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„Cucurbitacin E and Formononetin: Inhibition of Cell Migration“

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Abstract

Background: Cell migration is one of the crucial steps in metastasis. Migration is a coordinated mechanism where cells follow extrinsic and intrinsic signals. In order to develop strategies to prevent tumor cell migration and improve late-stage cancer treatment, it is crucial to study and understand the mechanisms that control cell migration in response to different stimuli in the tumor itself and in the TME. Several plant compounds have the potential to be anti-tumor or anti-cancer agents. Cucurbitacin E and Formononetin have been investigated for their ability to inhibit cell migration. Previous research has shown that these compounds act on different signaling pathways and key proteins involved in cell migration.

Methods: NIH-3T3 fibroblasts and 4T1 cancer cells were used as models in both monoculture and co-culture to observe different effects of the tested compounds. Growth curve measurements were performed to analyze the growth behavior of the cell lines in monoculture. The toxicity of Cucurbitacin E and Formononetin was investigated using the MTT assay to measure cell viability. The IC_{50} concentrations obtained were used to determine concentrations that were tested for effects on inhibiting cell migration. Cell migration was measured using the scratch assay.

Results: Cucurbitacin E showed a more pronounced toxicity on the cell lines tested and, in the co-culture, compared to Formononetin. Compared to 4T1 cancer cells, NIH-3T3 fibroblasts showed a higher tolerance to the toxic effects of Cucurbitacin E. Formononetin exhibited the same toxicity in all cells tested. Both substances inhibited cell migration at a concentration of 0.05 µM Cucurbitacin E and 40 µM Formononetin, respectively. Cucurbitacin E demonstrated better inhibition of cell migration for NIH-3T3 and the co-culture systems, while Formononetin E showed a slight advantage in inhibiting migration on cancer cells.

Conclusion: The experiments showed that fibroblasts, when used in co-culture, have a strong impact on cell migration. Therefore, substances that effectively inhibit fibroblast migration will be more successful in inhibiting migration in co-culture. In addition, the results showed that the cell-to-cell contact between the fibroblasts and the cancer cells can reduce the substance sensitivity of the cancer cells.

Zusammenfassung

Hintergrund: Zellmigration ist einer der entscheidenden Schritte bei Metastasierung. Bei Zellmigration handelt es sich um einen koordinierten Mechanismus, bei dem Zellen extrinsischen und intrinsischen Signalen folgen. Für die Entwicklung von Strategien zur Verhinderung der Migration von Tumorzellen und zur Verbesserung der Behandlung von Krebs im Spätstadium ist die Erforschung und das Verständnis der Mechanismen, die die Zellmigration als Reaktion auf verschiedene Stimuli im Tumor selbst und im TME steuern, von entscheidender Bedeutung. Diverse Pflanzeninhaltsstoffe zeigen das Potenzial, die Entwicklung von Tumoren oder Krebs zu hemmen. Cucurbitacin E und Formononetin wurden auf ihre Zellmigration hemmende Wirkung untersucht. Frühere Untersuchungen haben gezeigt, dass diese Substanzen auf verschiedene Signalwege und Schlüsselproteine einwirken, die an der Zellmigration beteiligt sind.

Methoden: NIH-3T3-Fibroblasten und 4T1-Krebszellen wurden sowohl in Monokultur als auch in Co-Kultur verwendet, um die unterschiedlichen Wirkungen der getesteten Substanzen zu beobachten. Es wurden Wachstumskurvenmessungen durchgeführt, um das Wachstumsverhalten der Zelllinien in Monokultur zu analysieren. Die Toxizität von Cucurbitacin E und Formononetin wurde als Messung der Zell -Viabilität, mit Hilfe des MTT-Assays, untersucht. Die erhaltenen IC_{50}-Konzentrationen wurden verwendet, um Konzentrationen zu bestimmen die keine toxische Wirkung aufweisen, jedoch die Hemmung der Zellmigration bewirken. Zur Messung der Zellmigration wurde das Scratch-Assay verwendet.

Ergebnisse: Cucurbitacin E zeigte, verglichen mit Formononetin, eine ausgeprägtere Toxizität auf die getesteten Zelllinien. Im Vergleich zu den 4T1-Krebszellen weisen die NIH-3T3-Fibroblasten eine größere Toleranz gegenüber der Toxizität von Cucurbitacin E auf. Formononetin wies bei allen getesteten Zellen die gleiche Toxizität auf. Beide Substanzen hemmten die Zellmigration bei der jeweiligen Konzentration von 0,05 µM Cucurbitacin E und 40 µM Formononetin. Cucurbitacin E zeigte eine bessere Hemmung der Zellmigration bei NIH-3T3 und bei dem Co-Kultursysteme, während Formononetin einen leichten Vorteil bei der Hemmung der Migration bei Krebszellen zeigte.

Schlussfolgerung: Die Experimente zeigten, dass Fibroblasten, wenn verwendet in Co-Kulturen, einen starken Einfluss auf die Zellmigration haben. Daher erweisen sich Substanzen, die die Fibroblasten-Migration wirksam hemmen, ebenfalls wirksam bei der Hemmung der Migration in Co-Kulturen. Darüber hinaus zeigten die Ergebnisse, dass der Zell-Zell-Kontakt zwischen Fibroblasten und Krebszellen die Empfindlichkeit der Krebszellen gegenüber den getesteten Substanzen verringern kann.

Table of content

Introduction

For centuries, various plants have been used in folk medicine as treatments. More than half of the medication available on the market today is obtained from plants or imitates ingredients of plants. [1][2] Through years of using medicinal plants, it was possible to observe the effects and side effects on a larger scale, which in turn can be seen as an advantage over chemically synthesized drugs. However, there is often a lack of precise information and understanding of the exact effect mechanisms of the active ingredients, as well as the influence of other substances that are also present in the plant extracts. In general, there are significantly fewer plant driven drugs on the market that are approved as therapeutics, than traditionally used medicinal plants. [2]

The anti-cancer activity of plant driven substances is starting to get more attention in research, with cancer causing 12% of the worldwide mortality. [3] Cancer is a frequently occurring and challenging disease, caused through a of series of gene mutations. These mutations lead to abnormal cell growth, due to uncontrolled cell division. [4] However, a positive outlook is provided by the fact that this great amount of potential tumor or cancer-fighting plant-based ingredients could contain effective candidates, which can one day successfully be used in cancer treatment. [1][5]
The common way of treating cancer patients is with drug treatment in form of chemotherapy, targeted cancer therapy or metastasis with radiation or removing the diseased tissue surgically, which often occurs with various side effects and resistance of the cancer against the treatment over time. [3][6] In particular, the mortality of various forms of breast cancer has decreased on average over the past 20 years. Still, this is not a complete solution, with certain variants of breast cancer being resistant to the prevalent treatments used, one of them being the triple negative breast cancer (TNBC). The TNBC type is characterized by the absence of estrogen (Erα) and progesterone receptors (PR) and shows a decreased expression of the human epidermal growth factor receptor-2 (HER2) receptors. This triple negative form occurs in approximately 10-15% of all breast cancer cases. Patients suffering from this type of breast cancer often receive the worst prognosis, due to this form of breast cancer being insensitive to the treatment with conventional endocrine therapy methods and HER-2 targeting drugs. [7][8][9] In this research, the 4T1 murine triple – negative breast cancer cells were used as a TNBC-model.

Tumor-stromal interactions are a hallmark of cancer, and the ratio in which they occur has become a prognostic factor for breast cancer patients. The tumor stroma ratio can indicate the potential risk of relapse for tumors with a high stromal content. Research has revealed that the molecular

markers of the tumor-stroma can provide information on the cancer's resistance to chemotherapy. [10]

Therefore, research has focused on understanding the tumor microenvironment (TME) to develop drugs that target not only the diseased tissue but also the area where cancer cells are found. Previous studies have shown that the proliferation of certain cancer types can be strongly influenced by the surrounding tissue. During tumor progression, cancer cells can transform the TME, leading to the development of a cancer-associated environment that supports the proliferation and invasion of cancer cells. [10][11] Fibroblasts, which are part of the connective tissue, are one of the most important cells in the TME. [10] Cancer-associated fibroblasts (CAFs) have been known to support the proliferation of tumor and are present in all stages of cancer. [10][12][13] On the other hand, research observed that normal fibroblasts (NFs) inhibit the progression of cancer and tumors in the early stages. [14] In healthy tissue, fibroblasts provide signal molecules that are part of the extracellular matrix (ECM). Some of these molecules, such as growth factors or cytokines, help maintain homeostasis and play a crucial role in wound healing. The interaction of tumor cells with stromal cells can result in increased secretion of these growth factors and chemokines, which can further contribute to tumor proliferation by supporting tumor survival or angiogenesis. To gain a better understanding of the effects of the interaction between healthy and tumor cells, co-culture cell systems were established to mimic the TME. [15] In this study, a two dimensional (2D) monolayer co-culture was used, where cancer cells and fibroblasts were in close contact to observe the interaction.

Cancer formation begins with an initiating mutation that allows unlimited proliferation, thereby creating a solid tumor. Those cells are not affected by defects in cell division or an unstable genome, but support other cell-autonomous functions that transform cells into oncogenic cells. [17][18] In the next step, called invasion, carcinoma cells that spread navigate through the tumor microenvironment, penetrate the basal membrane into the surrounding stroma until they reach the blood stream and can form metastases in organs afar from the origin. [16][17][18] The process of metastasis is a complex, multi-faceted mechanism involving changes in cell phenotype, cell migration and ongoing cell-cell interactions. [16] Cell migration is a coordinated mechanism in which cells are directed by both extrinsic and intrinsic factors. Integrins and other adhesion receptors sense mechanical signals, while chemokine and growth factor receptors sense chemical signals that influence tumor cell migration. [16] For cell migration the regulation of the β-actin network is crucial. The Arp2/3 complex can upregulate actin polymerization, which is essential for actin-based cell migration. Furthermore, increased expression and activity of focal adhesion kinase (FAK) has been associated with migration, invasion, and metastasis of tumor cells. Matrix

metalloproteinases (MMPs) have been identified as key molecules in cancer metastasis among the genes downstream of FAK. A prior study showed that the Src-FAK complex binds to MMP, which initiates ECM proteolysis and cell invasion. [18]

If the tumor cells survive in the circulation of the blood stream, they can attach themselves to the endothelium of the capillary and in the process of extravasation escape from the blood vessel. When the tumor cells reach the secondary organs, metastasis can be formed by proliferation of those cells. If any of these steps fail, the entire metastatic process can be compromised and is therefore a major target point in cancer therapy. Cell migration is considered to be one of the most important mechanisms for the cancer metastasis to be successful. [17][18] For that reason, understanding the mechanisms that guide cell migration in response to different stimulants in the tumor itself and the TME is crucial in order to develop strategies that prevent tumor cell from migrating and improve late-stage cancer treatment. [16]

Cucurbitacin E

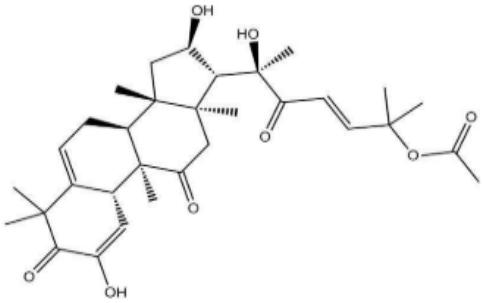


Figure 1: The chemical structure of Cucurbitacin E [3]

Cucurbitacin E (CuE) is one of two substances used in this study to test its effects on cell migration and proliferation, by using fibroblast and cancer cells. Fig. 1 shows the chemical structure of the compound.

The research of Cucurbitacins in the pharmaceutical field started in the 1960s, where the anticancer effect was observed on different cell lines and in vivo. The issues that this early research faced were that the cell toxicity was not specific, as well as the therapeutic index being low. The toxicity in question could have been caused by using the direct plant extract, which was not purified. [2]

The Cucurbitacins belong to the group of oxidized tetracyclic triterpenoids, which are found in the plant family Cucurbitaceae that consist of around 800 different species and is considered one of the most economically relevant plant families. Numerous plants of this family are often used in traditional Chinese medicine, for example, *Cucubita pepo cv Dayangua*. The Cucurbitacis are often characterized by their bitterness and toxicity. Regarding their structure, this group of triterpenes is

divided into twelve different components. Out of all the main Cucurbitacins, from Cucurbitacin A to Cucurbitacin T, studies have shown that Cucurbitacin B,D,E and I and their derivates showcase a potent anticancer effects in various cancer types, including breast cancer. [2][3][7][18]
The functionalization of bioactive plant derived ingredients is the most promising approach for the discovery of new drugs. In previous studies, the pharmacological effects of Cucurbicins have been observed. The research showed that the substances provide anti-inflammatory, antiviral, and anti-proliferative properties on certain cancer cell lines, for instance breast, lung, skin, or prostate cancer. [3][7][18]

The effects of Cucurbitacins on cancer cells are achieved by targeting the cyclooxygenase-2 (COX-2), fibrous-actin (F-actin), the Januskinase-2 (JAK-2) signal way and the signal transducer and activator of transcription 3 (STAT3), as well as inhibiting tumor angiogenesis through the interaction with the vascular-endothelial-growth-factor-receptor-2 (VEGFR-2). Research has also shown that these plant-based substances provide a synergistic effect, when combined with commonly used medication in chemotherapy. [3][7]

Especially the inhibition of cell adhesion and migration is achieved by CuE disrupting actin filaments of cells and suppressing the Arp2/3-dependent actin polymerization. Further, CuE also reduces the expression Arp3 and actin. [3][18]

Formononetin

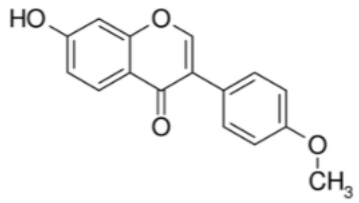


Figure 2: The chemical structure of Formononetin. [19]

The second substance observed in this research is Formononetin, also referred to as Biochanin B, an O-methylated isoflavone, which can be found in several medicinal plants. The chemical structure of the substance is shown in Fig. 2. This natural existing substance can be found in plants such as the red clover (*Trifolium pratense*), mongolian milkvetch (*Astragalus membranaceus)*, or in licorice *(Glycyrrhiza glabra*). [4][19][20] Formononetin is frequently used in the medical field, as well as in the food and cosmetics industry. [21]

Isoflavones are also classified as phytoestrogens, due to their structure mimicking endogenous estrogen, with the ability to bind to the estrogen receptors α and β. [4] Therefore, women that consume isoflavones experience less menopausal symptoms, according to studies from Japan. [21]

So far, the research on Formononetin has shown a variety of biological and pharmacological effects, with a few of them being properties that inhibit tumor growth, as well as inhibiting proliferation of cancer and growth inhibitory activity. [22]

Formononetin, was utilized in this study to investigate its potential in suppressing cell migration. Previous research has revealed its association with the inhibition of cell proliferation, cell cycle progression, and the induction of apoptosis in diverse cancer cell lines, primarily acting through the PI3K/AKT signaling pathway, where the expression MMPs is reduced. [20] MMPs play a crucial role in the process of extravasation, which involves the migration of tumor cells from the blood vessel to the target tissue. Tumor cells secrete MMPs to degrade the matrix, creating pathways of weakened and digested matrix that allow subsequent cells to migrate.
The main enzymes responsible for degrading collagen are MMP-2 and MMP-9, which are also overexpressed in breast cancer cells. Therefore, MMPs could serve as potential targets for inhibiting breast cancer invasion and metastasis. [16][20]

MTT-assay and cell viability

The MTT-assay is commonly used to assess the toxicity of exogenous substances by evaluating the proliferation of cells and measuring the metabolic activity of cells. The information gathered through this type of test is especially important for research of novel drug candidates, both plant-derived or chemically synthesized, to investigate their toxicity first on cancer cell lines. [23][24]

This colorimetric assay is based on the reduction of the yellow colored 3−[4,5−dimethylthiazol−2−yl]−2,5−diphenyltetrazolinum bromide, short MTT, to a purple colored formazan, as shown in fig. 3. The purple formazan is obtained as the product of the MTT reduction and can be easily dissolved in dimethyl sulfoxide (DMSO). [25]

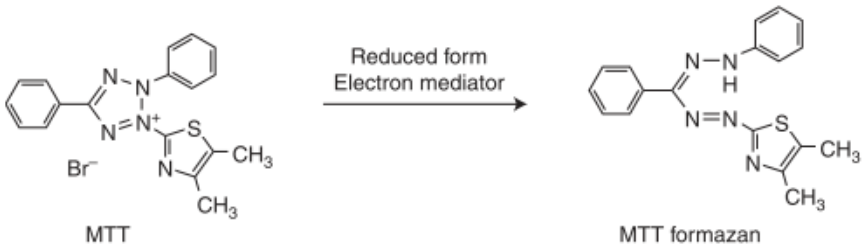


Figure 3: The conversion of MTT to formazan in the mitochondria of viable cells. [25]

This reaction only takes place in viable cells, where the active mitochondrial dehydrogenase reduces the MTT in high rates, whereas inactive or dead cells do not. Therefore, a linear relation between the amount of converted formazan and the number of viable and active cells is established, which allows a quantification of the proliferation rate of cells or the toxicity of a certain substance. This leads to the conclusion that a more intense purple color indicates a higher cell viability, whereas a declining purple color represents a lower cell number, which indicates cytotoxicity of the tested substance. To determine the viability of the cells, the formazan absorbance is quantified at a measurement wavelength of 540 nm. [23][24] The advantages of the MTT-method are the quick and convenient implementation, that does not require special technical equipment. [26]

Scratch-assay and cell migration

Cell migration and proliferation are the main processes that can be observed during the healing of a wound, with migration being the limiting factor. Therefore, migration assays are used to investigate wound healing, [27] besides the afore mentioned role in cancer progression. The wound healing assay or scratch assay is considered a standard in vitro method for analyzing and evaluating a collective cell migration. [28] The collective cell migration is described by the cells not moving individually, but in multicellular arrangements like clusters or sheets. [29] To perform a scratch assay, seeding a cell monolayer is the first step. When the appropriate confluency of the monolayer is reached, a wound, which is a cell-free area, is created either mechanically by using a sharp object like a pipette tip or a cell scraper or by thermal or chemical damage. The cells are now stimulated to migrate in the cell-free area to close the gap in the cell monolayer. This cell migration can be observed and evaluated. Pictures that are usually taken to document the process of wound healing are shown in fig. 4. [27][28]

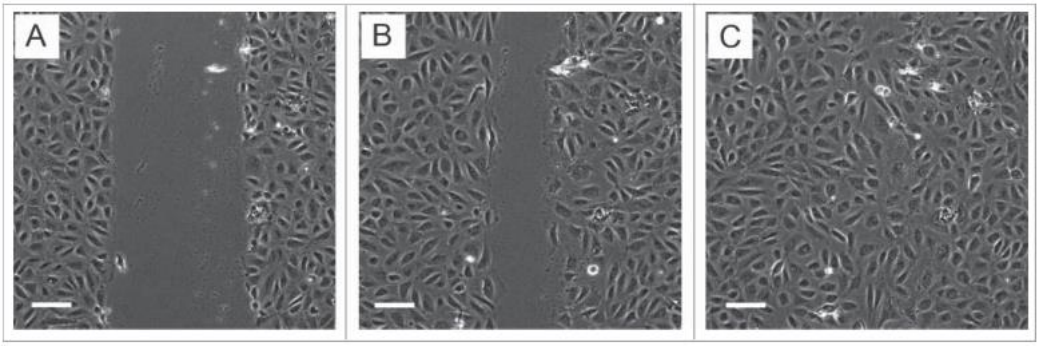


Figure 4: Scratch assay pictures taken at different time, showing the process of the cell free area (wound) closing. [28]

The wound healing assay examines the sheet migration, which is described as a form of collective migration. Epithelial and endothelial monolayers demonstrate such migration, where the cells are moving in two directions while maintaining the connections between their cells. Sheet migration is observed in various biological processes, including cancer metastasis, embryonic morphogenesis, and tissue damage. [28]

Commonly, a 2D cell monolayer is used for the scratch assay. The 2D format has become the most prevalent because it can be achieved by using conventional equipment found in most cell culture laboratories and is performed under standard cell culture requirements. As the 2D format gains popularity, it is important to consider its limitations. The process of wound healing involves mechanisms that cannot be properly represented in this format. [27] An evolved method, which provides a new approach to research is a three-dimensional (3D) model, where cells are seeded on scaffolds to depict a more complex mechanism of wound healing for analyzing cell migration. This 3D in vitro model can be used to investigate new aspects of scratch healing, such as interactions between cells or with the ECM and the TME [27] [13]

For this research the scratch assay, was used to analyze the effect of the substances CuE and Formononetin on the migration of cells. Further, to observe the effect of fibroblasts on cancer cells in cell migration, a monolayer co-culture system was developed, specifically for this assay. The purpose of a co-culture system was to mimic realistic metastasis, where healthy cells are in tight cell-cell contact with cancer cells and can also affect each other's behavior in migration. Fluorescent marked cancer cells were selected due to their ability to separate them visually from the fibroblasts via fluorescent imaging in a co-culture monolayer.

Objective

The objective of this master thesis is to investigate the impact of the substances Cucurbitacin E and Formononetin on cancer cell migration, using a triple-negative breast cancer cell line as a model system. To achieve this, monoculture and co-culture systems were set-up and analyzed. The co-culture system was established combining cancer cells with fibroblast cells in different proportions. The aim is to mimic the cancer environment, where healthy cells, like fibroblast, are in tight contact with cancer cells and to observe the effect of fibroblasts on cancer cells in a 2D system. Therefore, initially, the toxic effects of these two chemicals on cancer cells and fibroblast cells were examined, and concentrations for their use were calculated based on the IC_{50} value. These calculated values were afterwards utilized to suppress migration in both monoculture and co-culture systems. The impact of the chemicals on migration was observed using the scratch assay technique. The influence of monoculture and co-culture on migration was compared.

The information obtained through this research will provide results for transitioning from the commonly used 2D model to a more modern approach in research - spheroids and 3D co-culture systems.

Material & Methods

Substances

For this research the substances Formononetin (Sigma – Aldrich, LOT: BCCD9471) and CuE (Sigma – Aldrich, LOT: BCCD9471) were used. The chemicals were dissolved in DMSO (Sigma-Aldrich, Germany) at a concentration of 20,000 µmol/L to prepare a stock solution, subsequently subjected to further dilution for experimental use.

Cell culture

The immortalized adherent NIH-3T3, clonal mouse fibroblast cell line, derived from a randomly bred mice [30], (CLS cell lines, Eppelheim, Germany), were supplied from Andreas Teuschl-Woller lab (FH-Technikum Vienna, Austria), and used all throughout this study. Dulbecco's Modified Eagle's Medium (DMEM) – high glucose, with added 1% penicillin-streptomycin mixture (P/S), 1% L-glutamine (L-Glu) and 10% fetal calf serum (FCS) was used in cell culture for 3T3 cells. This medium was used when monoculture fibroblast cells were a part of an experiment or for cell cultivation.

The 4T1 murine triple – negative breast cancer cells cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) (supplemented with 10% FCS, 1% L-Glu and 1% P/S). 4T1 cells were lentiviral transduced with polybrene and sorted for iRFP720 expression on a FACS Aria III, as described in their earlier paper. [31][32] They were provided for this thesis from the Laboratory of Macromolecular Cancer Therapeutic (MMCT) (University of Vienna).

Cells were seeded in separate T75 cell culture flask with 15 mL medium. The flasks were incubated at 37°C with 5% CO_2 to provide a suitable environment for cell growth.

Cell culture was performed under a laminar air flow hood (Thermo Scientific, Germany).

Splitting

In order to facilitate optimal cellular growth and prevent overcrowding, lack of nutrients, and accumulation of waste products, cellular subculturing needed was performed two to three times per week.

The confluency of the cells in the cell culture flasks was checked with a microscope and cell splitting was conducted when confluency levels reached 80-90%. First, the old medium was removed with a serological pipette. Next, the cells were gently washed with 3 to 5 mL Dulbecco's Phosphate buffered Saline (PBS). Then, the cells were treated with 3 mL 0.05% Trypsin in

ethylenediaminetetraacetic acid (EDTA) solution, which was enough to cover the surface of the cell monolayer in the flask, and incubated for 5 minutes (37°C, 5% CO_2), to detach the adherent cells from the flask. By adding double the amount of medium the effect of the trypsin was neutralized, and the cell suspension was transferred in a 15 mL falcon tube. The suspension was centrifuged at 1500 rpm for 3 minutes and the supernatant removed. The pellet was resuspended in 5 mL fresh medium. A part of the cell suspension was seeded in a new T75 flask, while the rest was disposed. As a final step, the flask was filled up with the medium for a total amount of 15 mL.

Cell counting

The cell suspension was prepared as previously described in the cell splitting process. Further, the suspension was diluted using a ratio of 1:50, which was achieved by adding 10 µL cell suspension and 490 µL medium in a 1 mL tube. As cancer cells tend to build clusters, it is important to homogenize them by gently mixing the cell suspension. To determine the number of cells per milliliter within the suspension, a counting chamber, also known as hemocytometer (Neubauer), was used as shown in Fig. 5. 10 µL of cell suspension was applied twice and covered with a cover glass. The counting chamber was placed under a microscope and the cells were counted.

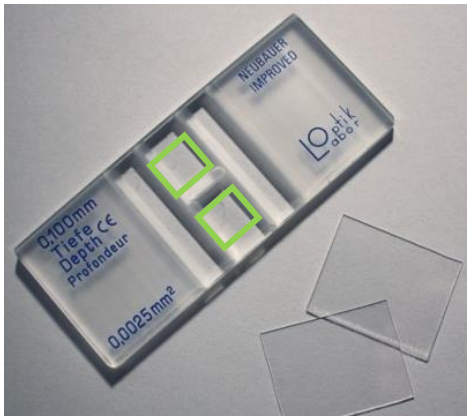
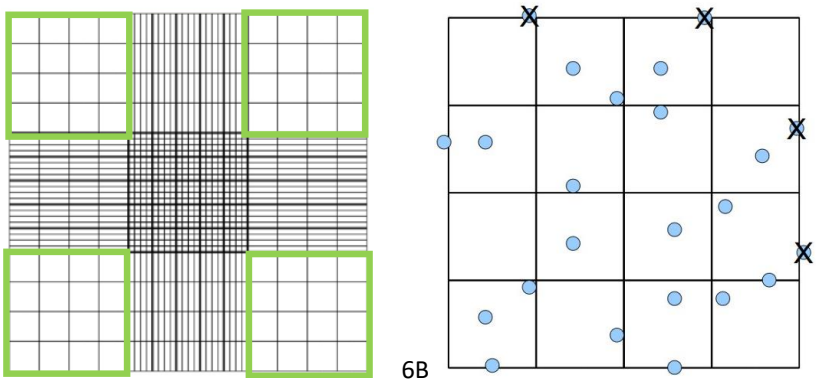


Figure 5: A hemocytometer or counting chamber made by the brand Neubauer. The spots marked green are where the cell suspension is applied. Before counting the cells under the microscope, the application spot is covered with glass, shown on the bottom right of the picture. [33]

The layout of the hemocytometer under the microscope is shown in Fig. 6A. It shows four large squares, which are built out of 16 smaller squares. Fig. 6B depicts the counting method. Cells that are touching the outline of the square are only counted on two sides. To calculate the mean cell count, all four squares are counted, and the value is then divided by four. If the suspension was diluted due to high cell count, the result is multiplied with a dilution factor, to consider the diluting steps. Last, the result is multiplied by 10,000 to estimate the cell count per milliliter.



6A 6B

Figure 6: 6A Layout of a hemocytometer. The squares marked green are counted, while the mean cell count represents the cell count per mL of cell suspension. Figure 6B displays the counting Method. If cells are on the outline of the square, only two sides of the square should be included, and all the other cells are counted. [33]

Growth-curve measurement

To assess and compare the growth patterns of different cell lines, a growth curve analysis was conducted. Cells were treated with Trypsin, centrifuged, and counted as described above. A cell suspension with a concentration of 5000 cells/mL was prepared for each cell line. Subsequently, the cells were seeded in 24-well plates, 1 mL suspension per well and incubated until the cell count starts decreasing, which was 17 days for NIH-3T3 cells and 18 days for cancer cells. Every 24 to 48 hours one row of the plate was counted (n=3). This involved removing the old medium, washing the cells with 200 µL of PBS per well and incubating with 200 µL Trypsin for 5 minutes. After the incubation, 800 µL of medium were added in each well, resulting in a total volume of 1 milliliter. The cell suspension was taken out with a pipette, transferred in tubes, and counted as described before. The medium, contained in the wells which still needed to be counted, was changed every other day for fibroblasts, and every day for cancer cells, due to latter's faster metabolism. Regular medium changes ensured that the cell count is not decreasing in number, which can be caused by undersupply of nutrients or toxins from the waste product. The whole process was repeated until all wells of the plate were counted or the number of cells per well started decreasing. The collected cell counts per well were later analyzed and a graph created by using Excel (Microsoft). The mean values, which are calculated from the triplicates, were presented as mean ± standard deviation (SD).

MTT-assay for evaluation of cell viability and IC_{50} determination

The MTT-assay is a colorimetric measurement, which permits the evaluation of the metabolic activity of cells. [24] The test is based on the reduction of the yellow MTT (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide), through the mitochondrial enzyme dehydrogenase in viable cell, into a purple formazan crystal. [26]

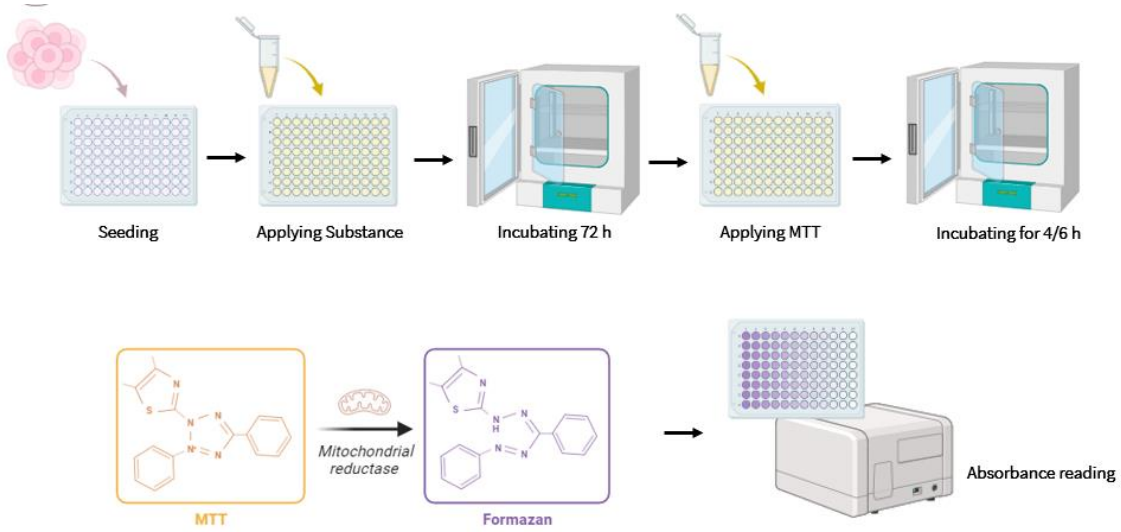


Figure 7: The workflow of the MTT assay. The Picture was created using the website BioRender.com [34]

Fig. 7 depicts an overview of the work steps of the MTT-assay, starting with cell seeding. NIH-3T3 fibroblast cells, 4T1-wt cancer cells, as well as 4T1-iRFP720 cancer cells were used for this assay. The cells were seeded in 96-well plates. Cell suspensions were prepared with a cell number of 2000 cells/100µL for the cancer cell lines and 5000 cells/100 µL for fibroblast cells per well. In order to generate triplicates, the cells were seeded three times for both the control and for each concentration of the series. The seeded plates were incubated for 24 hours at 37°C and a 5% CO_2. On the second day of the experiment, different series of dilutions of Cucurbitacin E and Formononetin were prepared, which was achieved by diluting the stock solution with a concentration of 20,000 µmol/L. Table 1 shows the concentration of Cucurbitacin E and Formononetin used for the experiment. Those are the final concentrations of the substances after application to the cells in the well-plates.

Table 1: Concentration series of Cucurbitacin E and Formononetin for different cell lines. Concentration series 1-3 were used for toxicity tests on NIH-3T3 fibroblast cells. Concentration series 2 was also used for the toxicity tests on 4T1 cancer cells. The shown Formononetin concentration series was used for both cell lines.

Conc. series 1 CuE [µmol/L] NIH-3T3	**Conc. series 2 CuE [µmol/L] NIH-3T3 and 4T1**	**Conc. series 3 CuE [µmol/L] NIH-3T3**	**Formononetin [µmol/L] NIH-3T3 and 4T1**
Control (0)	Control (0)	Control (0)	Control (0)
0.78	0.039	0.0000256	1.875
1.6	0.078	0.000128	3.75
3.1	0.16	0.00064	7.5
6.3	0.31	0.0032	15
12.5	0.63	0.016	30
25	1.25	0.08	60
50	2.5	0.4	120
100	5	2	240
200	10	10	480

The cells were first checked under the microscope to verify that all cells are attached to the plates. Then, the dilution series were applied to the wells which already contained 100 µL medium. Therefore, a 2-fold dilution step has to be considered, when preparing the dilution series. The plates were incubated for another 72 hours in the incubator at 37°C and 5% CO_2. After the respective incubation time, an MTT-stock solution, with a concentration of 5 mg/mL MTT in PBS, was further diluted in a ratio of 1:7 with medium. This MTT-medium differs from the medium used before, because it does not contain FCS, which would interfere with the absorbance measurement.

The cancer cell lines were incubated with the MTT solution for 4 hours and the fibroblast cells for 6 hours. Due to fibroblastic cells having a slower metabolism, the incubation was prolonged, so the mitochondrial reaction and the forming of the formazan crystals could be sufficiently achieved.

When the incubation time was over, the plates were examined to see if the formazan crystals were visible. After, the medium was taken off carefully by using a multichannel pipette, while making sure that the pipette tips are not removing any formed crystals or damaging the cell monolayer. The crystals were then dissolved by adding 100 µL DMSO. The plates were shaken gently to help with the homogenization of the now purple colored solution in the wells. After the crystals were fully dissolved the plates were placed in a plate reader (Tecan infinite M200PRO) and the absorption of the solution was measured at a measurement wavelength of 590 nm and reference wavelength

of 690 nm. The measured absorptions were further normalized and calculated using Excel (Microsoft). Due to the experiment being triplicated all values were presented as mean ± SD.

Co-culture seeding

The cells used to establish a co-culture are NIH-3T3 fibroblast cells and iRFP720-4T1 cancer cells. First, a fibroblasts cell suspension was prepared and seeded in a 24 well plate. The next day, after checking that the fibroblasts were attached on the plate, the medium was removed, and 1 mL of a cancer cell suspension was added on top of the fibroblasts. After another 24 hours, the co-culture could be further used for the wound healing assay.

It was important to seed the NIH-3T3 cells first. As a result of fibroblast cells` slower metabolism compared to that of cancer cells, it was assured that their cell count would not double in 24 hours and the calculated ratio of fibroblasts to cancer cells would not change.

Cell confluency experiment

Before the cell migration measurements could be started, it was important to determine the correct cell number per well as a sort of preliminary experiment. A range of different total cell counts per well were seeded in a 24 well plate, starting from 200,000 cells/well up to 1.5 million cells/well. The co-culture cell line was seeded in different ratios from 1:2 to 1:5 fibroblasts:cancer cells. Fig. 7 shows the exact seeding scheme of the experiment.

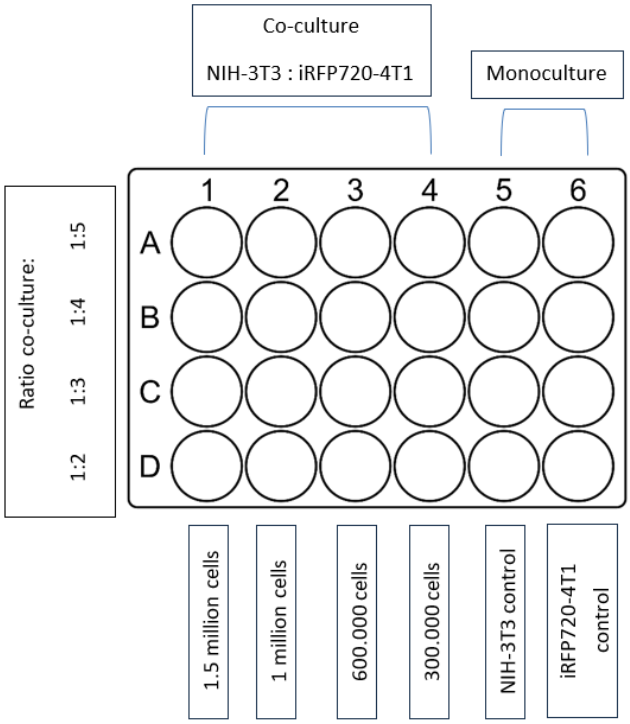


Figure 8: The seeding scheme of the confluency experiment. Cells were seeded in a 24-well plate, with rows 1 to 4 seeded as a co-culture, row 5 as a monoculture, containing only fibroblast cells NIH-3T3, while row 6 was also seeded as a monoculture with cancer cells iRFP720-4T1. The rows A to D consisted of different ratios of fibroblasts to cancer cells in the co-culture section of the plate. The total cell count per well was set in a range starting with 1.5 million cells per well ending with 300,00 cells per well, as can be seen in rows 1 to 4, still seeded as a co-culture. Row 5 and 6 also served as control groups, containing the same number of cells as in row 3 (600,000 cells per well), but seeded as a monoculture.

Table 2: Cells seeded per well for the confluency experiment in listed in detail.

Cell count Co-culture	**Cell line**	**1:2**	**1:3**	**1:4**	**1:5**
1,500,000 total	3T3	500,000	375,000	300,000	250,000
	4T1	1,000,000	1,125,000	1,200,000	1,250,000
1,000,000 total	3T3	333,333	250,000	200,000	166,667
	4T1	666,667	750,000	800,000	833,335
600,000 total	3T3	200,000	150,000	120,000	100,000
	4T1	400,000	450,000	480,000	500,000
300,000 total	3T3	100,000	75,000	60,000	50,000
	4T1	200,000	225,000	240,000	250,000
Control monoculture					
3T3		200,000	150,000	120,000	100,000
4T1		400,000	450,000	480,000	500,000

The cells were prepared for the seeding as described before, starting with the fibroblast. On the second day of the experiment the cancer cells were seeded for monoculture and co-culture. After 24 hours, pictures of the wells were taken with a microscope (EVOS FL Digital Inverted Fluorescence Microscope, Thermo Fisher Scientific). The microscope was first set up for bright field (BF) observation, with the phase annuli selector on BF and the light cub selector on transmitted. For the fluorescent images the phase annuli selector was placed on 4/10 PH and the light cube selector on Cy5 (628/40 nm Excitation; 692/40 nm Emission). 10x magnification was used for both settings. Those pictures where further analyzed using the program FIJI/ImageJ.

Scratch assay

The cell lines used for this cell migration assay were NIH-3T3 fibroblasts and iRFP720-4T1 breast cancer cells.

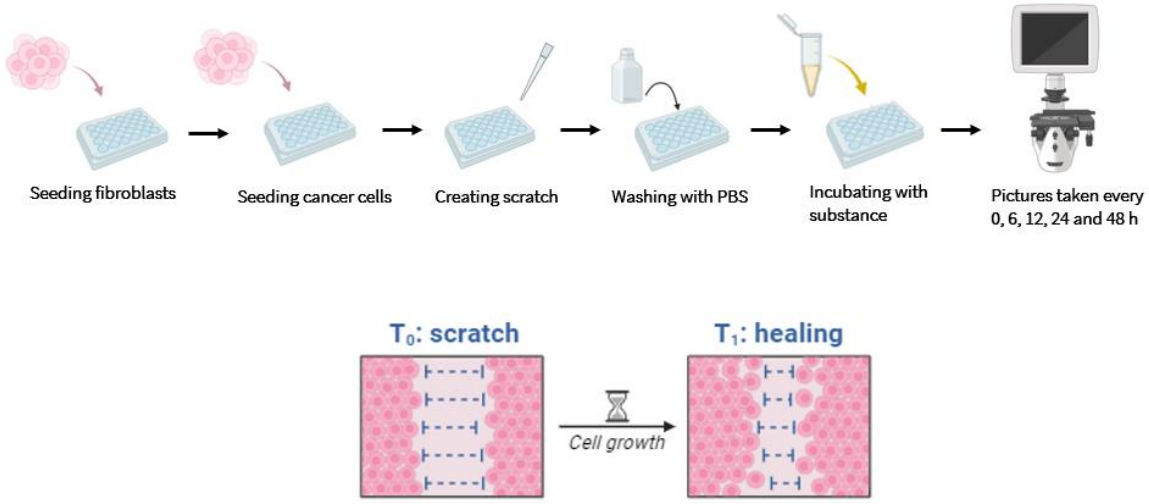


Figure 9: Overview of the work steps of the scratch assay. The picture was created using the website BioRender.com [34]

Fig. 9 should give and overlook of the steps included in the scratch assay. For this test, the cells were seeded on two different days in 24-well-plates, to create a monolayer co-culture system. On the first day, the NIH-3T3 fibroblasts were seeded in wells for the monoculture and in separate wells for co-culture in 1:3 and 1:4 ratio. The exact cells seeded per well are shown in table 3.

Table 3: Cell counts per well used for the scratch assay. Each well was seeded three times to create three replicates (n=3). The total cell count per well was 200,000 cells.

	3T3 cells/well	4T1 cells/well
NIH-3T3 monoculture	200,000	---
iRFP720-4T1 monoculture	----	200,000
1:3 co-culture	50,000	150,000
1:4 co-culture	40,000	160,000

The following day the fibroblasts were checked under a microscope. Afterwards, the medium was removed with a pipette and the cancer cells iRFP720-4T1 were seeded in wells for monoculture and on top of the fibroblast for co-culture. After 24 hours of incubation, the medium was removed, and a scratch was created with a 10 µL pipette tip. By creating the scratch, the cells detached and floated in the medium. These needed to be removed from the well, to prevent the cells from reattaching and closing the created gap. This was achieved with washing the wells by applying 200 µL PBS. For each cell line three groups were analyzed, the first being a control, in which the medium was added to the scratch. This was a RPMI-1640 medium which contained 1% L- glutamine, 1% penicillin-streptomycin, but only 0.5% FCS. The low FCS-concentration was chosen to prevent the cells from proliferation, while still enabling cell migration.

1 mL of a Cucurbitacin E solution, with a concentration of 0.05 µmol/L, was added in the second group of wells for each cell line. 1 mL of a 40 µmol/L Foromononetin solution was added to the third and last group. Right after the application of the medium and substances, pictures of the scratches were taken with a microscope and the exact spot was marked for each of the wells on the plate. This was considered the starting point (0 h) of the experiment. For Cucurbitacin E, as well as the control group, pictures were taken after 6 and after 12 hours. [18][20] The Formononetin group was incubated for a longer period, therefore pictures were taken after 12, 24 and 48 hours.

The image processing package Fiji/ImageJ was used to evaluate the cell movement. [35] First, the pictures from the starting point were analyzed. The measured areas were later normalized by using Excel (Microsoft), with the area of the gap at the beginning of the experiment being 100%.

Due to the experiment being triplicated all values were presented as mean ± SD.

Results and Discussion

Growth curve analysis

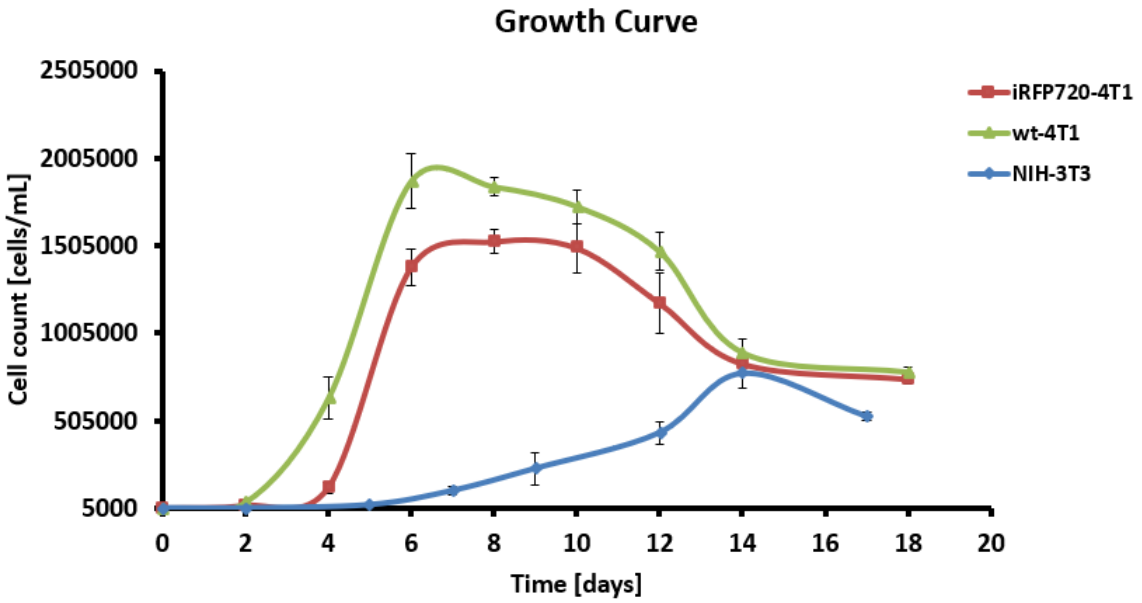


Figure 10: Growth curves of NIH-3T3 fibroblasts, 4T1-wt and 4T1-iRFP720 cancer cells grown in cell culture medium over 17 (NIH-3T3) and 18 days (4T1). Values are depicted as mean ± standard deviation of three replicates (n=3).

The differences in the rate of cell growth (Fig. 10), between the NIH-3T3 fibroblast cell line and the 4T1 cancer cell lines, wherein one of the 4T1 variants is wild type and the other is labeled with a fluorescent marker.

The growth curve of wt-4T1 cells is represented by the green line. The lag-phase of this cell line lasted for two days during which the cells attached to the surface after being seeded and began dividing. Following this, a log-phase began, where cells grew exponentially and doubled at a certain rate. This phase had reached its peak after six days, during which the maximum number of cells per well had been reached, approximately 2 million cells. From day six to ten, the wt-4T1 curve exhibited a stationary or plateau phase, where the growth of the cells slowed down and eventually stopped. After the tenth day, the declining or death phase began, during which the cell number decreased, indicating cell death.

The lag-phase of the iRFP720-4T1, presented with a red line, lasted for three days. After this, exponential growth began and reached the highest cell count at day seven, with around 1.5 million cells per well. The plateau phase lasted from day seven up to day 10, after which the number of viable cells began to decrease.

The NIH-3T3 cells, represented by the blue line, exhibited the longest lag phase, which persisted for 5 days. The exponential phase was prolonged compared to the other two curves, lasting from day

5 to day 14, indicating slower growth. The stationary phase is barely visible and lasted only one day. On day 15, the number of cells began to decline, initiating the death phase.

The results were expected, due to comparing previous studies. [36][37][38][39]

The variation in growth among the cell lines wt-4T1 and iRFP720-4T1 may have been caused by cells forming aggregates, such as clusters. This problem especially occurred while working with the fluorescent marked iRFP720-4T1, where difficulties appeared while homogenization of the cell suspension or while detachment of the cells with trypsin, due to the cells either building clusters or attaching strongly to the cell culture flasks. While cell counting, residues of cells were noticed in the 24-well plates, predominantly with the iRFP720-4T1 cells. This issue could have led to lower counts of this cell line compared to the wt-4T1.

The information, regarding the growing behavior, was further used to determine the right number of cells for the following experiments. As cell were seeded in 24-well plates for this analysis, it was possible to calculate seeding concentrations of cells/mL for other sized plates. For the subsequent MTT-assay 96-well plates were used, were 5000 cells/well fibroblasts and 2000 cells/well cancer cells were seeded.

MTT-assay

Toxicity of the solvent

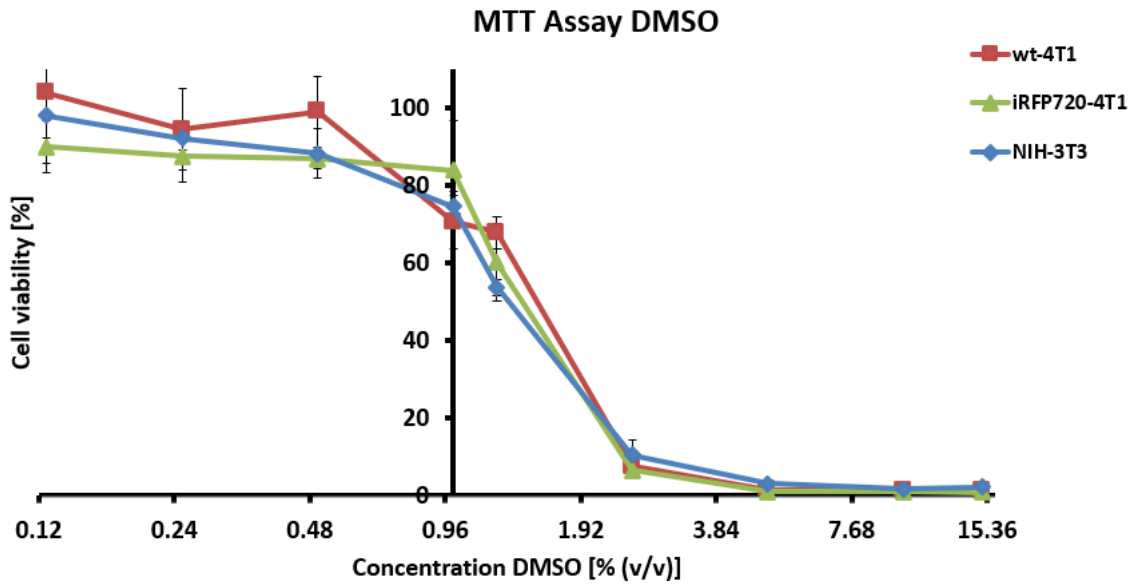


Figure 11: Toxicity measurements of DMSO. The viability (%) of cells treated with DMSO in a concentration range between 1 - 10 % (v/v) was determined by using the MTT-assay.

DMSO was used as a solvent for the substances CuE and Formononetin. In form of a preliminary experiment the toxicity of DMSO in a concentration < 10% (v/v) was tested with the MTT method. [40] The results of the measurement (Fig. 11) indicated that all cell lines tested had a similar response to treatment with DMSO. This test determined a No Observed Adverse Effect Level (NOAEL) of DMSO for the used cell lines of approximately 1% (v/v). This led to the result that not more than 1 % (v/v) DMSO can be used as a solvent for the substances in the dilution series of CuE and Formononetin.

Toxicity of Cucurbitacin E and Formononetin

To determine the limiting concentration of CuE for the subsequent scratch assay, the toxicity of CuE the NIH-3T3 cell line were tested. [41] Different concentrations were used for each dilution series (table 3) and the cell viability was measured using the MTT-assay (Fig. 12).

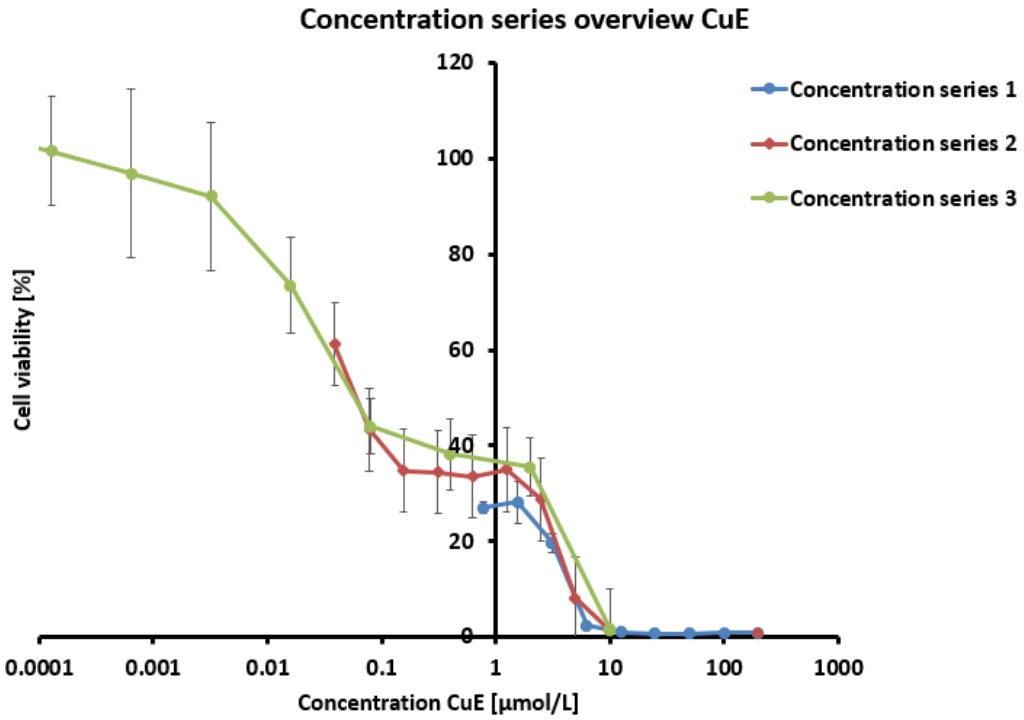
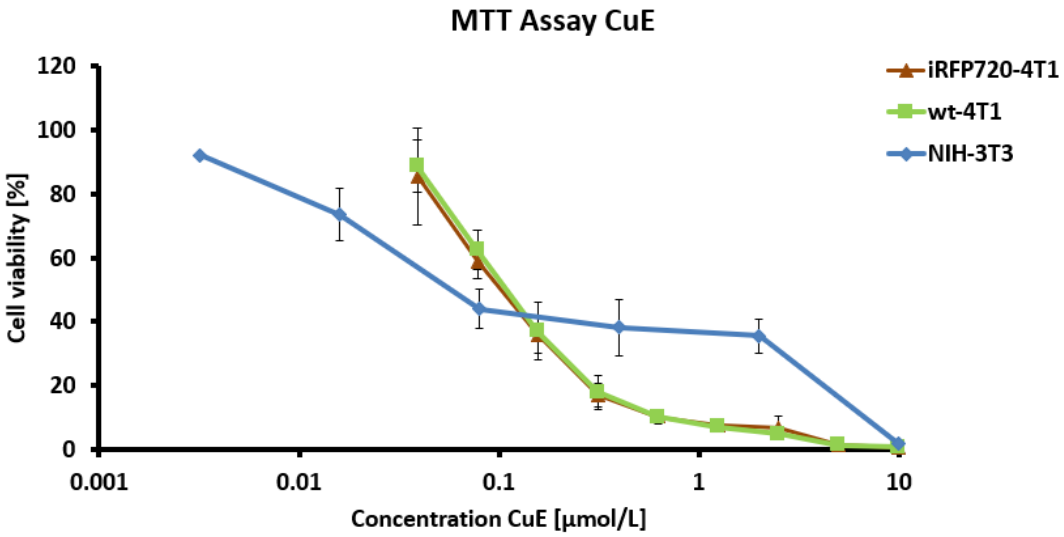


Figure 12: Overview of different concentration of CuE used to test toxicity on NIH-3T3 cells measured with MTT-assay. Values are presented as mean ± SD of three replicates.

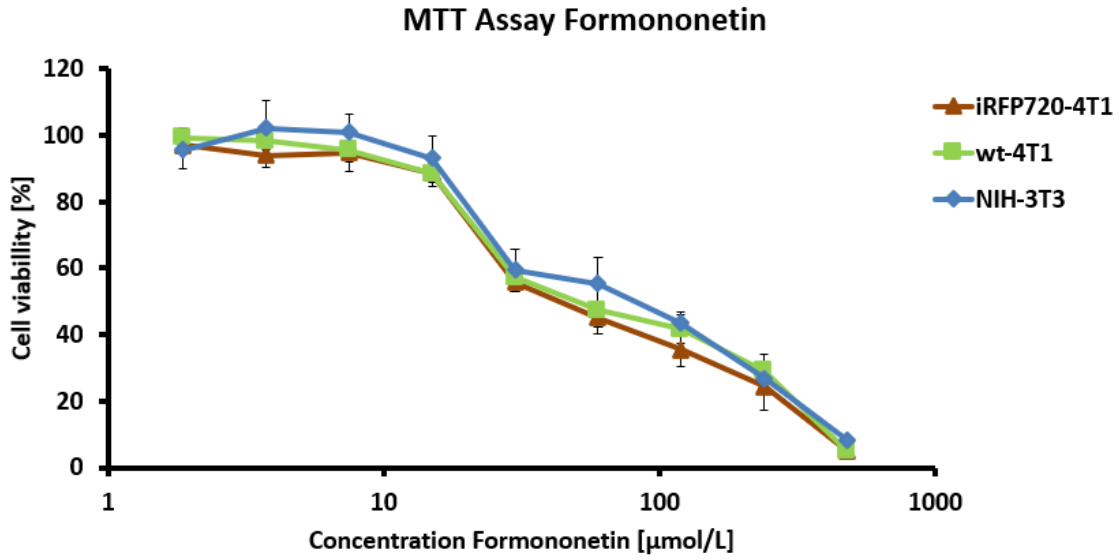
For the first concentration series, the highest concentration of 200 µM was chosen, based on published results, that a concentration of 100 µM CuE achieves cytotoxicity in most cells. [41] Based on the data presented in Figure 12, concentration series 1 at the lowest concentration (0.78 µmol/L) showed a cell viability of approximately 30%. A new dilution series was calculated based on the first tests and the MTT measurement was performed. Concentrations of concentration series 2 displayed a cell viability of 60 % in the lowest concentration (0.039 µmol/L). Therefore, when

calculating concentration series 3 modified dilution steps were used, with the range starting at 10 µmol/L CuE but diluting 1:5 each step, to create a wider range than in the test before.

Regarding the cell lines 4T1-wt and 4T1-iRFP720, in the first measurement, dilution series 1 (Table 2) was used, which resulted in high toxicity. In order to adapt these issues, dilution series 2 (Table 2) was used to perform a second test of cell viability.



A



B

Figure 13: MTT-assay results demonstrating the cell viability after incubation with Cucurbitacin E (13A) and Formononetin (13B) on different cell lines. The values are presented as mean ± SD of three biological and three technical replicates.

Fig. 13A gives an overview of the cytotoxic effect of CuE on the fibroblast and cancer cell lines. The toxicity of CuE on fibroblasts is presented by the blue curve, which is wider, and flatter compared to the green and red curves of the cancer cell line. The results indicated that cancer cells are more sensitive to CuE toxicity, particularly at higher concentrations. Using the results, an IC_{50} of 0.064 µmol/L was determined for CuE in relation to the fibroblast cell line, which shows 50% cell viability

at a lower concentration. The IC_{50} gave information of the amount of compound required to achieve 50% inhibition of a biological mechanism, or in this case 50% of cell viability. [32] Considering these results, a concentration below the IC_{50} of 0.05 µM was chosen for the following cell migration tests. To ensure cells were not exposed to a toxic concentration, the chosen concentration should only inhibit cell migration, so it had to be below the IC_{50}.

When comparing the viability of the cancer cell line, fluorescent marked and wild type, no difference was observed. This supported the use of iRFP720-4T1 cells in subsequent experiments, as the cell lines behaved similarly. The advantage of using fluorescently marked cells is the ability to differentiate cancer cells from fibroblasts through red fluorescence when used in co-culture.

In parallel, a concentration series for Formononetin was prepared with a modified range, as shown in table 2. [20][42]

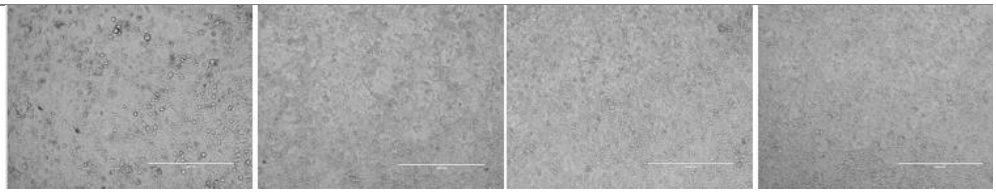
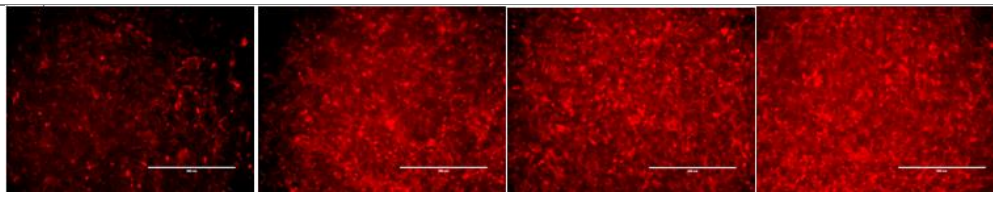
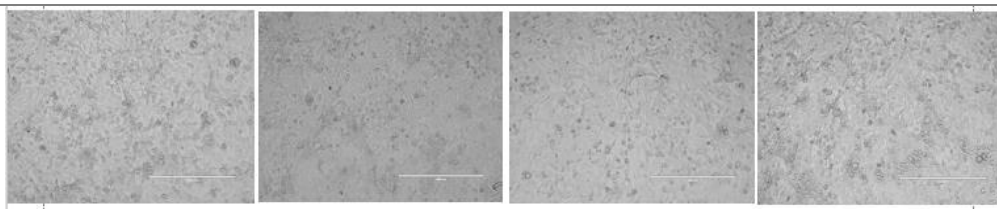
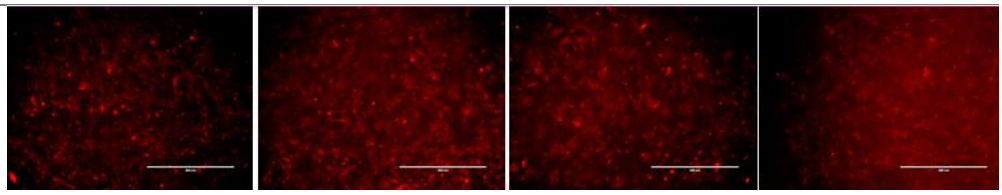
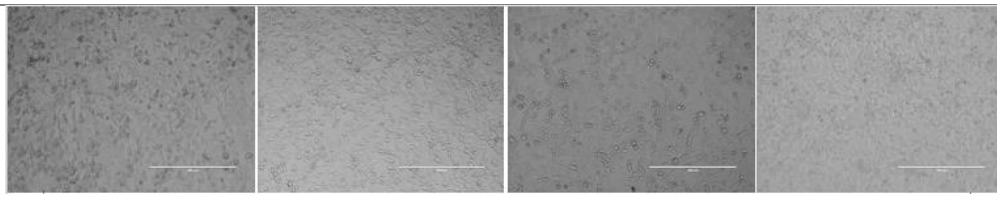
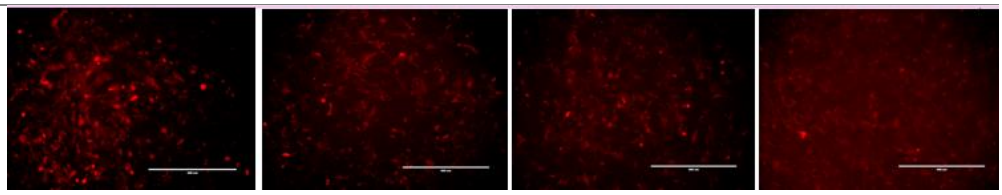
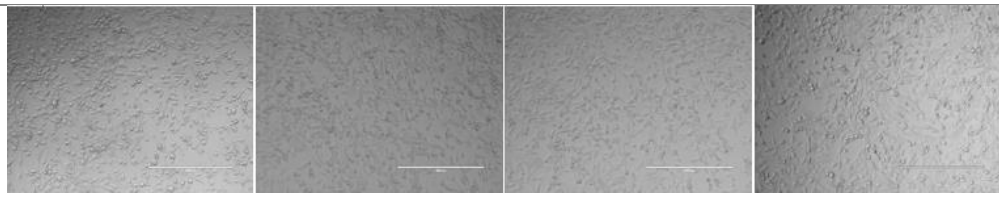
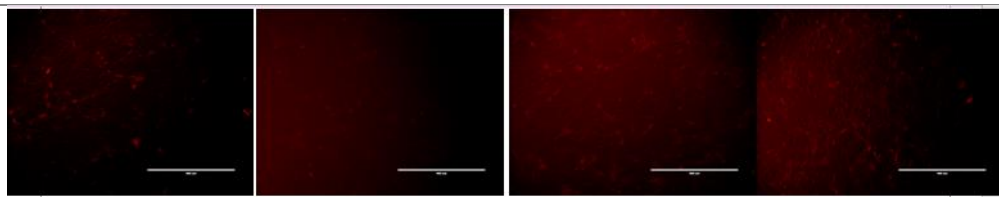
The results of the MTT measurement of Formononetin showed no difference in toxicity between NIH-3T3 fibroblasts, 4T1-wt, and 4T1-iRFP720, as all three curves had a similar course (Fig. 13B). An IC_{50} of 46 µM was determined from these results, and the concentration of 40 µM was chosen for the following scratch assay to avoid interferences of the assay, based on toxic effects of the compound. As the cancer cell line showed similar results, iRFP720-4T1 was used for subsequent experiments.

When comparing the MTT assay results of CuE and Formononetin, it was evident that CuE shows a stronger cytotoxicity. Therefore, a higher dose of Formononetin can be applied to the cells in the subsequent scratch assay, than for CuE. Another difference in toxicity between the substances is that Formononetin demonstrates the same effects on the cell lines tested, whereas CuE has varying effects on healthy cells, represented here by fibroblasts, compared to cancer cells, even when used at the same concentration.

Cell confluency experiment

A preliminary experiment was performed to determine the right cell count to achieve a confluency of 80%, when seeding the cells in co-culture.

Table 4: Results of the confluency experiment using co culture. The cells were seeded in a 24-well plate in four different ratios (1:2, 1:3, 1:4 and 1:5 fibroblasts:cancer cells) and in different overall cell counts per well (1.5 million, 1 million, 600,000 and 300,000). The pictures were taken in a brightfield (BF) and fluorescence (F) setting. The microscope's field of view was magnified to 400 µm.

Cell count		1:2	1:3	1:4	1:5
1.5 million	BF				
	F				
1 million	BF				
	F				
600,000	BF				
	F				
300,000	BF				
	F				

The brightfield images were used to observe differences in cell counts (table 4). The images of 1.5 million, 1 million, and 600,000 cells in all ratios show cells that are already detached, and cell debris is present, indicating a cell count too high. To achieve the desired 80% confluency and prevent cell overgrowth, the total cell count for the following cell migration assay was reduced to 200,000 cells. The fluorescent images were used to evaluate the different proportions of fibroblasts to cancer cells in co-culture. The 1:5 ratio shows an overpowering fluorescence, for all cell counts, which can cause issues when differentiating between fibroblasts and cancer cells. Therefore, ratios of 1:3 and 1:4 were preferred. Those ratios provide a sufficient cancer cell count, while still allowing for separation from fibroblasts. Creating co-culture systems with different stromal cells at various concentrations helps us better grasp how cells behave in the tumor environment.

Inhibition of cell migration

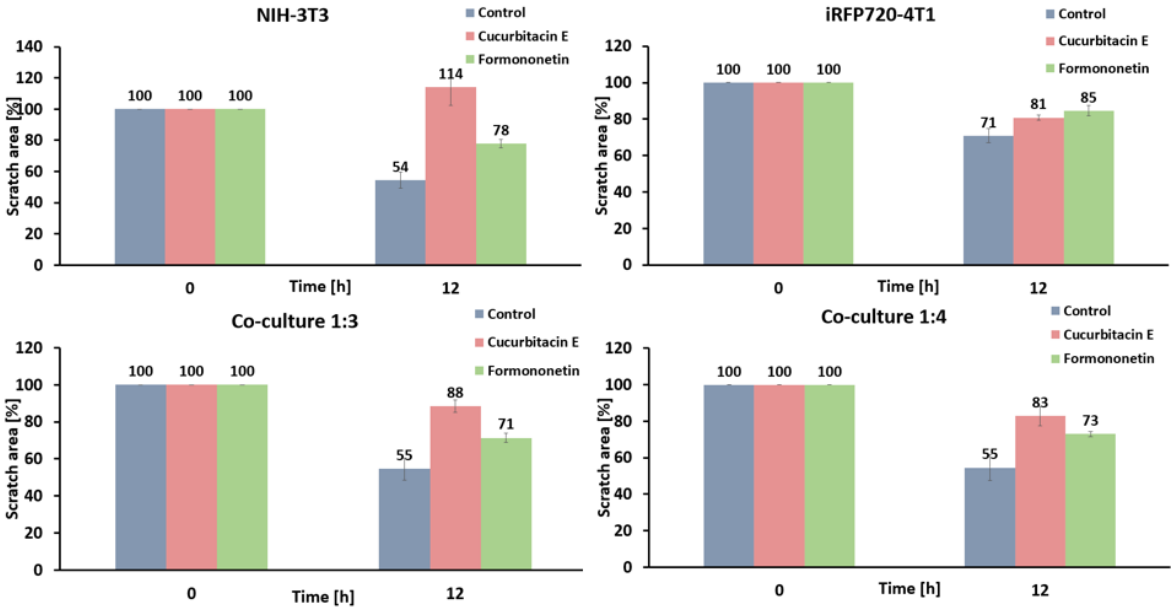


Figure 14: Results of the scratch assay. The bars in blue show the control group, pink bars represent the cells treated with CuE and the green bars display the effect of Formononetin. The bars are paired in two groups, which mark the start point at 0 h and the end of observation at 12 h. The results are presented as mean values of three biological and three technical replicated experiments ± SD.

To observe inhibition of cell migration, where cells were treated with CuE in a concentration of 0.05 µmol/L and 40 µmol/L Foromononetin. The test was performed on NIH-3T3 fibroblasts, iRFP720-4T1 cancer cells and co-culture of these cell lines in the proportion 1:3 and 1:4 (Fig. 14).

The scratch area was normalized to 100% for timepoint 0 h of the control. An inhibiting effect was measured when the control group exhibits a lower percentage than the treated group after 12 h.

Therefore, an inhibition of cell migration was observed for all cell lines and co-culture systems when treated with CuE and Formononetin.

The control groups of the co-culture in ratios 1:3 and 1:4 showed the same scratch area of 55% remaining after 12 h. When comparing the control groups of NIH-3T3 and iRFP720-4T1 after 12 h, the fibroblasts had an area of 54% and cancer cells 71%. It appeared that fibroblasts had migrated faster than cancer cells. This difference in migration behavior could be due to different migration mechanisms (Fig. 15). Fibroblasts tended to migrate with a few cells leading the process, whereas the cancer cells migrated as a whole sheet together. [11] Therefore, analyzing scratches of different cell lines can be difficult, particularly when determining the age of the gap and which cells to include or exclude.

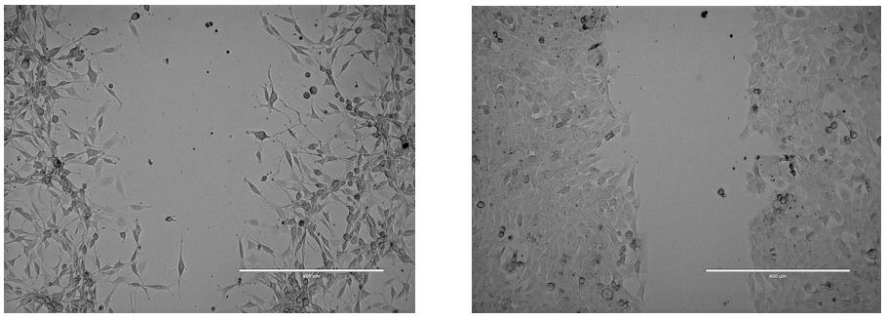


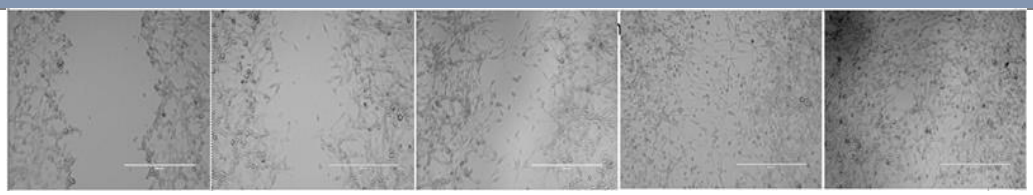
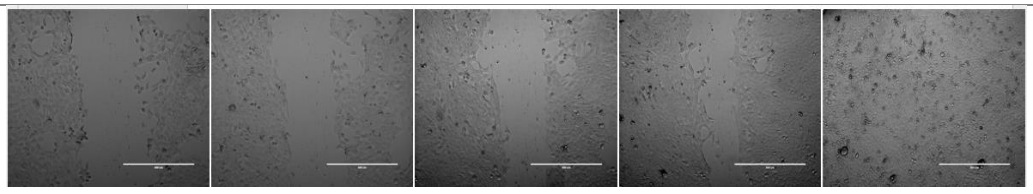
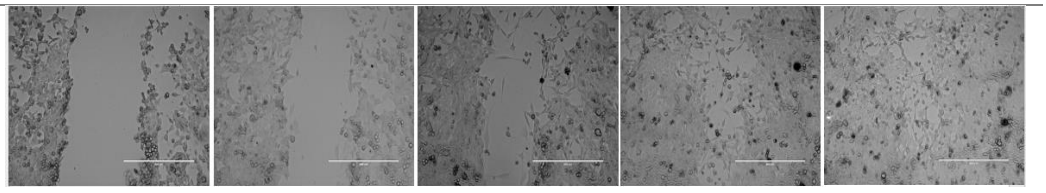
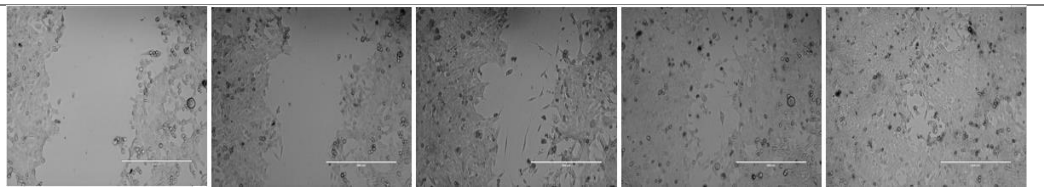
Figure 15: Differences in cell migration of NIH-3T3 fibroblasts and iRFP720-4T1 cells. Depiction of (left) NIH-3T3 and (right) 4T1 cells as control to show differences in migration behavior occurring either as single cell or collective migration, respectively, after 12 h.

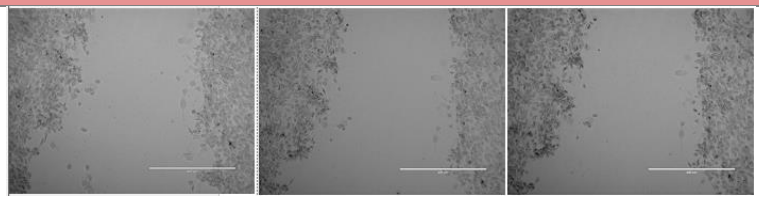
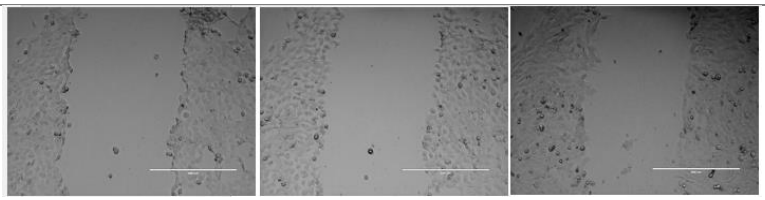
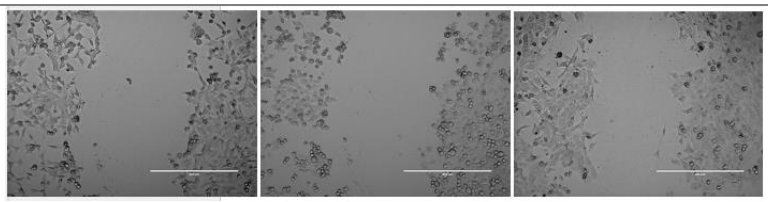
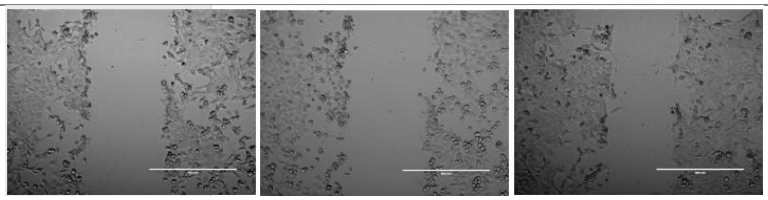
NIH-3T3 fibroblasts treated with CuE exhibited a larger scratch area after 12 hours of incubation, than at the start. This can be attributed to CuE disrupting actin, which is a crucial component of the cytoskeleton and involved in fibroblast adhesion. [43] When comparing the results of the two co-cultures, it was evident that the 1:3 ratio exhibited better inhibition of cell migration after 12 hours, with a percentage of 88%, compared to the 1:4 ratio which had 83% scratch area remaining. This difference could be attributed to the higher proportion of fibroblasts in the 1:3 ratio, which are more effectively inhibited in migration when treated with CuE. The lowest inhibiting effect of CuE was measured on the iRFP-720 cells with 81% area left.

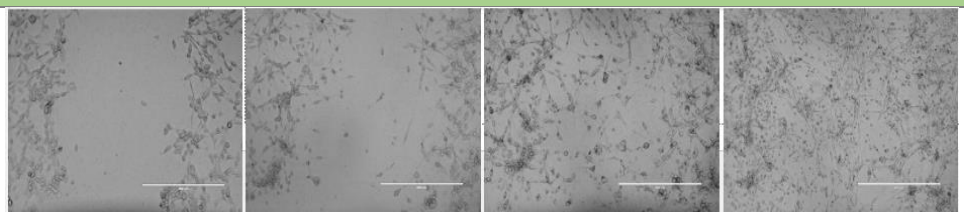
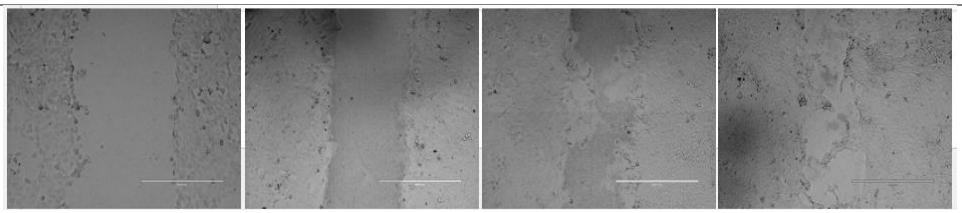
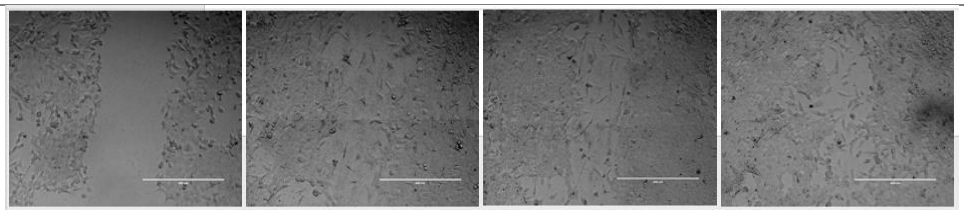
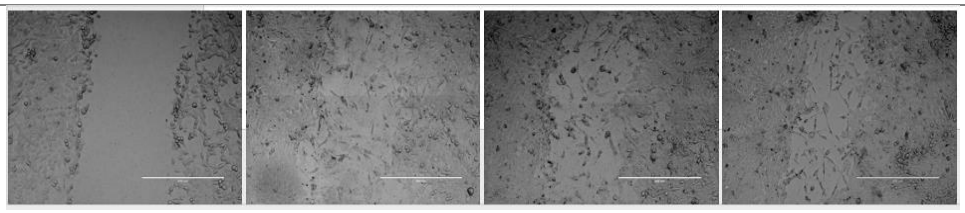
Formononetin most effectively inhibited cell migration in iRFP720-4T1 cancer cells, leaving 85% of the area after 12 hours of treatment. Fibroblasts exhibited a remaining area of 78% after exposure to Formononetin. There was no significant difference in the remaining area between co-culture ratios of 1:3 (71%) and 1:4 (73%). When comparing the four graphs, Formononetin inhibited cell migration in monoculture cell lines more effectively than the co-culture. As a result, the co-culture systems behaved more like the fibroblast cell line, than the cancer cell line.

Both substances tested have demonstrated an inhibitory effect on cell migration. CuE exhibited a stronger inhibition on fibroblasts in co-culture, while Formononetin appeared to have had a slight advantage in inhibiting the migration of cancer cells. The results for co-culture ratios of 1:3 and 1:4 showed similarities with the NIH-3T3 monoculture results, indicating that fibroblast cells highly influenced cell migration in co-culture. When inhibiting the migration of fibroblasts, such as with CuE that targets actin filaments, which are relevant for adhesion and therefore migration, the influence on co-culture was clearly visible as the scratch remained open at around 85%. Conversely, if the substance did not strongly influence the migration of fibroblasts, as was the case with Formononetin, the fibroblasts promoted cell migration in co-culture, causing the scratch to close further than in monoculture.

Table 5: Visual comparison of brightfield images of the scratch assay of NIH-3T3 and iRFP720-4T1 cells, as well as co-culture of the cells in ratios 1:3 and 1:4. Pictures of the control were taken after 0, 6, 12, 24 and 48 h after application of the scratch. The with 0.05 µM CuE treated scratches were photographed after 0 h, 6 h and 12 h. The last set of pictures shows scratched cell monolayers which were incubated with 40 µM Formononetin. Pictures were taken after 0, 12, 24 and 48 h.

Control	0 h	6 h	12 h	24 h	48 h
NIH-3T3					
4T1					
1:3					
1:4					

CuE	0 h	6 h	12 h
NIH-3T3			
4T1			
1:3			
1:4			

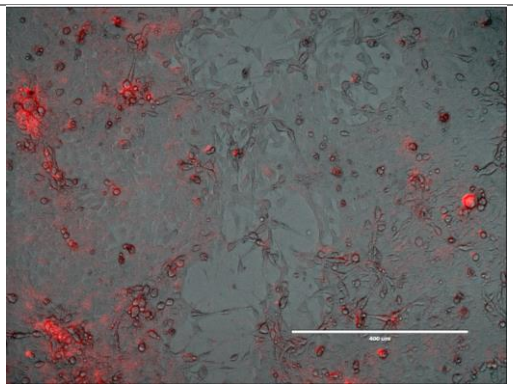
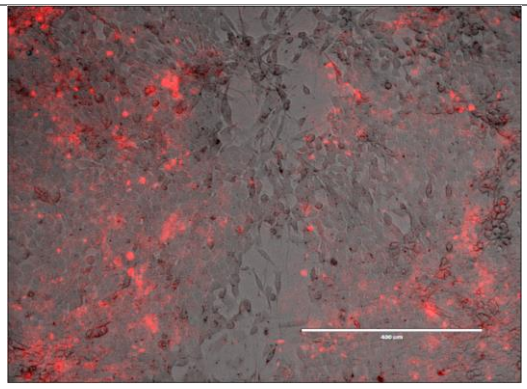
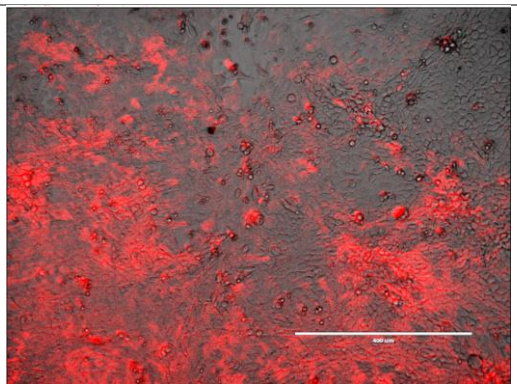
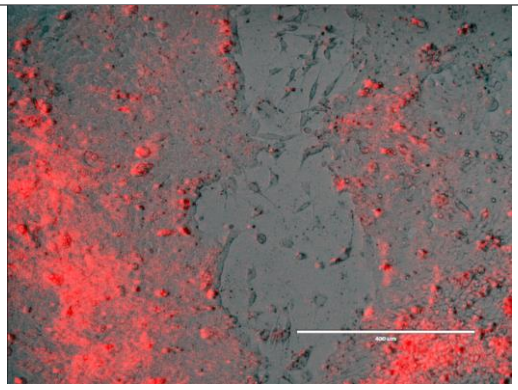
Formon o-netin	0 h	12 h	24 h	48 h
NIH-3T3				
4T1				
1:3				
1:4				

Comparison of scratch assay images (table 5), starting with the control group, with the main difference between the scratches being the type of migration between the cell lines. Fibroblasts showed single-cell migration, creating a more loosely closed gap, while cancer cells migrated in a tight sheet, closing the scratch more evenly. The gap-closing process appeared slower in 4T1 cells, but after 48 hours, the 4T1 scratch was fully closed, while the other three cell line scratches remained marginally open.

Furthermore, the groups treated with CuE still exhibited a visible scratch after 12 hours of incubation for all tested cell lines. The incubation time for CuE was limited to a maximum of 12 hours, as previous research has shown that longer incubation times can result in cell apoptosis [41]. Among all four groups, the cell morphology of fibroblasts appeared to be the most affected, with noticeable cell shrinkage.

When comparing to CuE, Formononetin did not inhibit cell migration as effectively. The scratches of the Formononetin-treated groups were not completely cell-free in the middle due to singular cells, mostly fibroblasts, migrating. This is further enhanced with the overlay images of table 6, which show that the cells migrating in the gap are mostly fibroblasts. In this direct coculture, it appeared that groups of cancer cells moved into the wound and these clusters were surrounded by fibroblasts. The fibroblasts were leading the migration process.
The group of 4T1 cells treated with Formononetin appeared to have a gap area without any single cell migration, indicating again that the main cells migrating into the gap in the co-culture were fibroblasts.
Increasing concentrations of Formononetin to inhibit migration further, is too close to the IC50 concentration. A higher concentration would not only inhibit migration but also prevent it from happening due to toxicity.

Table 6: Overlay images of co-culture scratches after 48 hours of incubation with medium (control) or 40 µM Formononetin. The images depict the different cell types used for co-culture and their migration behavior. The red fluorescence marks the cancer cells.

	Control	**Formononetin**
1:3		
1:4		

In general, scratches created by a mechanical tool such as a pipette tip show a higher inconsistency. If cells stick to the edge of a scratch or fold over the monolayer that cannot be removed by washing, they will reattach to the plate surface and close the gap faster than migration would. Furthermore, seeding cells in 24-well plates to create a homogeneous monolayer can be demanding, especially when seeding cells on top of each other in co-culture. While shaking the plate to evenly distribute the cells, there is the potential to disrupt the monolayer prior to seeding. These issues can lead to differences in the results of replicates. However, the method can be successfully used to obtain initial information on the cytotoxic effects of substances.

Conclusion

The results of the MTT assay demonstrated differences in the toxicity of CuE between fibroblasts and cancer cells. This suggested that the substance was less aggressive to healthy tissue, here represented by fibroblasts, than to cancerous tissue. Such differences in toxicity were not observed for Formononetin.

Migration of cells in co-culture was strongly dependent on fibroblasts, as shown by the scratch assay results. When substances such as CuE have a strong effect on fibroblasts and therefore successfully inhibit their migration, the effect will also be observed in co-culture, as seen in the results. In the case of Formononetin, the inhibition of fibroblast migration was not as effective. Therefore, the migration of cells in co-culture, when treated with Formononetin, was mainly driven by fibroblasts. Furthermore, the cell-cell-contact between fibroblasts and cancer cells in co-culture led to a reduced sensitivity to the inhibitory effect of Formononetin on cancer cells in co-culture.

This work has laid a foundation for observing the inhibition of migration induced by CuE and Formononetin. Further research in a 3D spheroid model could provide more information on the effects of the co-cultured cells on each other and the effects of the substances on them.

Index of Abbreviations

2D: two dimensional

3D: three dimensional 35; three-dimensional

CAFs: Cancer-associated fibroblasts

CuE: Cucurbitacin E

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: dimethyl sulfoxide

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

F-actin: fibrous-actin

FAK: focal adhesion kinase

FCS: fetal calf serum 1

JAK-2: Januskinase-2

L-Glu: L-glutamine

MMP: matrix metalloproteinase

MTT: 3−[4,5−dimethylthiazol−2−yl]−2,5−diphenyltetrazolinum bromide

P/S: penicillin-streptomycin 1

PBS: Phosphate buffered Saline 1

RPMI 1640: Roswell Park Memorial Institute 1640

SD: standard deviation

STAT3: signal transducer and activator of transcription 3

TME: tumor microenvironment

TNBC: triple negative breast cancer

VEGFR-2: vascular-endothelial-growth-factor-receptor-2

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List of Figures

List of Tables