cast from a micro mould plate*

Cells were allowed to adhere for 24 hr before the medium was added. They were grown at 37°C in a humidified atmosphere of 5% CO_2 prior to spheroid growth and cytotoxicity experiments.

5.2.Spheroid preparation

The moulds used to create the spheroid cell cultures were from the Microtissues Natural 3D company. The material used for casting was non-adhesive agarose gel, and casting was performed under sterile conditions according to the instructions of the manufacturer (Microtissues Natural 3D). The size of micro-mould used was the small-spheroid-producing

24-96 3D Petri dish, consisting of an 8 x 12 (total 96) well plate. The volume of agarose used was 330 µl and the cell seeding volume 75 µl (***Figs. 4. and 5.***).

Fig. 4. *96 well micro mould with 400 µm diameter wells as used to create the agarose non-adhering cell culture unit*

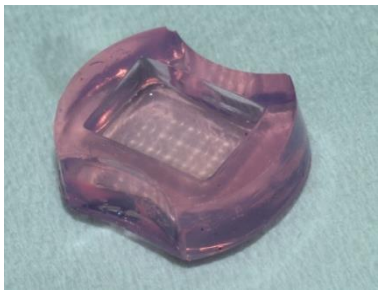


Fig. 5. *Solidified non-adhering agarose gel unit ready to use for culturing spheroids*

The cell cultures were then placed in an incubator for 7 days (37˚C, 5% CO_2). The cultures were checked every day, and the media surrounding the 3D petri dishes were changed regularly, every 3rd day.

5.3.Spheroid size measurement

The visible area of the spheroids was measured under a light microscope. Digital images of the spheroid cultures were taken on the 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, and 7^{th} days using a Canon D1100 camera (Canon Inc) connected to the microscope. The photographs were used to digitally compute the area of the spheroids visible in the images. Measurements were then carried out using ImageJ (Schneider et al., 2012) software, and we used a standard Burker-chamber for the standard size. The collected data was entered in to a table for easier comparison.

5.4.Exposure of IPEC-J2 cells to matriptase inducer

Before treatment IPEC-J2 cells were washed twice with plain medium. The matriptase activator, suramin, was added for different time intervals. After incubation, the cells were washed twice with plain medium before being subjected to the subsequent procedures.

The chemical structure of suramin as an inducer of matriptase activation can be seen in ***Fig. 6.***

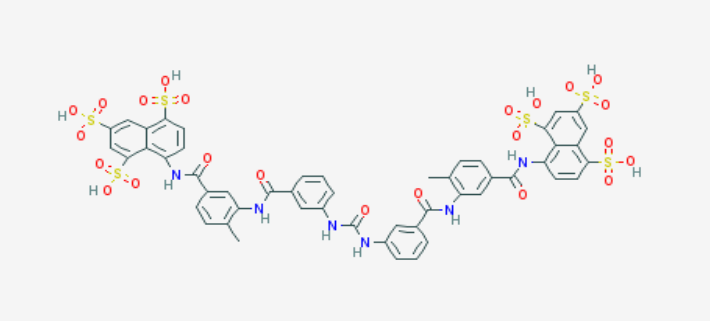


Fig. 6. *2D chemical structure of suramin*
(http://pubchem.ncbi.nlm.nih.gov/compound/suramin#section)

5.5.Exposure of IPEC-J2 cells to matriptase inhibitors

Before treatment, the confluent layers of IPEC-J2 cells were washed twice with plain medium. The non-selective broad-spectrum serine protease inhibitor AEBSF (***Fig. 7.***) was applied on IPEC-J2 cell monolayer at 25 µM. The solutions of the 3-amidinophenylalanine-derived matriptase inhibitors (***Table 1.***) in phenol red free DMEM at 50 µM were prepared freshly prior to each experiment from 10 mM stock solutions, which were stored at -20 °C. Matriptase inhibitors were added for different time intervals. After incubation, the cells were washed twice with plain medium before being subjected to the subsequent procedures.

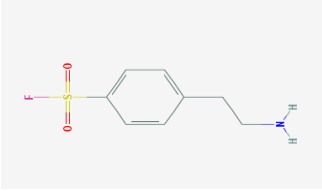
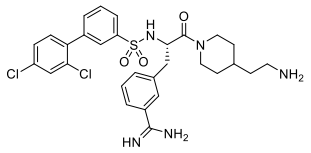
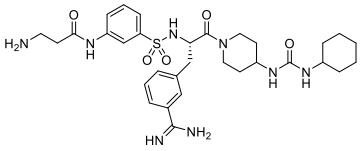
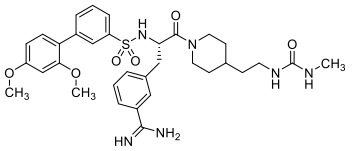
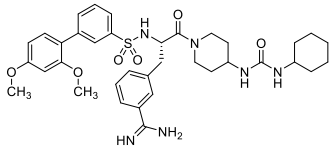


Fig. 7. *2D chemical structure of AEBSF* (http://pubchem.ncbi.nlm.nih.gov/compound/4-_2-Aminoethyl_benzenesulfonyl_fluoride#section=Top)

Table 1. *The chemical structures of the specific inhibitors used for the experiment, Ki values of the inhibitors are added relevant to matriptase and thrombin (Pászti-Gere et al., 2015).*

No	Structure	Ki (µM)	
		Matriptase	Thrombin
432		0.002	0.02
453		0.008	35.9
460		0.0018	0.051
463		0.0027	1.23

5.6.MTS assay for cell viability

The influence of the matriptase activator suramin (at 50, 100 and 200µM) in phenol red free DMEM was used to test the viability of the enterocytes. IPEC-J2 cells were seeded in a 96-well plate and incubated with suramin for up to 48 hr in treated groups. The control cells were incubated only with phenol red free DMEM. After removal of the medium and washing the cells 3 times with phosphate-buffered saline buffer (PBS), 20 µl of CellTiter96 aqueous one solution (Promega Corporation, Madison, WI ,USA) containing a tetrazolium compound, MTS, and an electron coupling reagent, phenazine ethosulfate, was pipetted into each of the sample-containing wells of the 96-well assay plate in 100 µl of culture medium. The plate was

incubated with the dye for 2 hr in a humidified atmosphere, containing 5% CO_2. Viability of IPEC-J2 cells was measured at 490 nm using an EZ Read Biochrom 400 microplate reader after 48 hr treatment with matriptase activators (***Fig. 8.***).

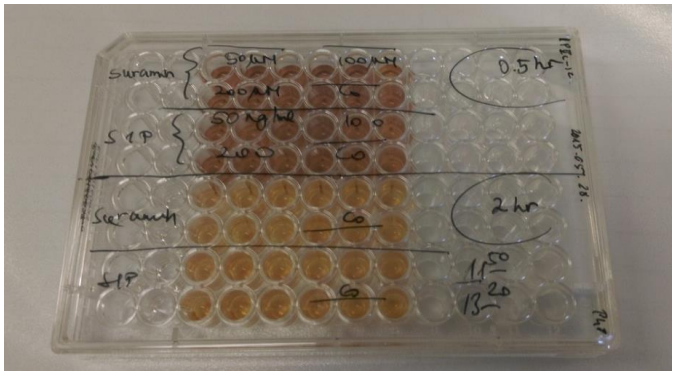


Fig. 8. *MTS cytotoxicity test performed on IPEC-J2 cell exposed to matriptase modulators in 96 well plates with the 6 x 8 layout.*

The cell inhibition %, as a marker of the extent of compound cytotoxicity, was then evaluated using MTS assay results from the spectrophotometry. The inhibition % was calculated as follows:

$$\text{inhibition\%} = 100 - ((\text{actual absorbance value}/\text{control absorbance value}) \times 100)$$

5.7.Statistical analysis

For statistical evaluation R 2.11.1 software package (2010) was applied. Differences between means were evaluated by one-way analysis of variance (one-way ANOVA) with post-hoc Tukey test, where data were of normal distribution and homogeneity of variances was confirmed. Differences were considered significant if the p value was <0.05.

6. Results

6.1. IPEC-J2 cell line

6.1.1. Assessment of IPEC-J2 cell viability after matriptase activation

Cell cytotoxicity assays were performed to evaluate the highest concentration of suramin which did not cause significant cell death. It was found that suramin at 100 and 200 µM concentrations decreased cell survival to a greater extent, indicating that only 50 µM suramin for 48 hr can be administered to the cell monolayers to see the effect of matriptase activation on barrier integrity without massive loss of IPEC-J2 cells (***Fig. 9.***).

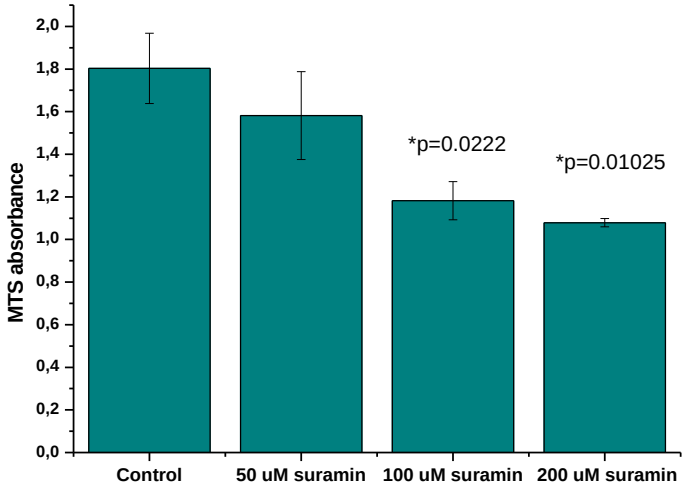


Fig. 9. *48 hr incubation of IPEC-J2 cells with suramin at different concentrations of 50, 100 and 200 µM. Values represent average absorbance values of produced MTS formazan in metabolically active cells± SEMs. Significant differences were found between the control groups and the cell monolayers treated with higher concentrations (100 µM, *p=0.0222 and 200 µM, *p=0.01025) of suramin.*

6.1.2. Assessment of IPEC-J2 cell viability after matriptase inhibition

IPEC-J2 cell groups were treated with 50 µM MI-432 and 50 µM AEBSF for different time durations and cell cytotoxic properties were checked at 24 and 48 hr time points.

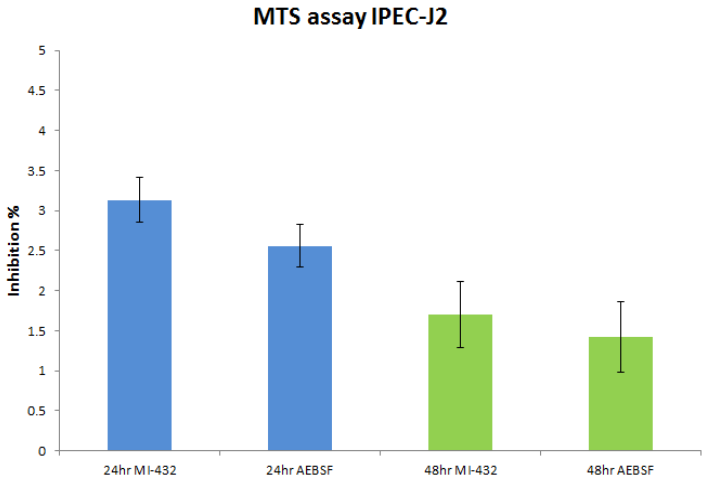


Fig. 10. *MTS assay of IPEC-J2 cells exposed to MI-432 and AEBSF. The results are represented as an average inhibition %s ± SDs, n=5.*

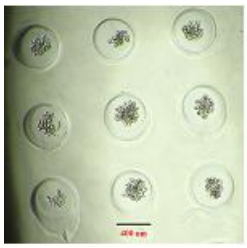
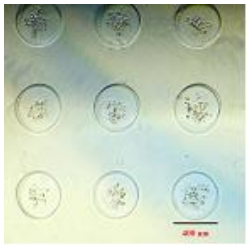
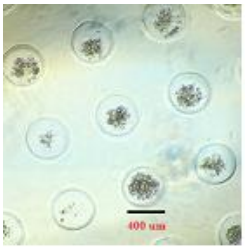
From the results in ***Fig. 10***, it was seen that the viability of the IPEC-J2 cells was not significantly affected by the administration of either 50 µM MI-432 or AEBSF. In the 24 hr group for MI-432, the average inhibition % was approximately 3.1± 0.3 %, in comparison to the 48 hr group, whereas the inhibition was approx. 1.7 ± 0.4 %. For AEBSF, in the 24 hr group, the inhibition % was approx. 2.5 ± 0.2 %, whereas in the 48 hr group it was approx. 1.4 ± 0.4%.

6.1.3. Treatment of IPEC-J2 cell aggregates with matriptase inhibitors

The IPEC-J2 cells were unable to form true spheroids, merely forming loose cell aggregates, with an average diameter after 3 days of growth of 150 µm.

Table 2. contains microscopic images taken of the control and treated IPEC-J2 cell cultures. It can be seen that the agglomerates of the IPEC-J2 cells were destroyed either by the selective matriptase inhibitor, MI-432 or by the non-selective serine protease inhibitor, AEBSF treatment. The connection between the cells seems to be greatly decreased, and the cells look to be separating from each other.

Table 2. *IPEC-J2 cell aggregate treatment with specific and non-specific matriptase inhibitors*

Control	MI-432	AEBSF
		

6.2.HT-29 cell line

6.2.1. Assessment of HT-29 cell viability after matriptase activation

HT-29 monolayer cancer cell lines were treated with 50, 100 and 200 µM suramin for 48 hrs (***Fig. 11.***).

After the application of the chemicals, the cell inhibition % was calculated.

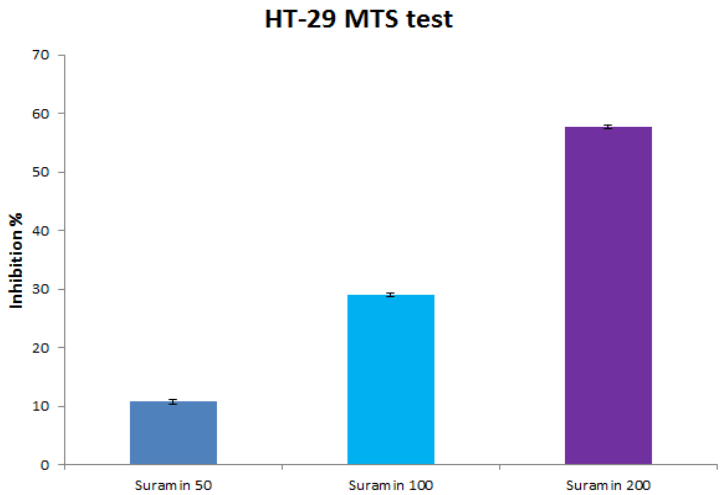


Fig. 11. *Concentration dependent cell viability response to suramin with HT-29 cells. As the concentration of suramin increases, so does the inhibition %. The results are represented as an average inhibition %s ± SDs, n=5.*

The cell viability of the HT-29 cells when subjected to suramin decreased as the concentration of suramin increased. At a concentration of 50 µM suramin, the inhibition % was approximately 10%. At 100 µM, the inhibition increased to approx. 30%, and at 200 µM, the inhibition was almost 60%. This is again indicative of the anti-tumorigenic effect of suramin in high concentrations.

6.2.2. Assessment of HT-29 cell viability after matriptase inhibition

To investigate the effects of matriptase inhibition, HT-29 monolayer cancer cell lines were used to assess the response of the cells to treatment by non-selective AEBSF- and selective- MI-432, MI-453, MI-460, MI-463, MI-469- matriptase inhibitors. (***Fig. 12.***)

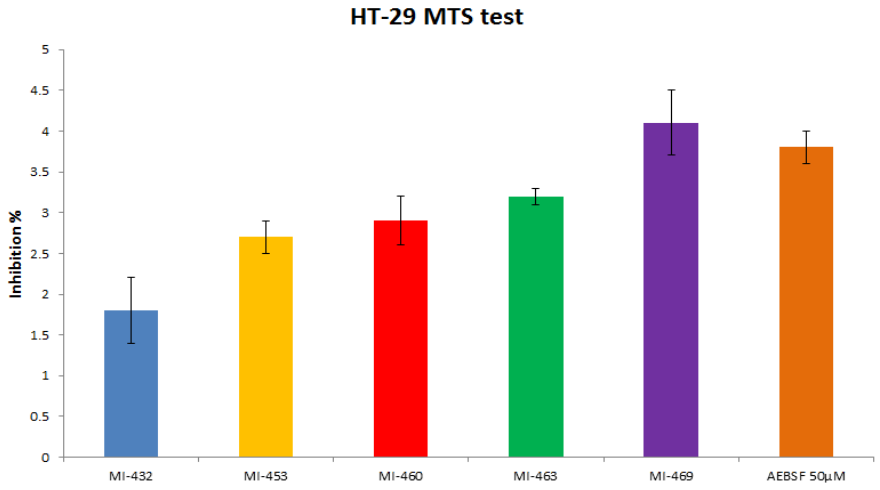


Fig. 12. *The effects of matriptase inhibitors on the viability of HT-29 cells. The average inhibition % calculated using MTS assay at a wavelength of 490 nm are shown here. The error bars demonstrate the standard deviations of each result (n=4). The results are represented as an average inhibition %s ± SDs.*

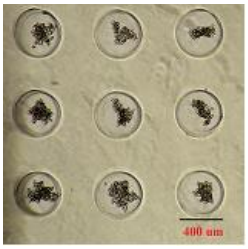
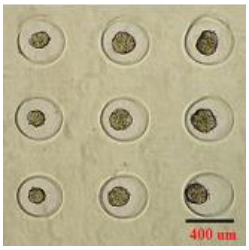
The viability of the HT-29 cells was not greatly affected by the matriptase inhibitors. Although there were slight variances between the groups in the level of inhibition %, they were all below 5%.

6.2.3. HT-29 spheroid development

As shown in ***Table 3.*** on the first day of the growth period for the HT-29 spheroids, the cells can be seen to begin to aggregate and form a spherical shape. By the third day, the HT-29 cells had formed spherically-shaped bodies, and which were increasing in size.

The average diameter of the HT-29 spheroids after a growth period of 3 days was 250-300 µm.

Table 3. *Microscopic images taken of the growth progressions of HT-29 spheroids over a period of 3 days.*

1st day	**2nd day**	**3rd day**
		

6.2.4. HT-29 spheroid treatment

In the case of treatment of the tumour cells, the effects of the selective and non-selective matriptase inhibitors were either smaller or absent in comparison to that caused by the inhibitors on the healthy IPEC-J2 cells. In the HT-29 cell group, the MI-432 treatment caused no visible effect to the spheroid structure, whereas the AEBSF treatment caused a mild destructive effect.

The suramin treatment had a destructive effect on the HT-29 cell spheroids. As can be seen in the images in ***Table 4.*** cells are detaching from the main spheroid mass, indicating that the adhesive structures between the cells have been highly damaged and weakened by the treatment.

Table 4. *Microscopic images taken of HT-29 spheroids after treatment with different matriptase activity regulators.*

Control	MI-432	AEBSF	Suramin
			

6.2.5. HT-29 spheroid growth curves

The HT-29 cell spheroid growth was investigated and measured, and the effects of the specific matriptase inhibitors on the area of the spheroids vs the incubation time was measured and compared to a control group. Each of the specific matriptase inhibitors, at a concentration of 50 µM, was used on HT-29 cell spheroids, and the area of the spheroids was measured over a 7 day period, and the results plotted against a control group, as shown in ***Fig. 13.***

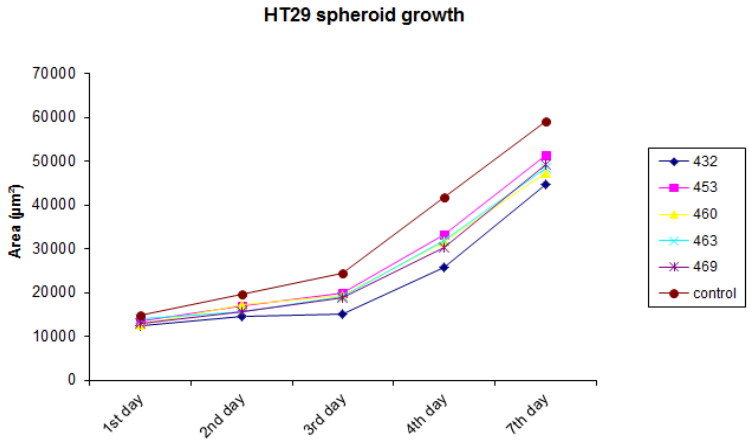


Fig. 13. *Spheroid growth curves over seven days of HT-29 cells in the presence of matriptase inhibitors (n=2). The average areas of the spheroids are shown in µm^2.*

As can be seen on the graph, the area of the spheroids in the control group was larger than that of the other groups which were treated with the specific matriptase inhibitors. The inhibiting agents did not have a strong detrimental effect on the growth of the spheroids, except for MI-432, which can be seen on the graph to have lower values for the average spheroid size each day than the rest of the inhibiting drugs.

6.3. C26 cell line

6.3.1. Assessment of C26 cell viability after matriptase activation

C26 monolayer cancer cell lines were treated with suramin at concentrations of 50, 100 and 200 µM for 48 hrs. The MTS assay was performed for cell viability and the inhibition % calculated using the same methods as were used with the HT-29 cells.

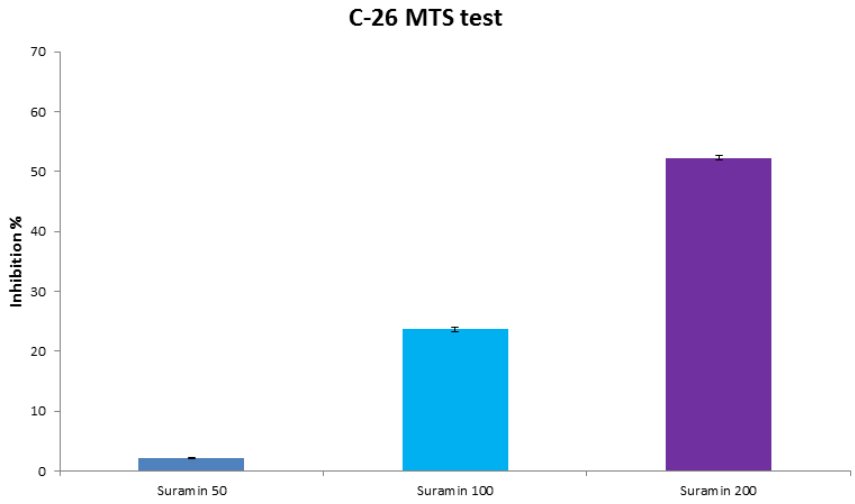


Fig. 14. *Concentration dependent suramin response on C26 cell viability. The inhibition % is greatly increasing as the concentration of suramin increases. The results are represented as an average inhibition %s ± SDs.*

Fig. 14. clearly shows that suramin at concentrations of 100 and 200 µM are powerful cytotoxic agents, with strong anti-cancer effects on the C26 cells. The 200 µM concentration of suramin had inhibitory effects of over 50%, meaning that more than half of the C26 monolayers exposed to suramin treatment for 48 hr at this concentration were no longer alive.

6.3.2. Assessment of C26 cell viability after matriptase inhibition

Fig. 15. shows the results when the C26 tumorigenic monolayer cell lines were treated with non-selective-AEBSF- and selective- MI-432, MI-453, MI-460, MI-463, MI-469 matriptase inhibitors.

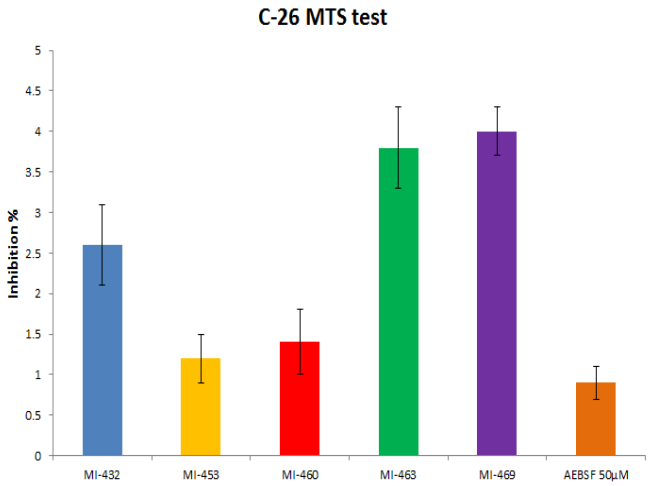


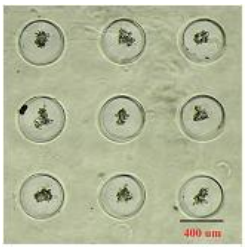
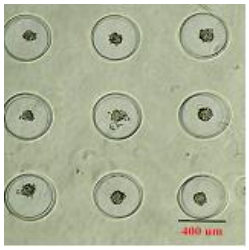
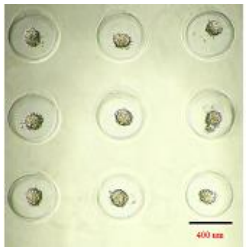
Fig. 15. *The effects of matriptase inhibitors on cell viability of C26 cells. The average inhibition % calculated using MTS assay at a wavelength of 490 nm are shown here. The error bars demonstrate the standard deviations of each result (n=4).*

As can be seen in ***Fig. 15.*** there was a strong variation in the cell viability between the cell groups treated by the selective matriptase inhibitors. The C26 cell groups treated with MI-432, MI-463 and MI-469 had less cell viability, with values closer to 5% inhibition in all those groups. In comparison, the groups treated with MI-453 and MI-460 had more viable cells, and values were below 2% inhibition. The group treated with AEBSF had the most viable cells, with approx. 1% inhibition.

6.3.3. C26 spheroid development

The growth pattern of the C26 spheroids (***Table 5.***) was very similar to that of the HT-29 spheroids over the 3 day growth period. However, the average diameter of the C26 spheroids after 3 days was 200 µm, which is smaller in comparison to that of the HT-29 spheroids.

Table 5. *Microscopic images taken of the growth progression of C26 spheroids over a period of 3 days.*

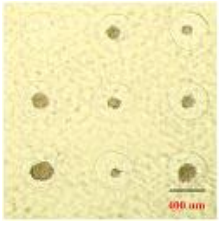
1st day	2nd day	3rd day
		

6.3.4. C26 spheroid treatment

As shown in ***Table 6.*** in the C26 cell group, the MI-432 inhibitor showed some destructive effects, although the AEBSF inhibitor showed no visible effects to the spheroid structure.

As with the HT-29 spheroids, the suramin treatment had a destructive effect on the C26 cell spheroids- in the images, cells can be seen detaching from the main spheroid mass.

Table 6. *Microscopic images taken of HT-29 spheroids after treatment with different matriptase activity regulators.*

Control	MI-432	AEBSF	Suramin
			

6.3.5. The effect of suramin treatment on C26 spheroids

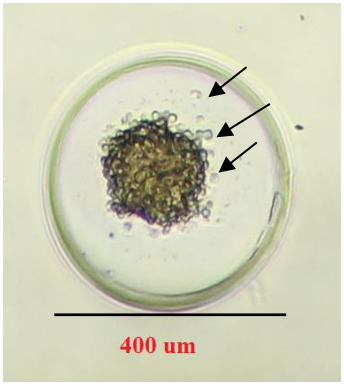


Fig. 16. *Microscopic image of C26 cell spheroid after treatment with suramin. Some cells can be seen detached from the main cell mass.*

In this higher magnification image, the superficial cells can be seen to be detached from the edge of the spheroid, floating freely in the culture medium.

6.3.6. C26 spheroid growth curves

The effects of specific matriptase inhibitors on the growth and area of the C26 cell spheroids was investigated and measured and compared to a control group, as with the HT-29 spheroids.

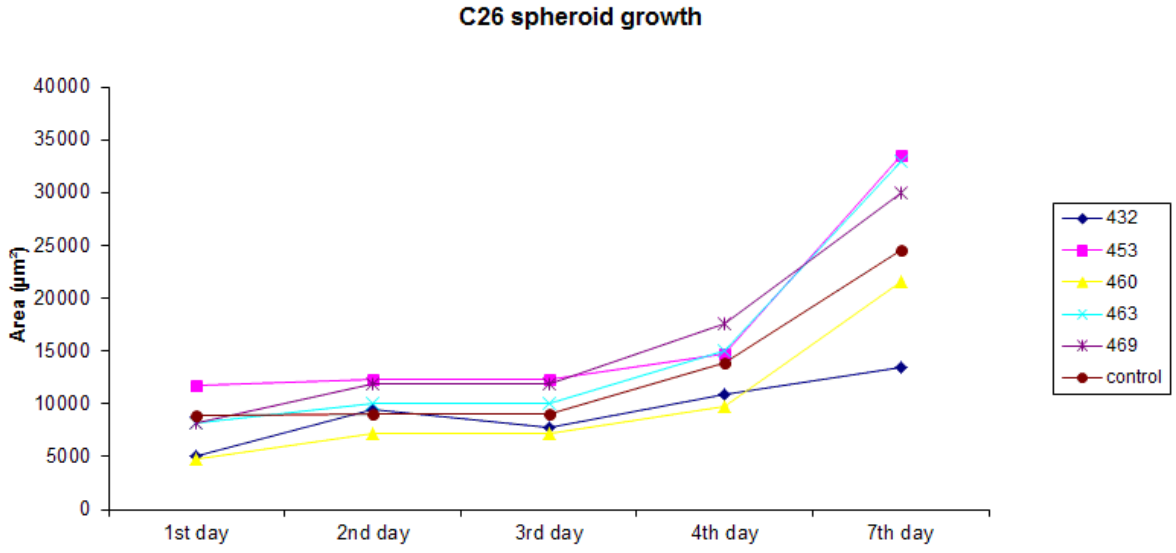


Fig. 17. *Spheroid growth rate of C26 in both the presence and absence of matriptase inhibitors. The growth of the spheroids was monitored for 7 days and the data is presented showing the average area (μm^2), $p<0.05$ ($n=2$).*

Each of the specific matriptase inhibitors, at a concentration of 50 μM, was used on C26 cell spheroids, and the area of the spheroids was measured over a 7 day period, and the results plotted against a control group, as shown in ***Fig. 17.*** The graph shows the inhibitor drugs MI-453, MI-463 and MI-469 did not inhibit the growth of the spheroids at all in comparison to the growth of the control group, as the areas of the spheroids treated by these inhibitor drugs were larger than that of the control group. However, the groups treated with MI-460 and MI-432 had smaller areas than the control group, indicating that the drugs applied inhibited the growth of these spheroids.

7. Discussion

Our results show that 3D spheroid cell culture systems are suitable models for testing anti-cancer drugs. It should be mentioned however that not any kind of cell type is capable of forming a stable spheroidal configuration, since according to the results, the healthy epithelial originated IPEC-J2 cell line was not able to form a solid spheroid, only small size agglomerates. These agglomerates were shown to be very sensitive to many kinds of external effects, since both the specific and non-specific matriptase modulating materials caused their deterioration even in small concentrations. Matriptase inhibitors (either broad-spectrum or selective types) did not affect cell viability to significant extent. On other hand the matriptase activator suramin also decreased the cell viability at a higher concentration. These results show that an increased artificial alteration in the matriptase levels via suramin addition could be harmful or even lethal for healthy cells.

The situation looks different if we see the results yielded from the tumour originated cell lines. The C26 cell line in monolayer culture was shown to be sensitive to the effects of suramin in a dose dependent manner and cell cytotoxic effects were observed. In spheroid configuration, the C26 cells formed a real 3D structure. In the growth rate examinations, MI-432 was shown to be capable of lowering the growth rate of the C26 spheroids..

In the case of the HT-29 cells, in monolayer culture the viability was not altered by specific matriptase inhibitors or the non-specific inhibitor AEBSF. The activator suramin showed again a dose dependent inhibitory effect in the same investigation. With regards to the 3D structure, HT-29 formed very compact spheroids, which had a higher growth rate than what we experienced in case of the C26 spheroids. After the experimental treatments, the specific and non-specific inhibitors did not cause any cytotoxic effects, only the enzyme inducer suramin showed cytotoxic properties.

Most likely, matriptase is involved in several physiological and pathophysiological functions. It has emerged as a potential target for the development of new anticancer drug agents (Lin et al., 1997; Santin et al., 2003; Santin et al., 2004; Riddick et al., 2005; Zeng et al., 2005; Cheng et al., 2006; Jin et al., 2006). However, based on our findings, selective inhibition of matriptase did not lead to reduction in cell viability of carcinogenic HT-29 and C26 cells. Further studies are planned to be performed to reveal if metastatic potential and invasiveness of the tumorigenic cells can be influenced with suppressed matriptase regulation. On the other

hand, the significant impact of suramin on desquamating the cells from the spheroids was observed which was supposed to be mainly attributed to its cytotoxic properties (Gan et al., 1998; Tayel et al., 2014) and not exclusively to the matriptase activation itself.

To sum up our results, we can conclude that the tumour originated cell lines were more resistant to drug effects than the healthy cell line we used. As for matriptase regulation, it seems that the matriptase inducer suramin is harmful to all the cell lines we used, although the inhibition of the enzyme showed diverse effects. The cause of this phenomenon needs to be clarified by further investigations.

Also, it is clear that the spheroidal structure provides protection to the cells against the harmful effects of the drugs. It is still unclear whether the differences in the results between the monolayer and the 3D cell cultures are caused by the ECM matrix or the stronger and more numerous connections between the cells. However the acquired data prove that a 3D spheroid structure works more similarly to the real *in vivo* solid tumours and has higher resistance against anti-cancer therapy than monolayer models. Hence using this type of cellular model can provide more reliable results during screening anti-cancer chemotherapeutic agent candidates than monolayers, without using living animal models. Although according to general experience, the 3D cell systems can never completely eliminate the use of live animals in drug research, these systems are very helpful to reduce the number of animals used in investigations.

8. Summary

Anticancer drug research requires suitable cell model systems to accurately screen and develop new treatment options capable of acting against tumour growth and metastasis. Currently the most suitable models are live animals, however nowadays, due to animal welfare and protection concerns, other potential replacement methods are being explored to minimise the use of live animals in pharmaceutical research. In 3D or multilayer *in vitro* cell cultures cells can produce their own extracellular matrix, which can influence the uptake and transport of drugs, thus better mimicking the *in vivo* conditions than the widely used monolayer cell culture systems.

Our goals in the experiments were to characterise the cells' viability using MTS assay, and the size and cell numbers of the spheroids. We used non-specific AEBSF and specific matriptase inhibitors such as MI-432, MI-453, MI-460 and MI-463 at a concentration of 50 µM, and a matriptase inducer, suramin, at concentrations of 50, 100 and 200 µM. During our research, we used three different cell lines- a porcine neonatal non-tumorigenic jejunal cell line, IPEC-J2, a murine colonic adenocarcinoma cell line, C26 and a human colon adenocarcinoma cell line, HT-29.

It was found that matriptase inhibitors did not cause cell death to a significant degree. However, suramin treatments lead to a dose-dependent reduction of cell viability at 100 and 200 µM in C26 and HT-29 spheroids, and in IPEC-J2 cell monolayers as well.

IPEC-J2 cells formed smaller sized loose cell aggregates. In contrast, the other two cell lines formed solid spheroids with diameters of approximately 200 µm for C26 and 250-300 µm for HT-29 after a growth period of 3 days. We found the control spheroids (formed from the cell lines C26 and HT-29) grew in an exponential rate during the total 7 day spheroid growth period. Matriptase inhibitors at a concentration of 50 µM did not alter the HT-29 spheroid growth rate within 7 days. In contrast, MI-432 caused a remarkable decrease in C26 spheroid growth. Suramin induced cellular detachment from the 3D spheroids, which can be attributed to its cytotoxic cell properties.

In conclusion, we used spheroids as part of a scientific system to investigate and evaluate potential anticancer properties of matriptase modulating drug candidates as well as to monitor the cancer cells' growth tendency during tumour cell attachment and formation *in vitro* when the cells had altered matriptase activity.

9. Összefoglalás

A tumorellenes gyógyszerek kutatása kapcsán szükségesek olyan sejtmodellek, melyek felhasználhatóak a gyógyszerjelölt vegyületek szűrésére, illetve új kezelési lehetőségek kifejlesztésére, amelyek eredményesen képesek gátolni a daganatok növekedését és a metasztázis képződést. Jelenleg a leginkább elfogadott ilyen rendszerek az állatkísérletek, de állatjóléti okokból egyre nagyobb az igény a felhasznált állatok számának csökkentésére. Az *in vitro* többrétegű vagy másképpen 3D sejttenyészetek lényegesen jobban utánozzák az élő szervezetet, mivel a sejtközötti állomány befolyásolja a gyógyszerek mozgását és sejtek általi felvételét.

Kutatásunk során vizsgáltuk a különböző matriptáz-aktivitást befolyásoló szerekkel kezelt sejtek életképességét MTS vizsgálattal illetve a kezelt szferoidok méretét és sejtszámát. A kezelésekhez nem specifikus (AEBSF) és specifikus (MI-432, MI-453, MI-460 és MI-463) matriptáz gátlókat 50 µM-, illetve suramint, mint enzimaktivátort alkalmaztunk 50, 100 és 200 µM koncentrációban. A kísérletben nem tumoros, sertés eredetű IPEC-J2, rágcsáló vastagbél adenokarcinóma eredetű C26 és emberi vastagbél adenokarcinóma eredetű HT29-es sejteket használtunk.

Az eredmények alapján a matriptáz gátlók nem okoznak jelentős mértékű sejthalált a kontroll csoporthoz képest. Mindazonáltal, a suramin kezelés a sejtéletképesség dózisfüggő csökkenését okozta mind a C26-os és a HT29-es sejtek mind pedig az IPEC-J2 sejtek egyrétegű tenyészetében.

Az IPEC-J2 sejtek csak laza szerkezetű aggregátumokat képeztek. Ezzel szemben a tumoros eredetű sejtek tömör szferoidokat alkottak a 3 napos inkubáció alatt, melyek C26 esetében átlagosan 200 µm, míg HT29 esetében 250-300 µm átmérőt értek el. A kontroll csoportok esetében mindkét sejttípus a teljes, 7 napos kísérleti idő alatt exponenciális növekedést mutatott. Az alkalmazott matriptáz gátlók nem befolyásolták a HT29 sejtekből álló szferoidok növekedését. Ezzel szemben C26 sejtek alkotta szferoidok esetében a MI-432 jelű anyag csökkentette a növekedési ütemet. Suramin hatására mindegyik szferoid esetében a felületről sejtleválást detektáltunk, amelyhez a hatóanyag citotoxicitása is hozzájárulhat.

Eredményeink alapján levonható az a következtetés, hogy az általunk alkalmazott szferoid kultúrák alkalmasak a matriptáz enzim aktivitását befolyásoló anyagok lehetséges rákellenes hatásainak vizsgálatára és a növekedési erély *in vitro* meghatározására megváltozott matriptáz aktivitás esetén.

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