Circumventing Gemcitabine resistance in pancreatic cancer using drug delivery systems

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ABSTRACT

Gemcitabine is the first line treatment of pancreatic cancer, a treatment that is relying on the expression of human Equilibrative Nucleoside Transporter 1 for cellular uptake. Combined with its short blood half-life and intracellular activation steps, Gemcitabine treatment has many limitations. In this project we tried to circumvent the need for cellular uptake as one step towards increasing the efficiency of Gemcitabine treatment. A liposomal drug delivery system was tested *in vitro* combined with chemically blocking the uptake route. Because of inconclusive results, the liposomal drug delivery system could not be fully evaluated.

Key words: Pancreatic cancer, Gemcitabine, hENT1, drug delivery, liposomes

List of abbreviations: DP – Dipyridamole; DZ – Dilazep; FBS – Fetal Bovine Serum; Gem – Gemcitabine; PC – Pancreatic cancer

INTRODUCTION

Pancreatic cancer (PC) is one of the most lethal cancer forms. Although it represents only 6% of all cancer deaths in the US, only 5 % of all patients with PC survive for more than 5 years. [1] The low survival rates can partly be explained by the high degree of resistance the tumor cells acquire and by the tumor environment, characterized by low vascularization and dense stroma, leading to a poor distribution of drugs to the cancer cells. [2] The stroma consists of various cell types, including endothelial cells, immune cells and fibroblasts. Evidence suggests that the stromal compartment together with the epithelium increases the aggressiveness of PC. PC cells distribute molecules stimulating the stroma, and the stroma in turn releases substances stimulating tumor growth. [3]

Most often, patients diagnosed with PC are over 40 years old, and the median age of diagnosis is 71 years of age. PC is more common in developed countries and is the eighth leading cause of death from cancer in men, while it is the ninth leading cause of death from cancer in women. The risk factors associated with PC are tied to smoking, obesity and long-term diabetes. [4]

PC is characterized by a lack of symptoms in early stages, making it difficult to diagnose in the early onset of the disease. Surgical options are often limited, [5, 6] and only 20% of patients diagnosed with PC are eligible for surgical removal of the tumor. [2, 7] In cases where surgery is not an option, other possible treatments consist of chemotherapy, radiation therapy or a combination of these. [7]

Gemcitabine (Gem), a cytidine analog, is commonly used as a standard treatment in patients with PC both in palliative and preoperative settings. [2] Inside the cells, Gem is phosphorylated to yield the active substance prior to incorporation into the DNA chain during DNA replication, forcing the cell into apoptosis by a process called “masked chain termination”. [2, 8]

The process of masked chain termination can be seen in Figure 1. The activated Gem is incorporated into the replicating DNA strand by DNA polymerase. Afterwards, a nucleotide is inserted, camouflaging Gem and preventing removal by DNA repair systems. Addition of a second nucleotide is now stopped, and chain elongation cannot continue. This forces the cell into apoptosis. [2]

Even though Gem is the standard treatment for PC, it has its limitations. One of the challenges regarding Gem-based treatment is the fact that Gem is a prodrug in its administered form and needs to undergo a series of phosphorylation steps to yield

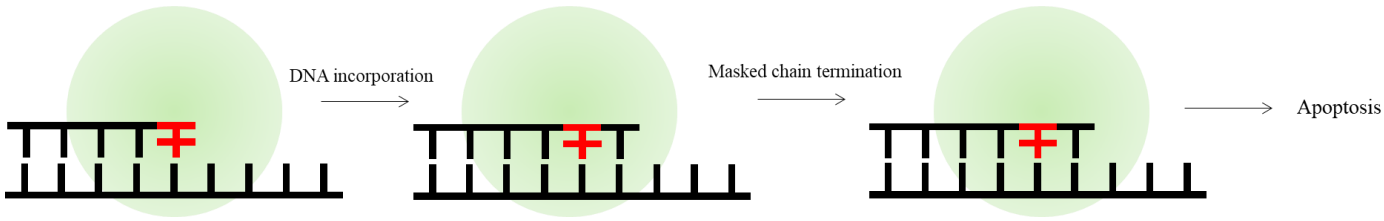


Figure 1. The mechanism of masked chain termination
First, the activated metabolite of Gem is incorporated into the replicating DNA chain. This is followed by incorporation of a nucleotide, masking Gem. This prevents removal of Gem by DNA repair systems. The incorporation of Gem also inhibits further incorporation of nucleotides, terminating chain elongation and forcing the cell into apoptosis.

the active substance. Intracellular resistance mechanisms can turn Gem into an inactive form, making it unable to exert its cytotoxic properties. Of the total amount of Gem administered, 77 % is excreted in its inactive metabolite or administered form trough renal excretion within 24 hours after administration. This creates the need to administer higher doses to reach desired effects. [2, 9, 10]

Moreover, Gem has a short blood half-life ($t_{1/2}$ between 15 [11] and 90 min [12]) and is rapidly converted to its inactive form in the plasma. Due to this short half-life, there is a need for frequent administration. [11] High dose in combination with frequent administrations give rise to more severe side effects. [2, 9, 10] Another challenge regarding Gem-based chemotherapy is, as previously mentioned, the low vascularization in the tumor, making it more difficult for Gem to reach the tumor cells. [2] This is not due to Gem but rather the nature of the tumor. However, in combination with the aforementioned limitations, this affects the efficacy of the treatment.

Another reason of concern when using Gem is its need for active cellular uptake by hENT1. [2] Gem is a hydrophilic drug, making diffusion across the cell membrane a slow process. Transport across the cell membrane is mediated by membrane transporters, where hENT1 is the major transporter for Gem. [13] The expression of hENT1 has been linked to the prognosis for PC patients treated with Gem, where a high expression of hENT1 is associated with a more optimistic prognosis than for PC patients with low hENT1-expression. [7] An earlier study on hENT1 as a biomarker studied tissue samples from patients with advanced pancreatic cancer treated with Gem. This study showed that patients with high hENT1 expression had a longer survival. [8]

hENT1 is a 50 kDa protein with 11 predicted trans-membrane domains [14, 15] and is responsible for uptake of nucleosides from the surrounding environment. [14-16] In previous studies, hENT1 has shown sensitivity towards the blocking effect of Dilazep (DZ), Dipyridamole (DP) and 6-*S*-[(4-Nitrophenyl)methyl]-6-thioinosine (NBMPR). [14-16] Functional studies of the protein has indicated that transmembrane domain 3-6 have great significance in inhibitor interactions, [14] as well as substrate binding. [15]

Equilibrative Nucleoside Transporters (ENT) have been found in most cell types throughout the body [14] and have been divided into two subgroups: ENT1 and ENT2. These subgroups are classified depending on their sensitivity towards inhibition by NBMPR. hENT1 show sensitivity to NBMPR at low concentrations, and is therefore classified as equilibrative sensitive (*es*), whereas hENT2 shows low sensitivity and is therefore classified as equilibrative insensitive (*ei*). [14, 17, 18]

Since the uptake of Gem is mainly dependent on the expression of hENT1, alternative uptake routes for the drug are needed to improve prognosis for PC patients with low hENT1 expression. In our group, we have been using liposomes as a drug delivery system in an attempt to circumvent the need for active cellular uptake of Gem, thereby decreasing the importance of hENT1 expression.

Liposomes are artificial vesicles consisting of at least one phospholipid bilayer. [19] The lipid bilayer of the liposome surrounds an aqueous interior, where hydrophilic drugs can be encapsulated. Hydrophobic drugs can be encapsulated in the phospholipid bilayer. Liposomal drug delivery can occur by the liposome fusing with the cell membrane of another cell, thereby releasing its contents in the intracellular compartment. It can also occur by endocytosis, where the liposome is internalized by the cell via endocytosis, followed by the release of the drugs encapsulated in the liposome. [20]

Initially, two cell lines were chosen for the experiment. The cell lines were human PC cell lines, chosen based on their expression of hENT1. Capan-1, a cell line with epithelial morphology was

derived from a liver metastasis in a 40 year old male, [21] has been shown to have high hENT1-expression. [22] PANC-1 is an epithelial cell line derived from the pancreatic duct of a 56 year old male, [23] and has been confirmed to have low hENT1 expression. [22] However, as can be seen in Figures 1 and 2, Appendix 2 PANC-1 show low inhibition of cell viability for the controls treated with only Gem. PANC-1 is known to display high resistance towards Gem due to its many resistance mechanisms, which reduces its sensitivity towards Gem. [24] This resistance is again proven by our results. Since PANC-1 showed lack of response towards Gem, the experiments on this cell line were discontinued and experiments were conducted on BxPC-3 instead. This epithelial human pancreatic cancer cell line is more sensitive to Gem than PANC-1 [24], but has similar hENT1 expression. [25]

The aim of this study was to see if the uptake of Gem could, through a liposomal drug delivery system, circumvent the need of active cellular uptake. In order to prove this theory, the uptake route of Gem (hENT1) was inhibited to simulate a low-hENT1 expression environment. From this low-expression scenario, we would try to circumvent this lack of uptake using liposomes as a drug delivery system.

MATERIAL AND METHODS

Cell culture Capan-1 was bought from ATCC (LGC, Manassas, VA, USA) and kept in Iscove's modified Dulbecco's medium (Gibco, Life Technologies, Grand Island, NY, USA) with 20% fetal bovine serum (FBS, Gibco, Life Technologies). 800 000 cells were seeded in one T75 flask and the cells were used between passages 25 and 40. BxPC-3 was obtained from ATCC and kept in Roswell Park Memorial Institute 1640-Glutamax Medium (Gibco, Life Technologies) with 10 % FBS. 300 000 BxPC-3 cells were seeded per T75 flask and the cells were used between passages 23 and 38. PANC-1, was obtained from ATCC and was grown in Dulbecco's Modified Eagle Medium (Gibco, Life Technologies) supplemented with 10% FBS. 300 000 PANC-1 cells were grown in one T75 flask and used for experiments between passage 3-10.

The media for all cell lines were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies). Cells were grown in T75 flasks in a 5% CO_2, 37^{o}C humidified environment. Cell media was changed twice a week and the cells were passaged once a week.

Before passaging, the cells were washed with PBS (Gibco, Life Technologies). TrypLE select (Thermo Fisher Scientific, Waltham, MA, USA) was used to detach the cells (5 min for PANC-1 and BxPC-3, and 10 min for Capan-1, respectively) before they were collected and centrifuged at 1400 rpm for 5 min. After dissolving the pellet in cell culture medium, cell concentration was determined using a Bürker chamber and 0.06 % Tryphan blue.

Expression of hENT1 Whole cell lysates for Western Blot were obtained by dissolving approximately 1.2 million cells in 1 ml of lysis buffer supplemented with Phosphatase inhibitor and Protease inhibitor (Thermo Fischer Scientific). The samples were incubated on ice for 10 minutes before they were frozen in aliquots.

Protein determination was carried out using NanoDrop 2000c. Whole cell lysate corresponding to 2.5 and 5 µg protein was diluted 1:1 with Laemmli buffer 2x (S3401, Sigma-Aldrich, St. Louis, MO, USA) and boiled for 5 min before they were placed in the gel. Samples were run on two different gels, BioRad 4-15 % Mini-PROTEAN® TGX™ Precast Protein Gel (4561086, BioRad) for detection of 5-200 kDa proteins and BioRad Mini-PROTEAN® Any kD™ Precast Protein Gel (4569033, BioRad) for detection of 10-100 kDa proteins. BioRad Trans-Blot Turbo Transfer System (1704155, BioRad) was used for transfer onto a nitrocellulose membrane (1704158, BioRad). Membranes were blocked in TBST/5 % milk powder (Sigma-Aldrich) for 2 hours in room temperature on a shaker, followed by 3 washes with TBST of 15 minutes each. The anti-hENT1-monoclonal antibody SP120 (SAB5500117, Sigma-Aldrich) was diluted 1:1000 in TBST/5% milk and mouse-anti-GAPDH (MAB374, Millipore, Billerica, MA, USA), used for housekeeping protein, was diluted 1:2000, also in TBST/5% milk. Membranes were incubated with the appropriate antibody on a shaker in 4^{o}C overnight.

After incubation with antibodies, the membranes were washed with TBST/5 % milk powder twice for 15 minutes, followed by a single wash with TBST, 15 min. Goat-anti-rabbit HRP diluted 1:2000 (A0545, Sigma-Aldrich) was used against SP120 and Goat-anti-mouse HRP (A4416, Sigma-Aldrich) 1:10000 for housekeeping protein. Both antibodies were diluted in TBST/5% milk. Membranes were incubated for 1 hour in 4^{o}C. The membranes were washed three times in TBST for

15 minutes each, before detection using SuperSignal West Femto Maximum Sensivitity Substrate (34095, Thermo Fischer Scientific) per manufacturer's instructions. Results were obtained using LiCor chemiluminescence Detection System.

Immunofluorescence For immuno-fluorescent staining, cells were seeded in 8-well chamber slides at a concentration of 15 000 cells/chamber. The cells were seeded in 200 µL medium and were left in the incubator for 72 hours before staining. After 72 hours, the chambers were rinsed with ice cold PBS before they were fixed in Paraformaldehyde (2% in PBS/0.2 % Triton x-100) on ice for 20 minutes. The cells were rinsed twice with ice cold PBS before blocking in block solution, containing 100 µL Donkey serum (Sigma Aldrich) and 1900 µL PBS/0.2 % Triton x-100, on ice for 30 minutes. Slides were washed twice with ice cold PBS before addition of primary antibody (SP120, 1:50, diluted in PBS/0.2% Triton x-100) and incubated for 1 hour in room temperature. This was followed by secondary antibody, donkey-anti-rabbit FITC (A21206, Thermo Fischer Scientific), after washing the chambers twice with PBS, and incubated for 50 minutes in darkness. Chambers were rinsed 3 times with PBS and stained with DAPI (Thermo Fischer Scientific) for 5 minutes before slides were rinsed with PBS and mounted. The stainings were carried out twice in duplicates.

Co-treatment Cells were seeded in 96-well plates with a concentration of 5 000 cells/well for 24 hours to allow cell adhesion before treatment with Gem (Sigma-Aldrich) and inhibitor. Stock solutions of NBMPR and DZ were prepared. NBMPR was dissolved in DMSO and DZ in dH20, with a final concentration of 100 mM. The stock solution was diluted to the desired concentrations tested in the experiment - 1, 50, 10 and 100 nM for NBMPR (Sigma-Aldrich) and 1, 10, 20 and 50 nM for DZ (Sigma-Aldrich). These concentrations were tested against 3 concentrations of Gem (Sigma-Aldrich, 10, 50 and 100 µM). All combinations were tested in triplicates (n=3). After 24 hour incubation, 100 µL Gem/inhibitor solution was added after removal of the medium by turning the plate upside down on paper tissues. Plates were incubated 24 or 48 hours before a cell viability measurement was done.

Pre-treatment Cells were seeded in 96-well plates with a concentration of 3 000 cells/well for 72 hours before treatment with inhibitor, followed by a treatment with both inhibitor and Gem. The concentrations for NBMPR and DZ were as previously described. A series of concentrations within micro molar range was also tested (0.5, 1, 5 and 15 µM). These concentrations were used for the treatments with DP (Sigma Aldrich). A stock solution was prepared, where DP was dissolved in DMSO to a concentration of 100 mM. This stock solution was then diluted to the desired concentrations. The Gem concentrations tested were the same as in Co-treatment described previously. After 72-hour incubation, 100 µL inhibitor diluted in growth medium was added after removal of the medium by turning the plate upside down on paper tissues. Plates were incubated for 24 hours before the wells were emptied as described previously. After emptying the plates, 100 µL Gem/inhibitor (n=3) were added to the wells and incubated for 24 or 48 hours. After 24 or 48 hours, a cell viability measurement was done.

Liposome preparation Liposomes of DPPC:Chol:DSPE-mPEG$_{2000}$ (80:20:5) molar ratios were prepared by the thin lipid hydration technique. [26] Briefly, phospholipids were weighed and dissolved in a round bottom flask using a (3:1 v/v) mixture of chloroform: methanol. In order to form a thin lipid film, organic solvents were removed under rotation and reduced pressure at 60ºC using a Büchi Rotavapor for 1 hour. Lipid films were further dried overnight in a Labconco Freezone Plus 6 lyophilizer (Labconco Corporation, Kansas City, MO, USA) in order to remove all traces of organic solvents. After drying, lipid films were hydrated with either Hepes Buffered Saline (HBS, pH 7.4) or a Gem solution under rotation at 60ºC for 1 hour and further vortexed until all lipid film dissolved. The multilamellar suspension was serially extruded 11 times through a 200 nm and 100 nm stacked pair of polycarbonate filters, each, to obtain a unilamellar suspension of small liposomal vesicles. Non-encapsulated Gem was removed from solution by gel chromatography using a Sephadex G25 column (GE Healthcare Life Sciences AB, Uppsala, Sweden), columns were equilibrated with HBS. Liposomes were prepared by the supervisor for use of the student in the aforementioned experiments.

GemLip Treatment 3 000 cells/well were seeded in 96-well plates for 72 hours before pre-treatment. After the 72-hour incubation time, the cell culture medium was removed by turning the plate upside down on paper tissues. The plates were pre-treated with the appropriate DP-concentration (0.5, 1, 5 or

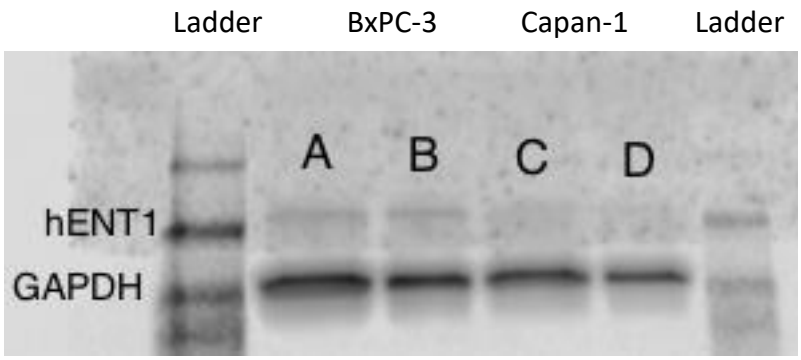


Figure 2. Western blot with hENT1 and GAPDH in BxPC-3 and Capan-1 cell lines
Whole cell lysates corresponding to 2.5 or 5 ug protein were run on the gel before transferred onto a nitrocellulose membrane. The membranes were blocked before stained with primary antibody (SP120 for hENT1, diluted 1:1000 and mouse-anti-GAPDH for housekeeping gene, diluted 1:2000) and secondary antibody (Goat-anti-mouse HRP for detection of hENT1, diluted 1:2000 and goat-anti-mouse HRP for detection of housekeeping gene, diluted 1:10000). SuperSignal West Femto Maximum Sensivitity Substrate was used according to manufacturer's instructions for detection. A. BxPC-3, 5 µg protein B. BxPC-3, 2,5 µg protein C. Capan-1, 5,0 µg protein D. Capan-1, 2,5 µg protein. The results show a weak band for hENT1 compared to the band of GAPDH. Despite the band for hENT1 being weak, it still shows the presence of hENT1. hENT1 is located in the upper bands and housekeeping gene (GAPDH) is located in the lower bands.

15 µM) for 24 hours before GemLip treatment and control treatments.

After 24-hour Pre-treatment incubation, the medium was removed as described previously. The cells were treated with a combination of DP and GemLip, where all DP concentrations were tested against all GemLip concentrations. GemLip, Gem, Lip and DP were tested alone, and a combination of DP/Gem and DP/Lip were tested. 100 µL treatment solution was added to each well and incubated for 24 or 48 hours. After 24 or 48 hours, a cell viability measurement was done.

Cell viability measurement Cell viability measurement was performed with WST-1 (Sigma-Aldrich). WST-1 was added to the wells followed by 4 hour incubation. After the 4 hour incubation time, the absorbance was read at λ=450 nm for cell viability and λ=660 nm for background.

Statistical analysis Co-Treatment, Pre-Treatment and GemLip-treatment assays are presented as means in per cent ± standard deviation in per cent. Statistical analysis was performed with Ordinary one-way ANOVA by use of GraphPad Prism 7.

RESULTS

Western blot In order to demonstrate the presence of hENT1, a Western blot was performed. As can be seen in Figure 2, Western Blot showed traces of hENT1 in both Capan-1 and BxPC-3, even though the bands are weak. Samples were loaded onto the gel with two different protein amounts and as can be seen in the Figure, as the bands are weaker with decreasing protein amount. Compared to the housekeeping protein (GAPDH), the hENT1 expression is lower.

Immunofluorescence As a complement to Western blot in the process of demonstrating the presence of hENT1, immunofluorescent staining targeting hENT1 was performed. Capan-1 and BxPC-3 were seeded and stained as described in the method section and the fluorescent stainings are shown in Figure 3. Negative control for Capan-1 is displayed in A, showing no fluorescence. Capan-1 did show staining on some of the cells, but not on all. The results indicate hENT1 being present since some fluorescence is visible, even though fluorescence cannot be seen around all cells. The same goes for BxPC-3, which also shows fluorescent staining for hENT1 on some of the cells. The negative control for BxPC-3 does not show the green fluorescent staining for hENT1.

Pre-Treatment In order to obtain an optimal blocking protocol, cells were treated with inhibitor for 24 hours before addition of inhibitor and Gem, as previously described in the Pre-Treatment section. As shown in Figure 4, BxPC-3 cells treated with a combination of DP and Gem display a inhibition of Gem uptake after 48 hours than cells treated with Gem only. This is true for all cells independent of the Gem concentration they were treated with (10, 50 or 100 µM). According to these results, a higher DP concentration correlates with higher inhibition of Gem, indicated by higher cell

Capan-1

BxPC-3

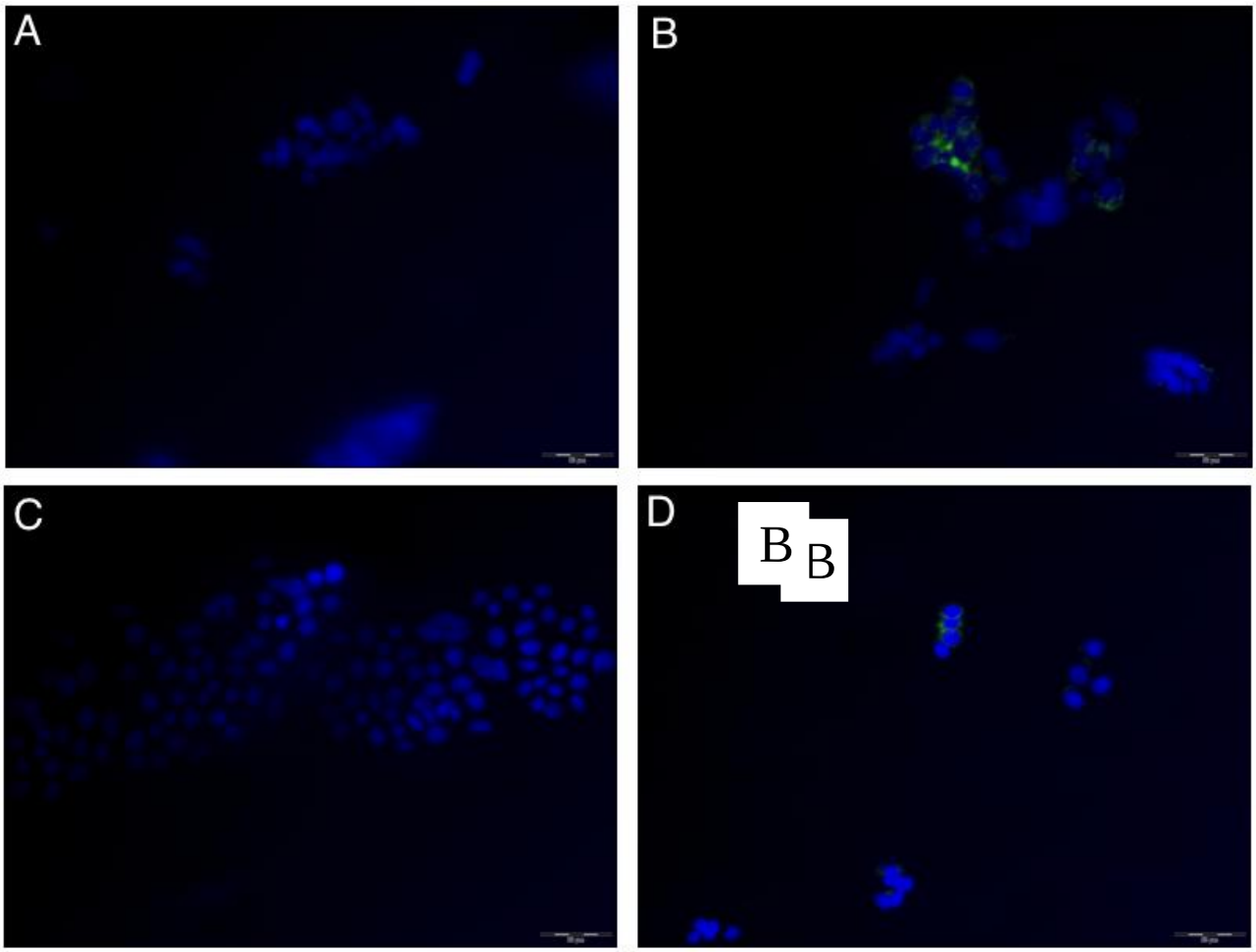


Figure 3. hENT-1 immunoreactivity in Capan-1 and BxPC-3 cells
Capan-1 cells and BxPC-3 cells were grown for 72 hours before fixation in in 2% paraformaldehyde/0.2% Triton-X 100. Cells were stained with (B, D) or without (A, C) the SP120 antibody directed towards hENT1 (Green staining, diluted 1:50). All cells were stained with DAPI (A-D, blue) for detection of the cell nuclei. Control cells (A, C) were not stained with SP120 but rather with secondary antibody and DAPI only. The scale bars represent 50 µm. The figure shows representative results (n = 4). Capan-1 and BxPC-3 negative controls (A, C) does not show green fluorescence for hENT1, whereas the staining using the SP120 antibody (B, D) show green fluorescence.

viability. This inhibition of Gem uptake cannot be seen after 24 hours.

Capan-1 cells (Figure 5) were treated as described previously in the Pre-treatment section. The cells treated with a combination of DP and Gem do not show an inhibition of Gem uptake compared to the controls treated with only Gem. on the contrary, after 48 hours, the cell viability for cells treated with only Gem is higher than for cells treated with DP and Gem, indicating a still functioning Gem uptake, namely an unsuccessful blocking of hENT1.

GemLip-Treatment As shown in Figure 6, the inhibition of Gem seen in Figure 4 for BxPC-3 cells is not present here. This is indicated by the cell viability for the cells treated with only Gem was similar to the cell viability of the cells treated with DP and Gem. This strongly suggests that the inhibition of Gem uptake seen in the Pre-Treatments are not present here. Furthermore, Figure 6 also indicates either a successful Gem delivery to the cells by the liposomes or a leakage of Gem from the liposomes to the surrounding environment. This is indicated by the control for free Gem being similar to the control for liposomal Gem (GemLip). The cells treated with a combination of DP and GemLip as well as the cells treated with only GemLip show similar cell viability as
the cells treated with only Gem.

Similar to what was observed with experiments using BxPC-3, no inhibition of hENT1 was seen using Capan-1, Figure 7. These results correlate with the results in Figure 5 where there was a poor blocking of hENT1. The cell viability for the cells treated with a combination of DP and Gem show similar cell viability as the cells treated with Gem only, which does not indicate inhibition of Gem uptake as a consequence of blocking hENT1. Furthermore, the results here just like the results in Figure 7 indicate either a successful Gem delivery by the liposomes, or a leakage of Gem from the liposomes to the cell media. This is again indicated by the fact that the cell viability for cells treated

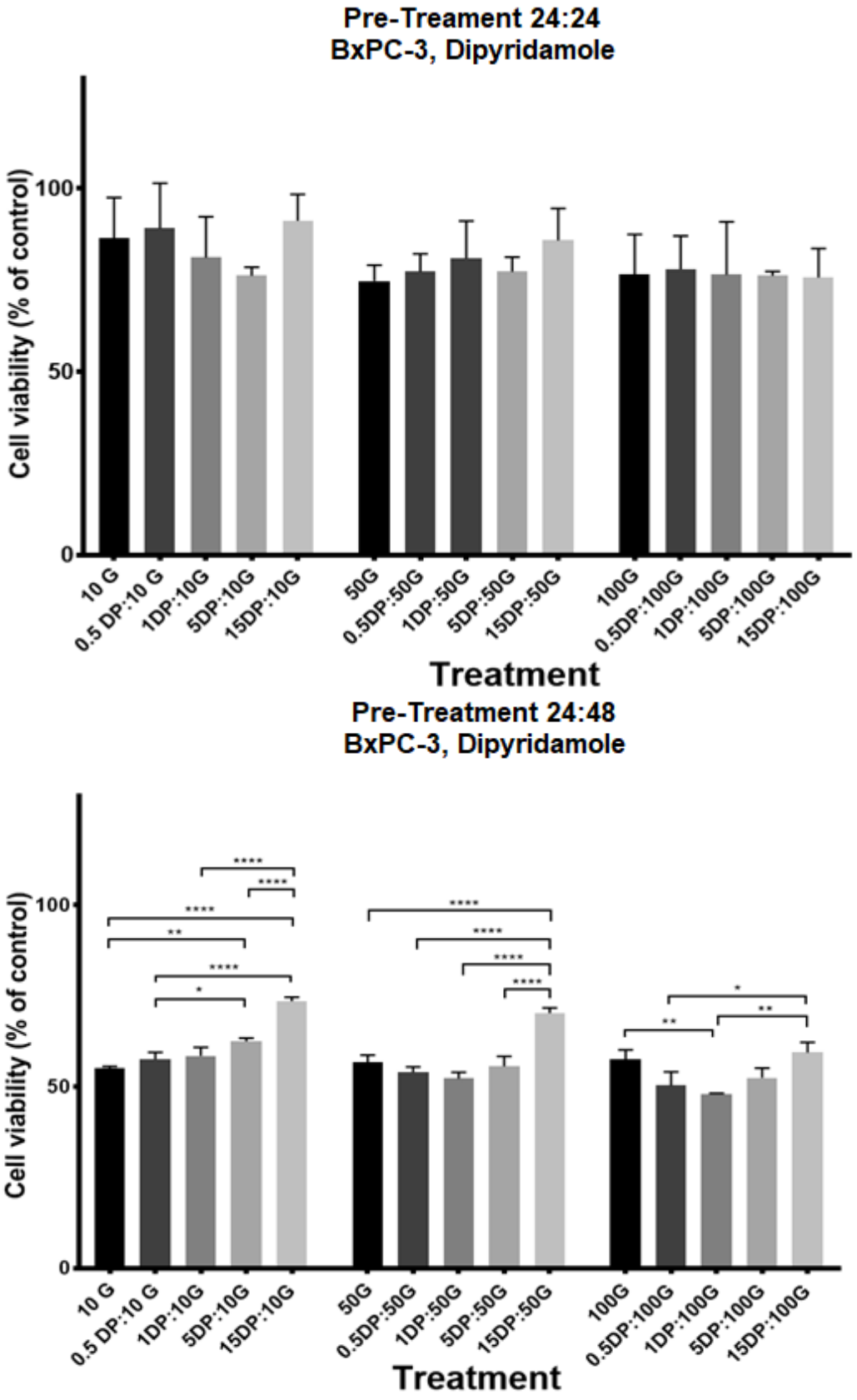


Figure 4. Pre-Treatment with Dipyridamole (DP) on the BxPC-3 cell line
BxPC-3 cells were grown for 72 hours followed by a 24-hour treatment with DP. This was followed by a 24- or 48-hour co-treatment with DP and Gemcitabine. The results displayed in the figure show decreased cell viability after 48 hours (B) for cells treated with DP. After 24 hours, an increase in cell viability for cells treated with DP is not seen. From left to right: Gem (black bar), 0.5 µM DP + Gem, 1 µM DP + Gem, 5 µM DP + Gem and 15 µM DP + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem. 24:24 – 24 hour Pre-Treatment followed by a 24 hour Co-treatment. 24:48 – 24 hour Pre-Treatment followed by a 48 hour Co-Treatment. For all conditions, data is expressed as mean cell viability in percent of control ± standard deviation in per cent, n=3 * = $P \leq 0.05$, ** = ≤ 0.01, *** = $P \leq 0.001$, **** = ≤ 0.0001

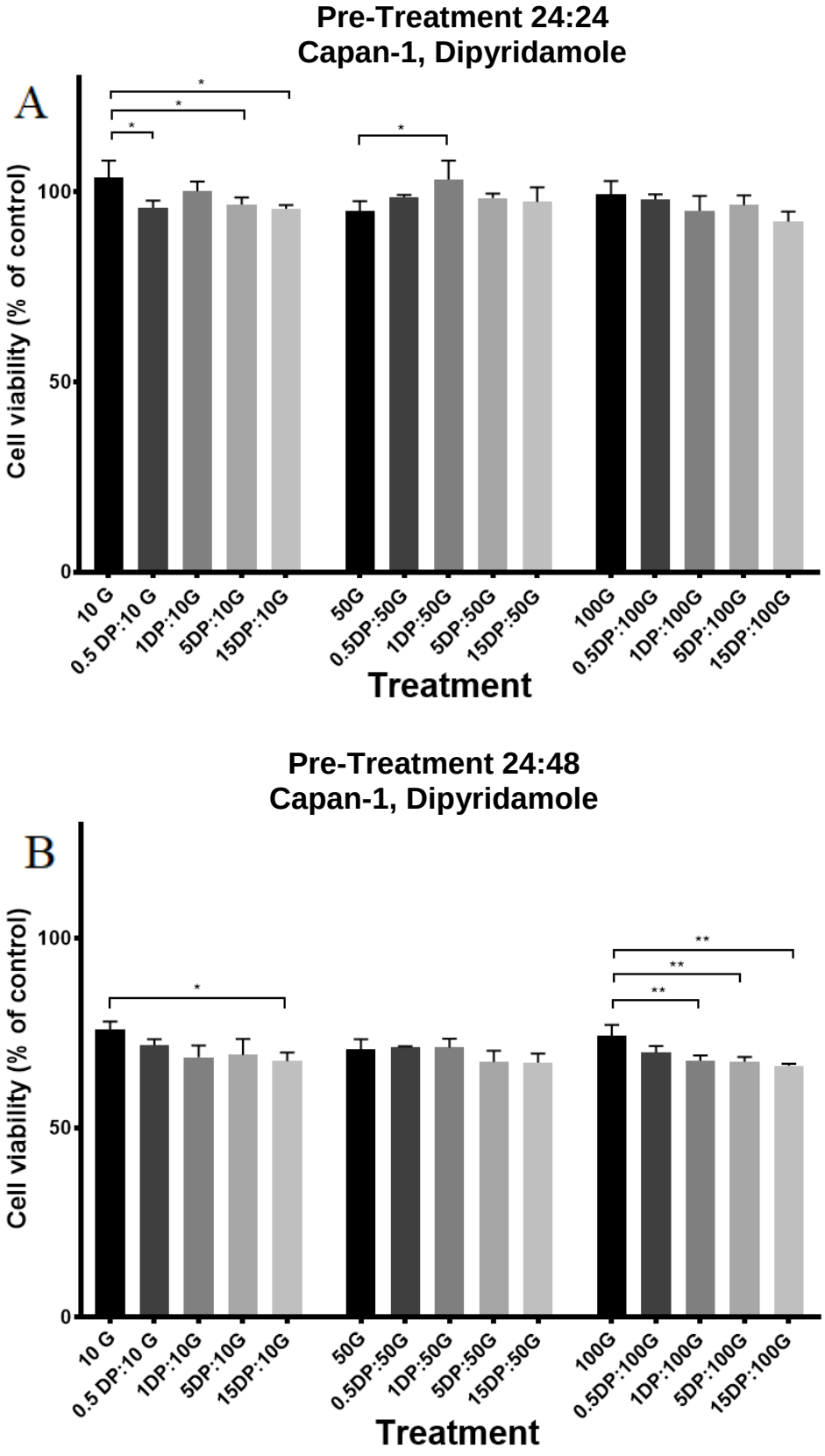
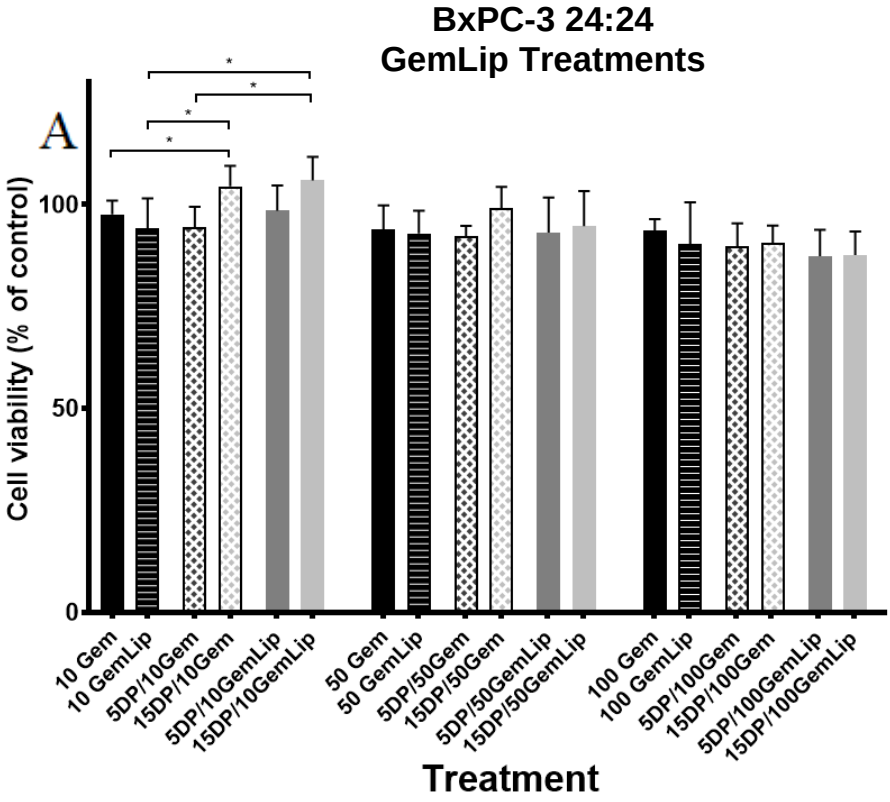


Figure 5. Pre-Treatment with Dipyridamole (DP) on Capan-1 cell-line
Capan-1 cells were grown for 72 hours followed by a 24-hour treatment with DP. This was followed by a 24- or 48-hour co-treatment with DP and Gemcitabine. These results from the treatments using DP on Capan-1 cells do not show decreasing cell viability for cells treated with DP, both after 24 and 48 hours. From left to right: Gem (black bar), 0, 5 µM DP + Gem, 1 µM DP + Gem, 5 µM DP + Gem and 15 µM DP + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem. 24:24 – 24 hour Pre-Treatment followed by a 24 hour Co-treatment. 24:48 – 24 hour Pre-Treatment followed by a 48 hour Co-Treatment. For all conditions, data is expressed as mean cell viability in percent of control ± standard deviation in per cent, n=3. * = $P \leq 0.05$, ** = ≤ 0.01, *** = $P \leq 0.001$, **** = ≤ 0.0001



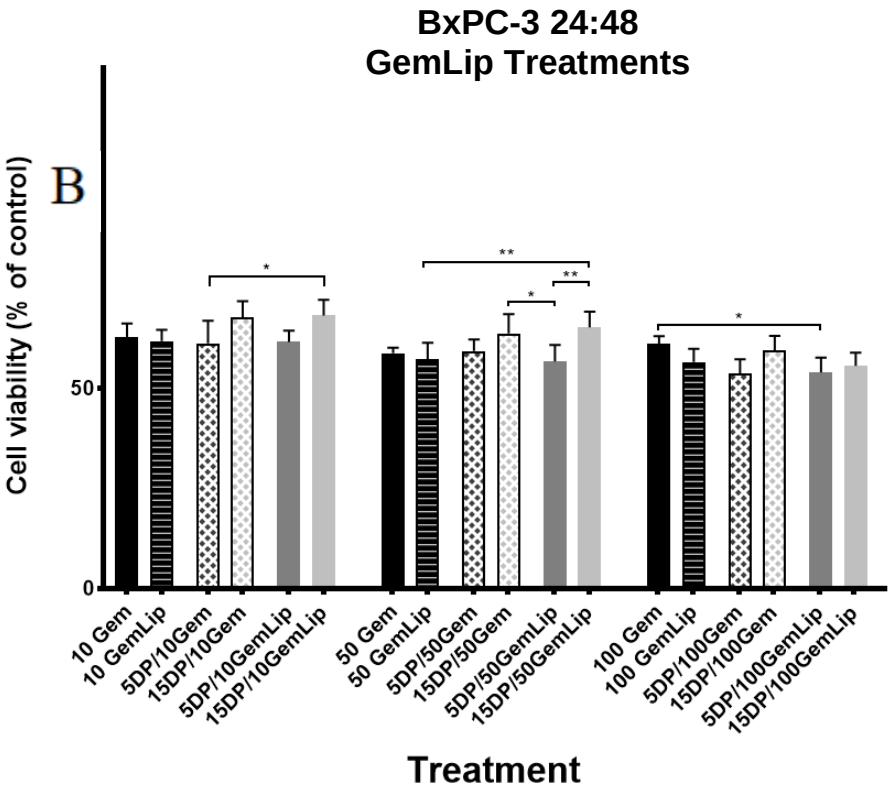


Figure 6. Pre-Treatment with Dipyridamole (DP) followed by a 24- or 48-hour Co-Treatment with GemLip using the BxPC-3 cell line

BxPC-3 cells were grown for 72 hours before a 24-hour treatment with DP. This was followed by a 24- or 48-hour treatment with either free Gemcitabine of liposomal Gemcitabine before cell viability was measured. When comparing cells treated with DP and Gem with cells treated only with Gem, the cell viability does not differ between the treatments. Also, when comparing the cell viability presented by the cells treated with a combination of DP and GemLip with the cells treated with only GemLip, they show similar cell viability. The cell viability for DP/Gem treatments does not differ from DP/GemLip treatments. From left to right: Gem, GemLip, 5 µM DP/Gem, 15 µM DP/Gem, 5 µM DP/GemLip, 15 µM DP/GemLip. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem. 24:24 – 24 hour Pre-Treatment followed by a 24 hour Co-treatment. 24:48 – 24 hour Pre-Treatment followed by a 48 hour Co-Treatment. For all conditions, data is expressed as mean cell viability in percent of control ± standard deviation in per cent, n=3. * = $P \leq 0.05$, ** = ≤ 0.01, *** = $P \leq 0.001$, **** = ≤ 0.0001

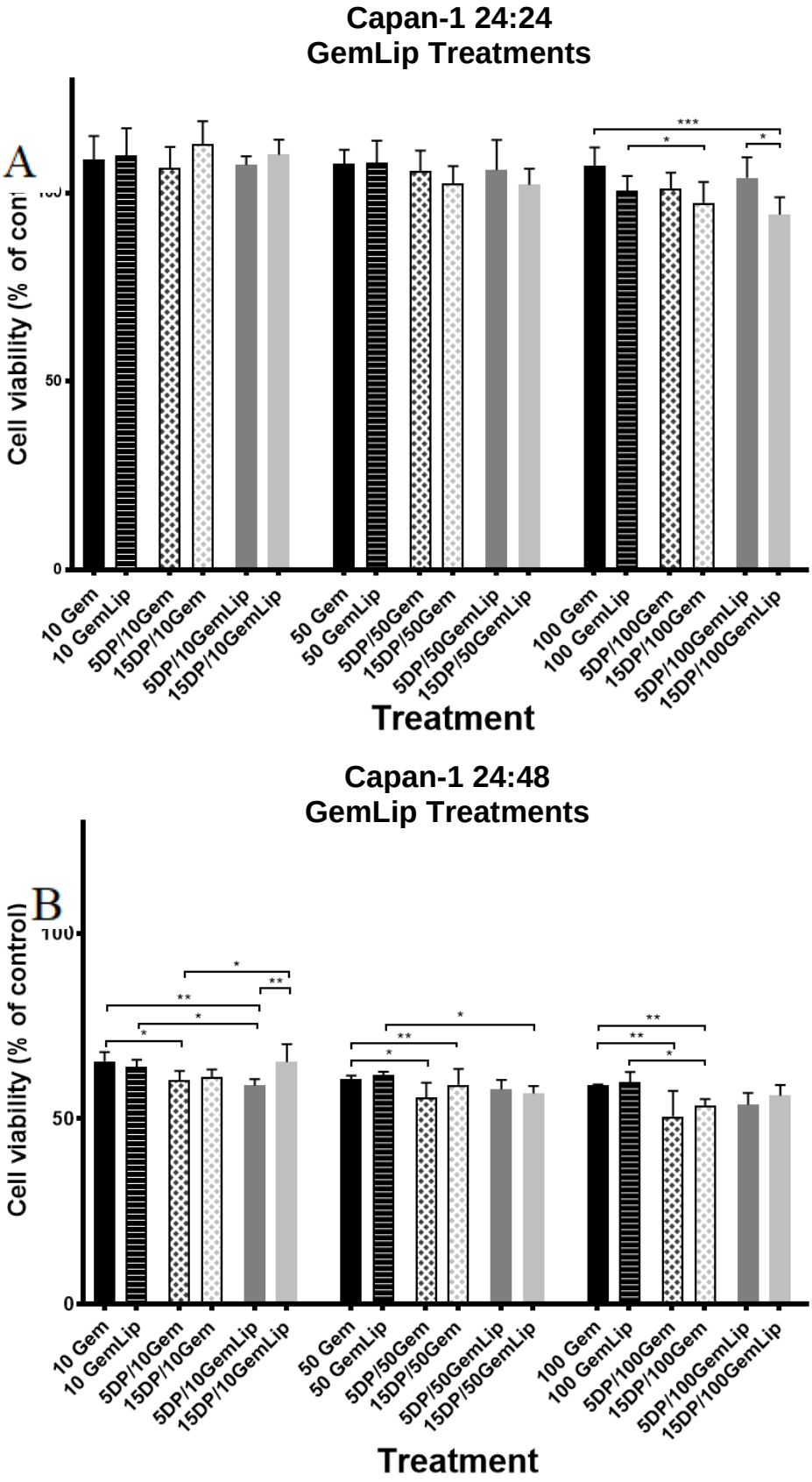


Figure 7. Pre-Treatment with Dipyridamole (DP) followed by a 24- or 48-hour Co-Treatment using Capan-1 cell line.

Capan-1 cells were grown for 72 hours before a 24-hour treatment with DP. This was followed by a 24- or 48-hour treatment with either free Gemcitabine of liposomal Gemcitabine before cell viability was measured. When comparing cells treated with DP and Gem with cells treated only with Gem, the cell viability does not differ between the treatments. Also, if one compares the cell viability presented by the cells treated with a combination of DP and GemLip with the cells treated with only GemLip, they show similar cell viability. The cell viability for DP/Gem treatments does not differ from DP/GemLip treatments. From left to right: Gem, GemLip, 5 µM DP/Gem, 15 µM DP/Gem, 5 µM DP/GemLip, 15 µM DP/GemLip. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem. 24:24 – 24 hour Pre-Treatment followed by a 24 hour Co-treatment. 24:48 – 24 hour Pre-Treatment followed by a 48 hour Co-Treatment. For all conditions, data is expressed as mean cell viability in percent of control ± standard deviation in per cent, n=3. * = $P \leq 0.05$, ** = ≤ 0.01, *** = $P < 0.001$, **** = < 0.0001

with only GemLip is similar to the cell viability for cells treated with only Gem.

DISCUSSION

The transmembrane transporter hENT1 is important for Gem sensitivity as it has been identified as the main uptake route of Gem. [2, 8] The expression of hENT1 is particularly important as it has been correlated to prognosis and helps predict patient response to Gem treatment. [9] As such, finding a way to circumvent the need for active cellular Gem uptake by hENT1 is a step towards a less transport-dependent uptake and a more efficient treatment.

hENT1 inhibition To create a model of low hENT1-expression, the function of hENT1 needed to be inhibited. This was possible in two ways; pharmacological inhibition of hENT1 or by siRNA gene silencing. It has previously been reported that NBMPR, DZ and DP inhibit hENT1 at nanomolar concentrations. [27] Based on this, NBMPR and DZ were initially chosen for the pharmacological inhibition experiments. NBMPR in particular was chosen due to its role in the definition of *es* and *ei* nucleoside transporters. [14, 17, 18] NBMPR has been reported to inhibit transport mediated by hENT1 at low concentrations (Inhibitory constant, K_i = 10 nM). [14] DZ has also been reported to inhibit the nucleoside transport trough hENT1 at low concentrations (K_i = 19 nM). [27] Therefore, NBMPR and DZ were chosen for the experiments.

The K_i values previously mentioned for both DZ and NBMPR were used as a starting point for the concentrations which were later used in the experiments. However, as can be seen in Appendix 2, DZ and NBMPR did not reach satisfactory inhibition of hENT1. The concentrations were raised from nanomolar to micromolar to see if results could be obtained by using higher concentrations. As these results too were inconclusive, this lead to the decision to use DP instead of NBMPR and DZ.

DP is an ENT-inhibitor, just as DZ and NBMPR, and it has been reported to inhibit hENT1 (K_i = 48 nM). [27] The reported K_i for DZ is lower than the K_i reported for DP, and therefore DZ was initially chosen over DP. The micromolar concentrations previously used for DZ and NBMPR were used for DP as well, and as can be seen in Figure 5 and 6, this gave an inhibition of approximately 20% compared to the untreated cells, in the highest inhibitor concentrations.

For both NBMPR and DP, DMSO was used as a solvent. It is known that DMSO is toxic to cells by interaction with the cell membrane and the cells' metabolism, causing cell damage. [28] However, the stock solution prepared with DMSP was of the highest possible concentration to allow as much dilution of DMSO as possible. To achieve the desired concentration, this means a 100x dilution was needed to reach a concentration of 100 μM before further dilution to reach the concentrations used in the treatments. This resulted in the DMSO concentration being lower than 0.1% and its possible cytotoxic effect was not taken into consideration.

It has been reported that siRNA gene silencing indeed does reduce the amount of hENT1 expressed. [29] However, there is no mention regarding the degree of silencing, making it difficult to predict the amount of silencing that can theoretically be achieved. Because of this insufficient information, and the complex protocols for gene silencing, we chose to first use chemical inhibition ahead of siRNA gene silencing.

hENT1 expression Western blot was made to demonstrate the presence of hENT1, just as the immunofluorescence. Western Blot shows very weak bands even for Capan-1 which is known to have a high hENT1 expression, [22] as mentioned in the introduction. However, the sample run was a whole cell lysate and no purification steps were conducted before electrophoresis. This means that the amount of protein measured in the sample, and loaded on to the gel, were all the expressed proteins and not only hENT1. As a consequence, of the total amount of proteins loaded on to the gel only a smaller fraction of the proteins were hENT1, which could explain why the bands on the gel are very weak. The reason for running a whole cell lysate instead of purifying the membrane bound proteins is because of the process being both a complicated and a time-consuming task. What could have been done to increase the protein concentration in the cell lysate was to increase the amount of cells per ml of lysis buffer.

One cannot exclude the antibody function as a reason for the weak bands displayed in the western blot. The only type of control used for the western blot was GAPDH, which means that the antibody targeting hENT1 was not tested against a positive control consisting of hENT1. What could have been done was to buy hENT1 and load the protein in one well on the gel to test the antibody against a positive control of the same protein that was

targeted. Therefore, one could see if the weak bands were because of a low amount of hENT1 or because of the function of the antibody.

Immunofluorescence was performed to detect the presence of hENT1. hENT1 was stained using SP120, a rabbit-anti-hENT1 monoclonal antibody. [30] Previous studies have used SP120 for immunohistochemistry with 1:50 [30, 31] and with 1:100 dilutions. [31] With the information previously mentioned, we chose to use one antibody dilution, 1:50. As Figure 3 shows, hENT1 is visible through its green fluorescence, and cell nuclei are stained blue. Figure 3 demonstrates that for both BxPC-3 and Capan-1, there is staining for hENT1 but not on all cells. Some cells only display the blue color for cell nuclei. Previous reports have used the SP120 anti-hENT1 antibody for immunohistochemistry and not immunofluorescence, which can be part of the explanation as to why the staining is only partial.

hENT1 inhibition experiments Initially, a Co-Treatment was performed. In this Co-Treatment, inhibitor and Gem was mixed and added to the cells simultaneously. The cell viability was measured after 24 or 48 hours. As can be seen in Figures 1-4, Appendix 2 there is no evident difference between the cells treated with Gem and the cells treated with a combination of the inhibitor and Gem. These results led to the suspicion that there was not enough time for the inhibitor to bind to hENT1 and limit Gem uptake before a toxic amount of Gem was taken up.

To give the inhibitor some time to bind to hENT1 before addition of Gem in combination with the inhibitor, a series of incubation times for a Pre-Treatment was tested. Figures 5-12, Appendix 2 show the results from the different incubation times for the different inhibitors and cell lines. These results are inconclusive but still more promising than the Co-Treatment. Since the first two inhibitors chosen for the experiment did not work, DP was tested in the same Pre-Treatment setting as DZ and NBMPR had been. DP was added 24 hours prior to the addition of a combination of DP and Gem. These results can be viewed in Figures 4 (BxPC-3) and 5 (Capan-1). The results for BxPC-3 clearly state that at the highest concentration of DP (15μM) the inhibition of cell viability decreases from ~55% to ~75% for the two lower Gem concentrations, compared to control.

Cell culture When cultured, manufacturer's instructions were followed. BxPC-3 were grown in medium supplemented with 10% FBS, [32] whereas Capan-1 was grown in medium supplemented with 20% FBS. [21] FBS is a combination of proteins, growth factors, vitamins and other components essential for cell growth. [33] Therefore, one cannot exclude that the differences in FBS concentration in the growth medium affected the results. Capan-1 has twice the amount of FBS compared to BxPC-3, and therefore a different environment. The cells were also grown in two different growth medium, where BxPC-3 were grown in RPMI and Capan-1 in IMDM. The cells therefore had different conditions for growth. The manufacturer's intrigues were followed, but this causes a difference between how the two cell lines are grown and thus a difference between the cells in the experiment.

GemLip experiments The results from the GemLip experiments are inconclusive. This because the inhibition seen in the Pre-Treatment was lost during the GemLip treatments. The cells treated with a combination of DP and Gem did not indicate an inhibited Gem uptake and the blocking of hENT1 by DP are not demonstrated in Figures 6 and 7. The data from the cells treated with GemLip indicates two things; a successful Gem delivery or a possible leakage of Gem from the liposomes. When comparing the cells treated with only Gem to the cells treated with only GemLip, these display similar cell viability, indicating a similar inhibition of proliferation. No assays determining the leakage of Gem from the liposomes were done.

Identifying the malfunctioning component in the experiment is not feasible based on the data available. 12 passages differ between the cell lines used for the Pre-Treatment and the results from GemLip. GemLip were tested on cells at between passages 35 and 40, and Pre-Treatment was performed 12 passages earlier. Based on the data available, one cannot rule out changes in the cell's gene expression, which may have changed the amount of hENT1 expressed, or may even have altered the protein. Changes in mRNA levels could have been analyzed with qRT-PCR, an analysis that could have given more insight into what went wrong with the GemLip experiments.

Because the cells used had high passage number, a GemLip treatment was performed on new cells. These cells passage number was below 10. The results can be seen in Figure 3, Appendix 1 (BxPC-3) and Figure 5, Appendix 1

(Capan-1). The cell viability values for the experiments can be seen in Table 3, Appendix 1 (BxPC-3) and Table 4, Appendix 1 (Capan-1).

To exclude inhibitor function as a reason for the inconclusive results, an assay evaluating inhibitor binding could provide additional details. A binding assay, which evaluates the amount of inhibitor bound to the cells, could provide information regarding the possible change in the amount of inhibitor bound to the cells at the time for the Pre-Treatment and at the time for the GemLip treatments. If there was a change in inhibitor binding, a binding assay may have provided that information.

The quality of the inhibitor cannot be excluded from the equation. It is possible that the inhibitor lost its function partly or completely while stored. A stock solution was frozen (-20^{o}C) in aliquots, and new aliquots were thawed for each experiment. Therefore, freeze-thaw cycles can be excluded as a reason for this loss of function. However, product information sheets mentioning stability for a DP solution recommends storing the DP solution in aliquots at -20 ^{o}C for use within a month. [34]

CONCLUSION AND FUTURE PERSPECTIVES

Based on the results generated by the experiment, it is difficult to draw a conclusion. The results indicate that GemLip either delivers Gem to the cells or that there is leakage of Gem from GemLip. One reason cannot be excluded for the benefit of another based on the data obtained during the course of the project.

If GemLip successfully delivers Gem to the cells, this Gem delivery does not inhibit proliferation to a greater extent than free Gem. Gem is still needed for the treatment of PC, a treatment that is complicated by the complexity and aggressiveness of PC. More research on the subject can provide more insight into liposomal Gem and its positive effects in the treatment of PC. Optimizing chemotherapy treatment in combination with more effective screening methods and diagnostic tools is a crucial step in prolonging life, to provide better quality of life for affected individuals, but is also a step in the process of decreasing the mortality rate displayed by PC. Finding biomarkers and creating an accessible and accurate diagnostic tool for early diagnosis is important. However, it is also important that the treatment for PC is more effective, and therefore further research in drug delivery systems is of uttermost importance.

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Appendix 1

The cells were treated as mentioned in the GemLip-section. Figures 1 and 2 show controls for the GemLip experiments shown in the Results section. The results in Figures 1 and 2 show cell viability for cells treated with DP, empty liposomes or a combination of these. This was done to see if DP or the liposomes are toxic alone or in combination. Figure 1A and 1C show DP-treated cells in comparison to untreated cells and cells that were given empty liposomes in comparison to untreated cells. These Figures show that DP and empty liposomes are not toxic to BxPC-3 when not given in combination. Figure 2A and 2C show the same results for Capan-1 – DP and empty liposomes are not toxic when given separately.

Figure 1B and 1D show cell viability for the cells treated with a combination of DP and empty liposomes. This in order to see if the inhibitor and the drug delivery system in itself were toxic to the cells combined. As can be seen in the Figure, DP and empty do not display toxicity when combined. The same goes for Capan-1, Figure 2B and 2D. Together, Figure 1 and 2 show that DP and empty liposomes are not toxic when given separately or in combination with one another. Therefore, the toxicity shown for the cells treated with GemLip has to be mediated by Gem only. Supplementary data for these experiments can be seen in Table 1 and 2 for BxPC-3.

Figure 3 show the results from GemLip on new BxPC-3 cells. The results are similar to the results displayed by the old cells. Inhibition and GemLip treatments are inconclusive, and the Figure indicated a successful Gem delivery, but an unsuccessful hENT1 inhibition by DP. It is again proven, in Figure 4, that DP and empty liposomes display no toxicity alone or in combination.

Figure 5 show the results from GemLip-treatments on new Capan-1 cells. These results differ from the results displayed by the old Capan-1 cells. The cell viability is significantly lower than previously demonstrated. The cells also behaved differently in the cell culturing flask, growing much faster than the old cells did. Figure 6 show that this increase in toxicity is not influenced by DP or the liposomes, which means that the decreased cell viability is a consequence of Gem. No inhibition is displayed by the cells in Figure 5, similar to previous experiments. Because of the differences in behavior by the cells, the results are not comparable to the previous results on the old Capan-1 cells. Supplementary data can be seen in Table 3 and 4.

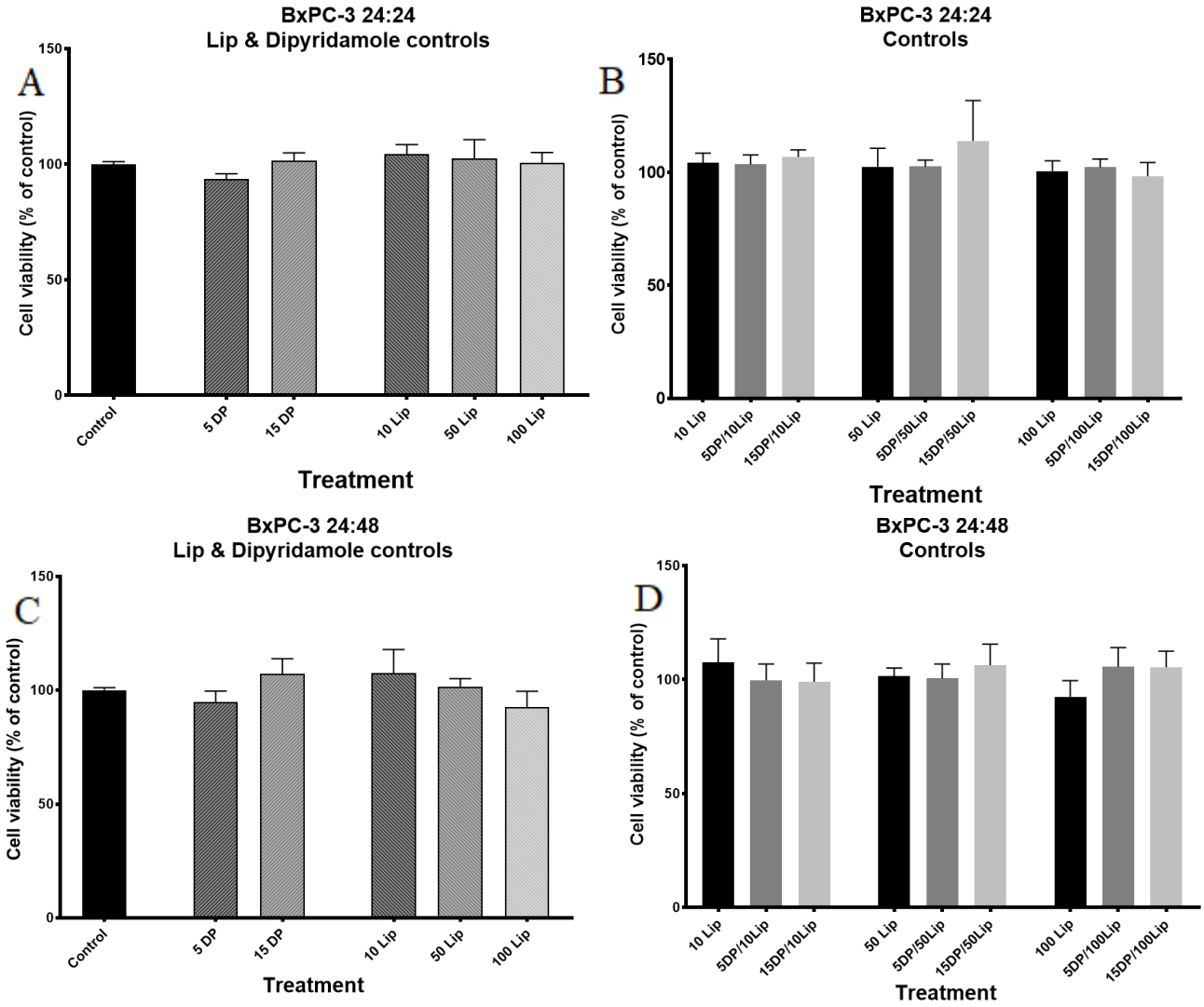

Figure 1. Controls for the GemLip experiments on old BxPC-3 cells.
The Figure show controls for the GemLip experiments. A and C display a comparison between untreated cells and cells treated with DP and empty Liposomes. A display cell viability after 24 hours and C after 48 hours. B and C show cell viability for the combination of DP and empty Liposomes in the different concentrations. These controls were done in order to see if the inhibitor and the liposomes were toxic alone or in combination. The figure shows that DP and empty Liposomes were not toxic, both alone and in combination.

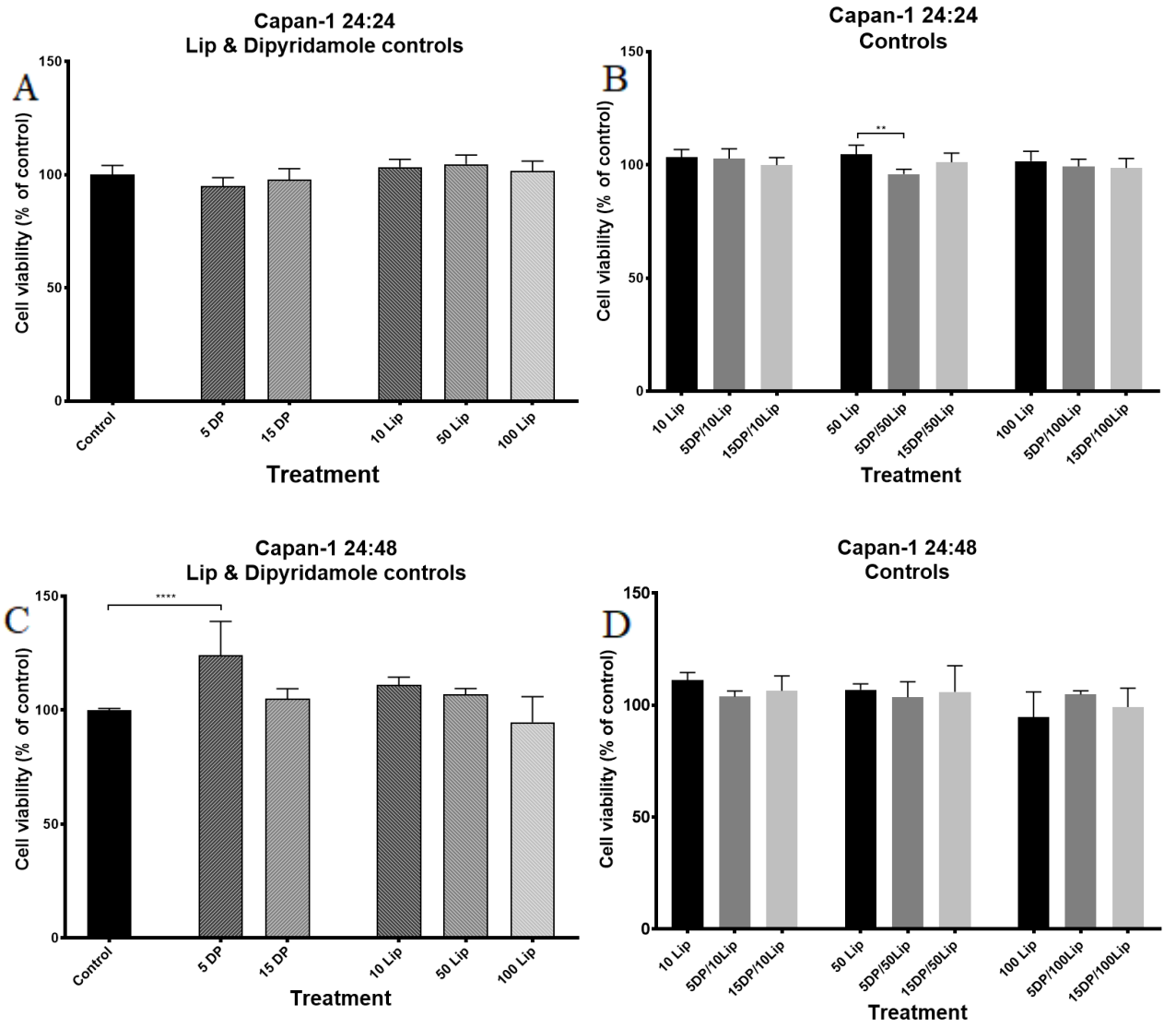

Figure 2. Controls for the GemLip experiments on old Capan-1 cells.
Shown in the Figure are controls for the GemLip experiments. A and C display a comparison between untreated cells and cells treated with DP and empty Liposomes. A do display cell viability after 24 hours and C after 48 hours. B and C show cell viability for the combination of DP and empty Liposomes for the different concentrations. These controls were done in order to see if the inhibitor and the liposomes were toxic alone or in combination. The figure shows that DP and empty Liposomes were not toxic, both alone and in combination.

Table 1. The values for the GemLip experiments conducted on old BxPC-3 cells. The values are the mean cell viability in per cent ± standard deviation in per cent.

	10 GemLip		5DP/10GemLip		15DP/10GemLip	
24:24	98.6 ± 6.3	89.7 ± 3.9	95.9 ± 4.6	101.2 ± 5.3	107.0 ± 2.2	104.9 ± 6.6
24:48	61.7 ± 2.1	62.0 ± 5.5	63.7 ± 3.3	59.6 ± 1.1	71.4 ± 1.4	65.1 ± 3.6
	50 GemLip		5DP/50GemLip		15DP/50GemLip	
24:24	92.6 ± 2.8	93.4 ± 7.2	100.4 ± 3.8	86.0 ± 2.2	102.3 ± 0.7	87.2 ± 3.7
24:48	58.4 ± 8.6	56.2 ± 2.3	60.1 ± 2.8	53.4 ± 1.2	67.4 ± 6.1	63.2 ± 1.4
	100 GemLip		5DP/100GemLip		15DP/100GemLip	
24:24	98.7 ± 3.8	82.1 ± 5.7	92.7 ± 3.6	82.0 ± 0.7	92.2 ± 3.6	83.0 ± 1.8
24:48	56.9 ± 7.3	56.3 ± 1.2	55.8 ± 2.3	52.1 ± 7.3	59.3 ± 2.7	53.0 ± 2.7
	10 Gem		5DP/10Gem		15DP/10Gem	
24:24	99.1 ± 1.2	96.2 ± 3.8	98.4 ± 1.6	90.6 ± 3.1	108.6 ± 2.8	100.4 ± 0.3
24:48	63.6 ± 4.0	62.1 ± 5.6	65.0 ± 1.4	57.6 ± 8.7	70.5 ± 4.9	64.9 ± 1.5
	50 Gem		5DP/50Gem		15DP/50Gem	
24:24	98.7 ± 0.6	89.3 ± 4.1	93.9 ± 1.9	90.8 ± 1.7	103.5 ± 1.3	94.8 ± 2.4
24:48	59.1 ± 1.2	58.4 ± 2.7	59.8 ± 4.3	59.0 ± 4.4	67.9 ± 1.5	59.6 ± 3.4
	100 Gem		5DP/100Gem		15DP/100Gem	
24:24	95.3 ± 2.3	92.2 ± 1.7	94.2 ± 2.8	85.4 ± 3.3	94.3 ± 1.9	86.7 ± 0.8
24:48	62.0 ± 0.4	60.4 ± 3.5	54.6 ± 8.0	52.9 ± 1.8	62.5 ± 2.8	56.4 ± 1.5
	10 Lip		5DP/10Lip		15DP/10Lip	
24:24	104.5 ± 3.6	104.1 ± 3.8	103.1 ± 2.2	104.0 ± 4.6	109.0 ± 1.0	104.7 ± 2.0
24:48	114.4 ± 7.4	100.8 ± 3.4	101.0 ± 7.2	98.4 ± 5.4	103.4 ± 8.3	102.4 ± 0.4
	50 Lip		5DP/50Lip		15DP/50Lip	
24:24	104.9 ± 0.8	100.1 ± 10.0	100.3 ± 1.9	104.7 ± 0.9	109.1 ± 3.7	118.5 ± 18.4
24:48	99.8 ± 3.5	103.4 ± 1.2	98.2 ± 6.9	94.9 ± 1.2	110.2 ± 9.7	106.3 ± 2.1
	100 Lip		5DP/100Lip		15DP/100Lip	
24:24	104.4 ± 1.0	96.8 ± 2.1	103.0 ± 1.3	101.4 ± 4.2	95.3 ± 1.4	101.3 ± 6.4
24:48	98.2 ± 3.5	86.8 ± 2.6	105.0 ± 10.1	102.9 ± 3.3	106.1 ± 8.3	104.8 ± 2.1
	5 DP		15 DP			
24:24	95.4 ± 1.4	91.7 ± 0.4	102.4 ± 2.6	100.9 ± 3.2		
24:48	95.0 ± 6.2	94.4 ± 2.6	110.0 ± 6.2	104.5 ± 3.2		

Table 2. The values for the GemLip experiments conducted on old Capan-1 cells. The values are the mean cell viability in per cent ± standard deviation in per cent.

	10 GemLip		5DP/10GemLip		15DP/10GemLip	
24:24	115.6 ± 1.7	104.3 ± 4.0	108.5 ± 1.3	106.3 ± 2.1	110.4 ± 3.4	109.9 ± 3.3
24:48	64.0 ± 2.7	72.4 ± 2.3	59.0 ± 2.6	69.8 ± 4.8	65.3 ± 6.8	72.7 ± 3.6
	50 GemLip		5DP/50GemLip		15DP/50GemLip	
24:24	105.6 ± 1.7	110.6 ± 5.8	106.2 ± 2.6	106.0 ± 9.3	105.4 ± 2.4	99.0 ± 1.8
24:48	61.8 ± 1.4	67.0 ± 8.5	58.0 ± 3.9	61.6 ± 5.0	56.8 ± 3.3	69.1 ± 4.3
	100 GemLip		5DP/100GemLip		15DP/100GemLip	
24:24	102.1 ± 3.9	98.9 ± 2.3	105.0 ± 3.6	102.9 ± 5.5	92.1 ± 5.3	96.4 ± 1.7
24:48	59.9 ± 4.2	68.4 ± 3.7	53.8 ± 5.4	63.8 ± 3.0	56.2 ± 4.7	63.7 ± 4.0
	10 Gem		5DP/10Gem		15DP/10Gem	
24:24	109.4 ± 7.1	108.5 ± 1.5	110.8 ± 1.9	102.7 ± 3.4	113.4 ± 5.2	99.0 ± 2.9
24:48	65.5 ± 3.4	70.9 ± 3.4	60.3 ± 3.9	69.1 ± 2.9	61.2 ± 3.1	75.2 ± 2.2
	50 Gem		5DP/50Gem		15DP/50Gem	
24:24	108.9 ± 2.6	106.7 ± 3.2	104.8 ± 2.2	106.7 ± 6.1	105.7 ± 2.9	101.5 ± 2.3
24:48	60.7 ± 1.4	68.6 ± 5.6	55.7 ± 6.7	63.0 ± 7.2	59.2 ± 6.5	69.3 ± 6.3
	100 Gem		5DP/100Gem		15DP/100Gem	
24:24	109.7 ± 1.7	104.6 ± 4.7	103.7 ± 2.8	98.4 ± 3.0	99.3 ± 5.8	100.6 ± 2.1
24:48	59.0 ± 0.3	69.3 ± 3.3	50.6 ± 12.4	62.9 ± 5.9	53.5 ± 3.1	63.4 ± 2.2
	10 Lip		5DP/10Lip		15DP/10Lip	
24:24	103.3 ± 3.0	103.5 ± 3.0	103.8 ± 4.6	101.7 ± 2.7	99.3 ± 3.4	99.0 ± 2.9
24:48	111.1 ± 2.8	102.2 ± 4.6	103.8 ± 2.2	106.2 ± 4.0	106.3 ± 5.8	102.2 ± 4.6
	50 Lip		5DP/50Lip		15DP/50Lip	
24:24	107.2 ± 3.0	102.1 ± 5.4	94.1 ± 1.1	100.5 ± 2.6	103.4 ± 3.0	101.5 ± 2.3
24:48	1.608 ± 2.2	108.6 ± 1.8	103.5 ± 6.1	104.2 ± 3.8	105.9 ± 10.1	108.6 ± 1.8
	100 Lip		5DP/100Lip		15DP/100Lip	
24:24	102.0 ± 1.3	101.3 ± 5.4	97.1 ± 1.5	97.8 ± 0.4	96.6 ± 4.3	100.6 ± 2.1
24:48	94.7 ± 10.8	105.5 ± 2.9	104.7 ± 1.5	104.8 ± 4.3	99.2 ± 7.7	105.5 ± 2.9
	5 DP		15 DP			
24:24	97.1 ± 2.0	92.6 ± 3.6	97.4 ± 5.9	98.5 ± 1.8		
24:48	124.1 ± 10.9	96.3 ± 1.3	105.0 ± 3.8	93.6 ± 14.5		

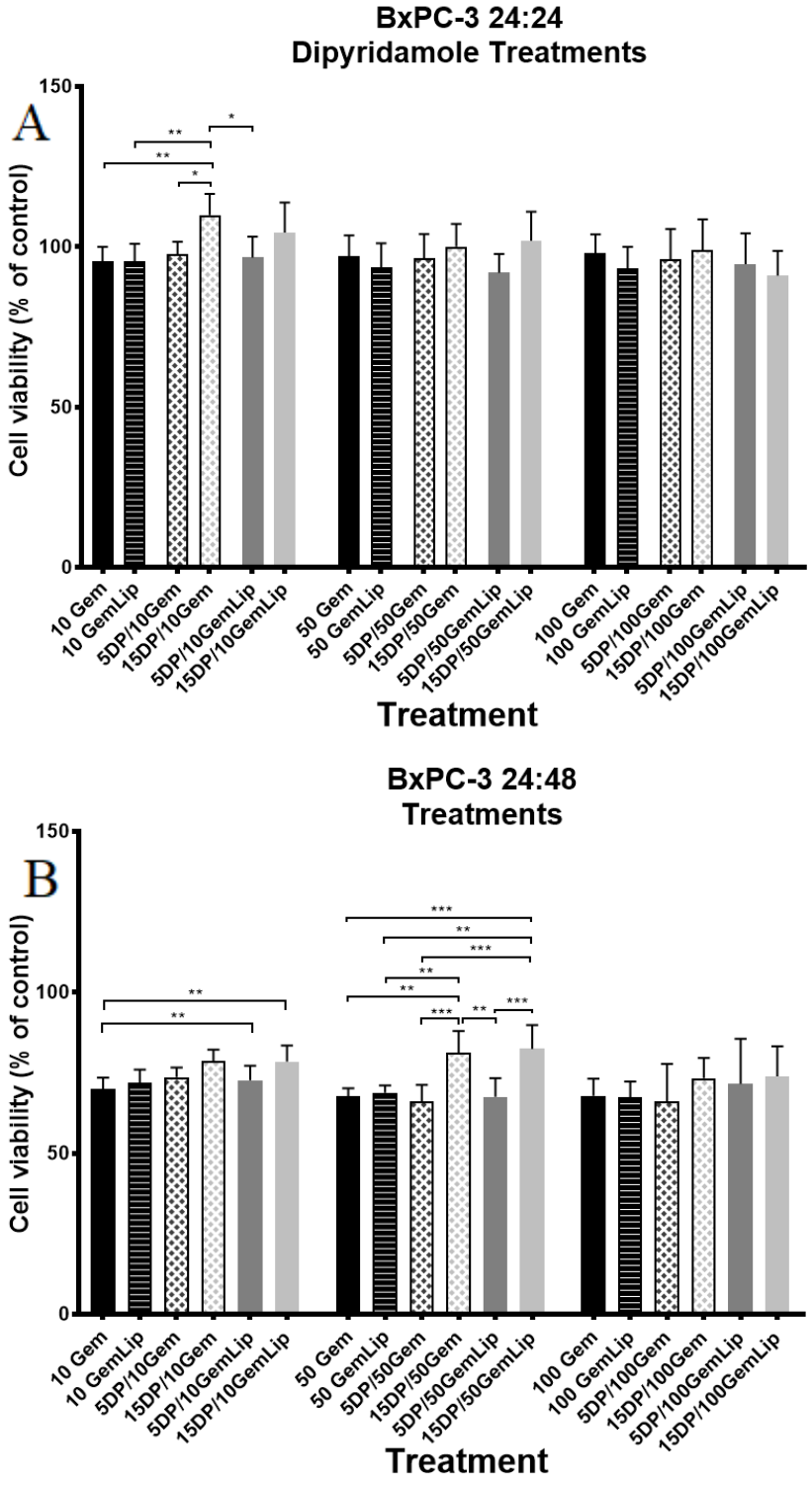

Figure 3. 24 hour Pre-Treatment with DP followed by a 24- (A) or 48-hour (B) co-Treatment with GemLip and DP using new cells from the BxPc-3 cell line.
The results displayed in this set of figures are similar to the previous results displayed by the older cells from the same cell line. Overall, the results are inconclusive with inhibition being present at some places and absent at other. The results indicate successful Gem delivery by GemLip, since the cell viability for the cells treated with GemLip has decreased.

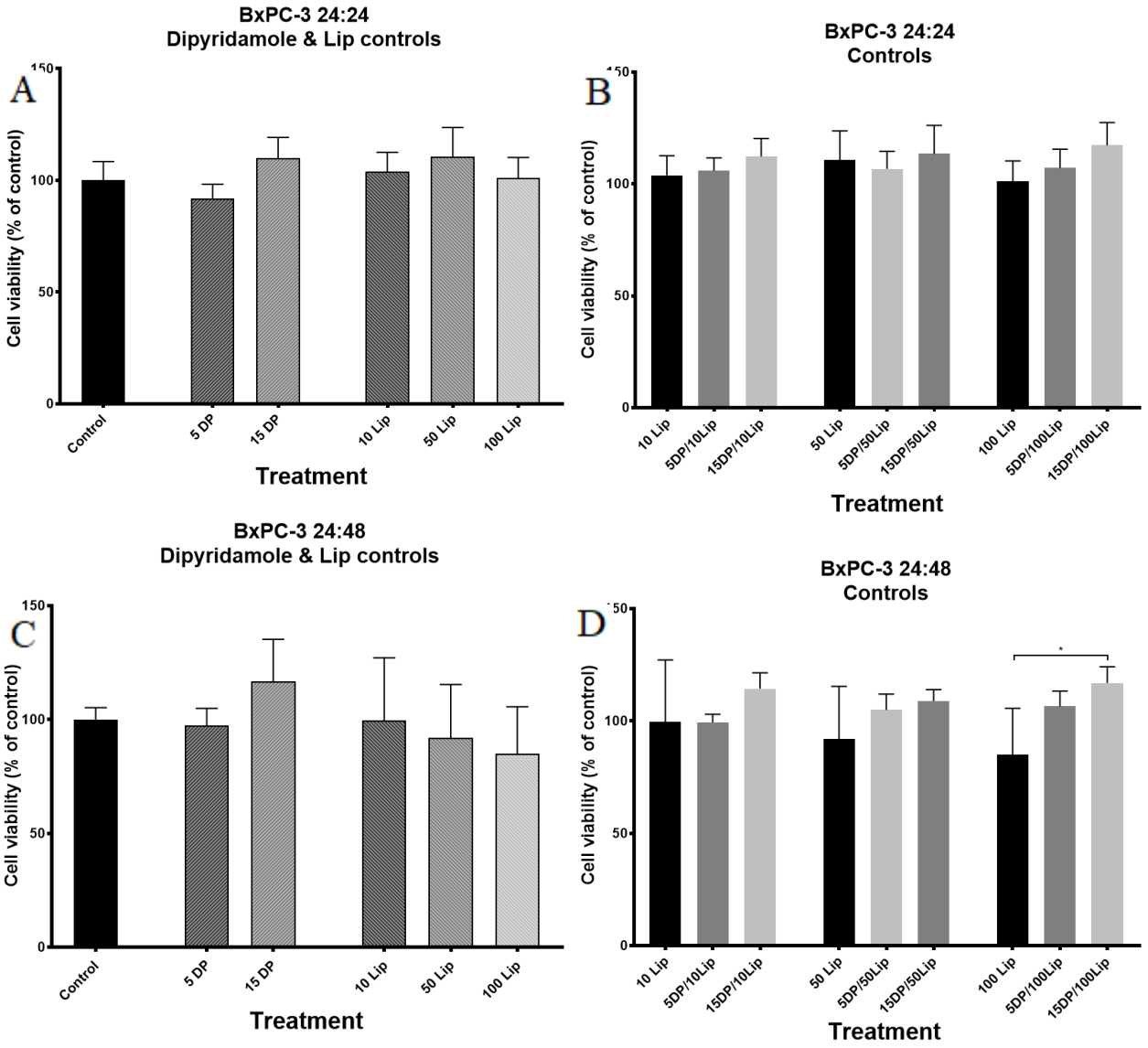

Figure 4. Controls for the GemLip experiments on new BxPC-3 cells.
Shown in the Figure are controls for the GemLip experiments. A and C display a comparison between control (cells only given growth medium) cells and cells treated with DP and empty Liposomes. A display cell viability after 24 hours and C after 48 hours. B and C show cell viability for the combination of DP and empty Liposomes for the different concentrations after 24 or 48 hours. These controls were done in order to see if the inhibitor and the liposomes were toxic alone or in combination. The figure shows that DP and empty Liposomes were not toxic, neither alone or in combination.

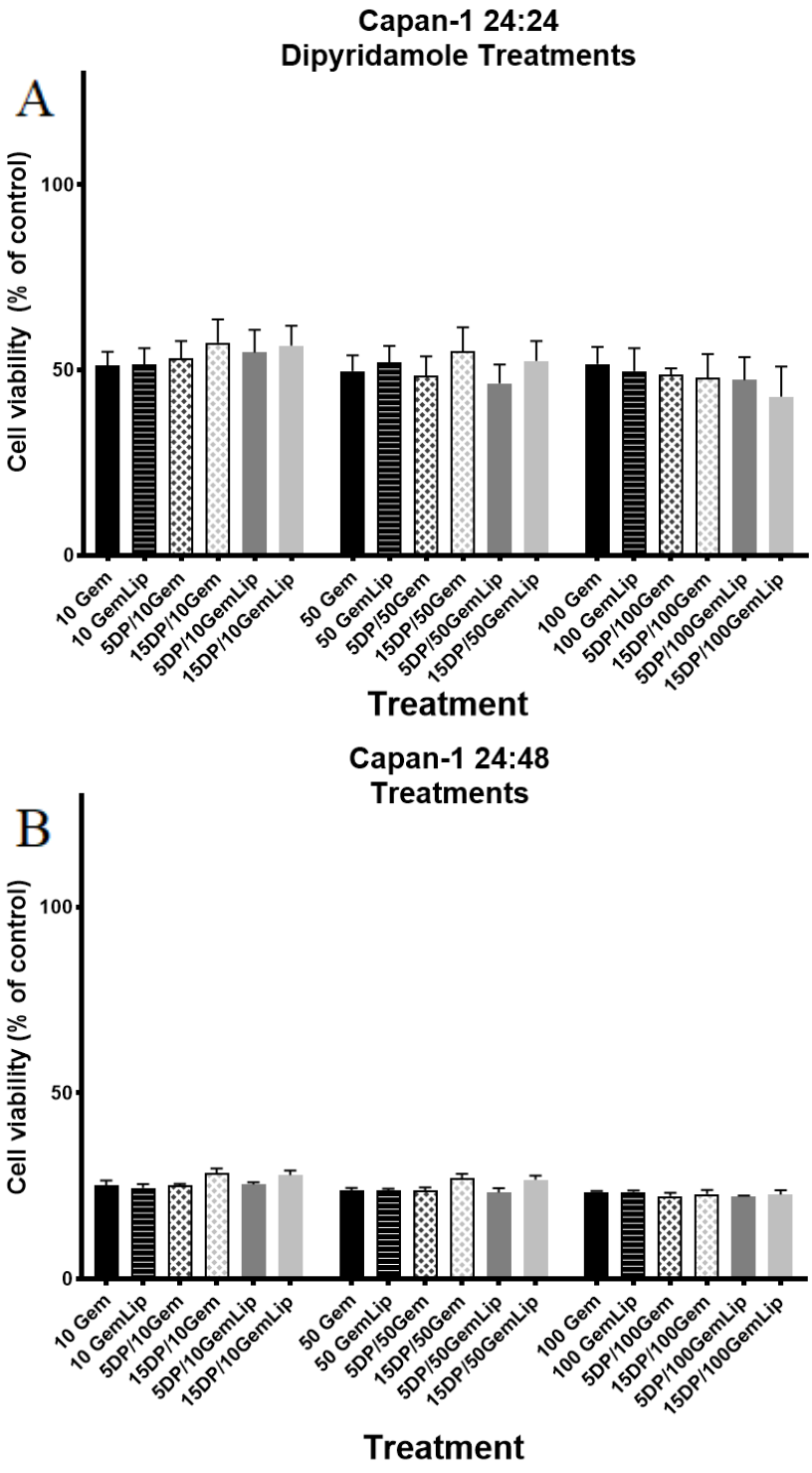

Figure 5. 24 hour Pre-Treatment with DP followed by a 24- (A) or 48-hour (B) co-Treatment with GemLip and DP using new cells from the Capan-1 cell line.
The results displayed in this set of figures differ from the previous results displayed by the older cells from the same cell line. Overall, the results are inconclusive with inhibition being absent. The results indicate successful Gem delivery by GemLip, since the cell viability for the cells treated with GemLip has decreased. The cell viability for all cells are substantially lower than the cell viability displayed by the old Capan-1 cells.

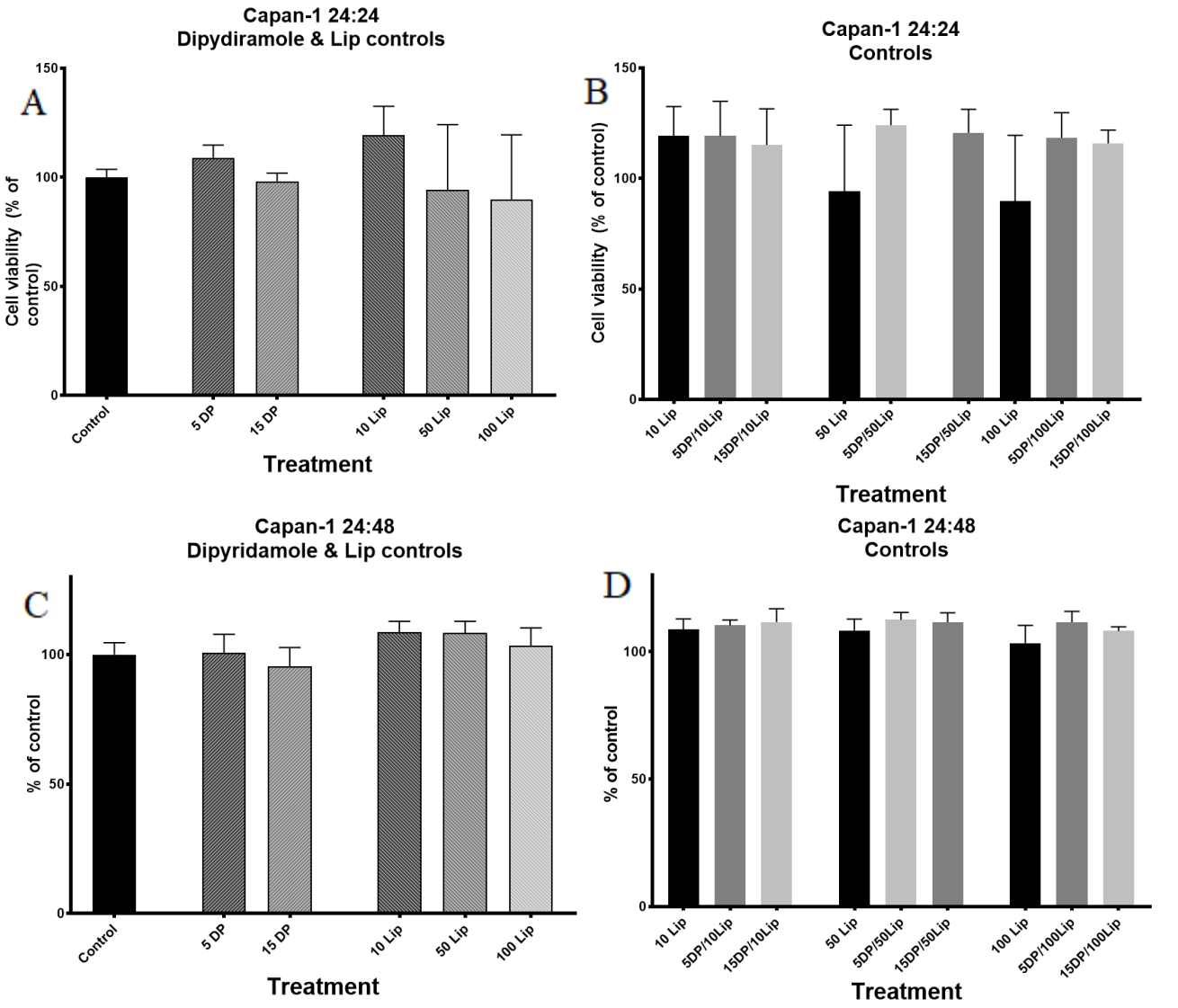

Figure 6. Controls for the GemLip experiments on new Capan-1 cells.
Shown in the Figure are controls for the GemLip experiments. A and C display a comparison between control (cells only given growth medium) cells and cells treated with DP and empty Liposomes. A display cell viability after 24 hours and C after 48 hours. B and C show cell viability for the combination of DP and empty Liposomes for the different concentrations after 24 or 48 hours. These controls were done in order to see if the inhibitor and the liposomes were toxic alone or in combination. The figure shows that DP and empty Liposomes were not toxic, neither alone or in combination.

Table 3. The values for the GemLip experiments conducted on new BxPC-3 cells. The values are cell viability in per cent ± standard deviation in per cent.

	10 GemLip		5DP/10GemLip		15DP/10GemLip	
24:24	100.0 ± 2.8	91.1 ± 1.1	102.4 ± 1.8	91.0 ± 1.1	111.4 ± 3.8	97.4 ± 5.7
24:48	73.2 ± 6.6	70.5 ± 1.2	73.2 ± 7.8	72.1 ± 1.7	74.4 ± 3.7	82.5 ± 1.0
	50 GemLip		5DP/50GemLip		15DP/50GemLip	
24:24	99.5 ± 4.4	87.7 ± 2.2	95.6 ± 5.6	88.1 ± 2.0	108.8 ± 5.1	95.0 ± 3.1
24:48	70.0 ± 3.1	67.6 ± 0.6	72.4 ± 2.4	62.5 ± 3.7	89.0 ± 1.8	75.9 ± 1.5
	100 GemLip		5DP/100GemLip		15DP/100GemLip	
24:24	98.5 ± 4.8	88.3 ± 0.5	102.6 ± 4.1	86.6 ± 3.2	97.8 ± 2.7	84.2 ± 0.8
24:48	70.5 ± 5.1	64.3 ± 4.0	83.7 ± 4.2	59.3 ± 7.6	80.7 ± 8.6	67.0v 2.9
	10 Gem		5DP/10Gem		15DP/10Gem	
24:24	99.1 ± 2.0	92.1 ± 2.1	100.8 ± 2.3	94.5 ± 1.2	113.5 ± 1.2	107.5 ± 6.1
24:48	72.4 ± 3.7	67.4 ± 1.2	74.8 ± 2.0	72.5 ± 4.3	76.3 ± 3.9	81.1 ± 0.5
	50 Gem		5DP/50Gem		15DP/50Gem	
24:24	102.9 ± 1.0	91.2 ± 1.1	102.7 ± 3.9	90.0 ± 0.7	104.7 ± 6.1	95.5 ± 0.8
24:48	68.6 ± 4.1	66.6 ± 0.9	70.1 ± 3.8	62.1 ± 3.3	85.3 ± 6.9	77.1 ± 3.3
	100 Gem		5DP/100Gem		15DP/100Gem	
24:24	102.3 ± 3.8	93.9 ± 2.5	104.4 ± 2.8	88.0 ± 1.8	106.6 ± 2.3	91.6 ± 6.0
24:48	69.4 ± 9.2	66.2 ± 1.7	76.1 ± 5.6	55.9 ± 5.2	77.5 ± 6.6	68.9 ± 3.0
	10 Lip		5DP/10Lip		15DP/10Lip	
24:24	110.5 ± 3.6	97.1 ± 4.9	110.1 ± 3.8	101.8 ± 1.6	119.3 ± 1.5	105.2 ± 2.2
24:48	114.5 ± 25.0	84.6 ± 2.3	96.2 ± 0.5	102.4 ± 1.8	117.5 ± 6.6	111.2 ± 2.1
	50 Lip		5DP/50Lip		15DP/50Lip	
24:24	120.4 ± 8.0	100.9 ± 1.7	113.6 ± 0.8	99.7 ± 2.4	124.5 ± 3.0	102.4 ± 3.0
24:48	102.1 ± 26.2	81.6 ± 1.8	107.9 ± 7.2	101.9 ± 2.1	106.0 ± 4.8	111.6 ± 1.4
	100 Lip		5DP/100Lip		15DP/100Lip	
24:24	107.8 ± 5.9	94.2 ± 3.2	114.2 ± 3.1	99.9 ± 1.5	125.7 ± 2.0	108.7 ± 4.5
24:48	93.6 ± 25.3	76.3 ± 2.6	107.7 ± 6.1	105.3 ± 5.3	115.5 ± 7.8	118.2 ± 1.6
	5 DP		15 DP			
24:24	96.7 ± 2.3	86.9 ± 4.7	115.9 ± 7.3	103.8 ± 1.9		
24:48	104.1 ± 1.6	90.4 ± 1.4	126.1 ± 15.7	107.6 ± 1.5		

Table 4. The values for the GemLip experiments conducted on new Capan-1 cells. The values are cell viability in per cent ± standard deviation in per cent.

	10 GemLip		5DP/10GemLip		15DP/10GemLip	
24:24	49.7 ± 9.7	53.2 ± 3.9	52.0 ± 11.3	57.5 ± 6.2	55.3 ± 10.0	57.9 ± 6.5
24:48	25.1 ± 3.2	23.8 ± 2.2	25.7 ± 2.5	25.3 ± 0.3	28.9 ± 1.7	27.0 ± 1.9
	50 GemLip		5DP/50GemLip		15DP/50GemLip	
24:24	53.4 ± 9.6	50.7 ± 4.1	43.6 ± 11.6	49.3 ± 3.2	52.0 ± 13.1	53.0 ± 2.2
24:48	23.9 ± 2.1	23.8 ± 0.8	23.6 ± 0.7	22.9 ± 6.0	27.0 ± 3.6	26.5 ± 3.3
	100 GemLip		5DP/100GemLip		15DP/100GemLip	
24:24	45.3 ± 10.8	54.2 ± 1.7	46.2 ± 16.8	48.5 ± 2.4	36.3 ± 13.2	49.3 ± 3.7
24:48	23.4 ± 1.1	23.4 ± 1.6	22.0 ± 0.6	22.3 ± 1.1	23.3 ± 5.0	22.0 ± 1.9
	10 Gem		5DP/10Gem		15DP/10Gem	
24:24	48.0 ± 1.7	54.6 ± 1.2	52.2 ± 9.9	54.3 ± 4.5	55.9 ± 10.1	58.9 ± 9.2
24:48	25.8 ± 3.7	24.3 ± 4.3	25.0 ± 2.1	25.3 ± 1.3	28.7 ± 2.5	28.4 ± 4.7
	50 Gem		5DP/50Gem		15DP/50Gem	
24:24	46.7 ± 6.2	52.7 ± 4.0	46.4 ± 12.6	50.7 ± 2.3	54.8 ± 15.1	55.5 ± 1.6
24:48	24.3 ± 1.8	23.5 ± 1.4	23.9 ± 1.8	23.8 ± 3.8	27.8 ± 2.5	26.5 ± 2.3
	100 Gem		5DP/100Gem		15DP/100Gem	
24:24	50.7 ± 10.8	52.6 ± 3.4	49.2 ± 4.2	48.3 ± 1.2	49.5 ± 12.7	46.2 ± 10.9
24:48	23.3 ± 1.5	23.4 ± 0.6	22.4 ± 3.7	22.2 ± 3.5	23.1 ± 4.9	22.6 ± 3.0
	10 Lip		5DP/10Lip		15DP/10Lip	
24:24	121.0 ± 12.5	117.8 ± 6.4	107.7 ± 14.8	126.8 ± 3.2	111.3 ± 18.2	119.0 ± 2.0
24:48	109.2 ± 3.0	108.2 ± 3.9	109.9 ± 2.4	110.8 ± 0.4	114.3 ± 4.1	109.0 ± 2.8
	50 Lip		5DP/50Lip		15DP/50Lip	
24:24	120.9 ± 7.0	67.4 ± 2.8	123.2 ± 6.8	124.9 ± 3.1	117.6 ± 10.9	123.6 ± 2.0
24:48	108.8 ± 4.0	108.0 ± 3.5	112.9 ± 1.9	112.3 ± 2.7	111.9 ± 3.7	111.2 ± 2.2
	100 Lip		5DP/100Lip		15DP/100Lip	
24:24	116.4 ± 6.1	63.4 ± 3.2	114.4 ± 11.3	122.5 ± 2.8	113.4 ± 6.0	118.4 ± 0.3
24:48	99.5 ± 5.8	107.4 ± 3.6	110.0 ± 4.1	113.3 ± 1.8	107.0 ± 0.9	109.5 ± 0.5
	5 DP		15 DP			
24:24	104.2 ± 1.9	113.8 ± 2.3	97.7 ± 2.6	98.7 ± 4.0		
24:48	100.2 ± 9.1	101.2 ± 1.3	91.9 ± 5.1	99.2 ± 6.3		

Appendix 2

Co-treatment

Cells were treated as mentioned in Material & Methods. All in all, the Co-Treatment shows modest results, with lack of inhibition of hENT1. The values for PANC-1 shown in Figure 1 and Figure 2 indicate that no effect from Gem is visible after 24 hours. Some effect is observed after 48 hours, but the Figures also indicate the absence of inhibition of hENT1, and thereby Gem uptake. The cells treated with Gem do not demonstrate lower cell viability than cells treated with a combination of Gem and inhibitor. These inconclusive results indicate a malfunctioning inhibition. Important to note, as mentioned in the discussion, is that PANC-1 has resistance mechanisms which lower the sensitivity towards Gem. These results may therefore be the result of a combination of the resistance to Gem shown by PANC-1, and a lack of effect of the inhibitor in its role of inhibiting hENT1.

Cells treated with only Gem show a cell viability >80% after 48 hours. Because of this observed resistance, the hypothesis was that the differences between cells treated with the inhibitor and Gem, and control cells treated only with Gem would not be large enough to indicate a successful treatment. This because the cell line is not affected as much as would be needed to display differences. In other words, cells with functioning uptake (hENT1 not inhibited) still have intracellular resistance mechanisms as protection against the function of Gem. Therefore, the experiments on PANC-1 were discontinued and BxPC-3 was tried instead.

The results for Capan-1 in the Co-Treatment setting were similar to the results of PANC-1, with the exception that the amount of resistance demonstrated by PANC-1 was not demonstrated by Capan-1. The results in Figures 3 and 4 indicates a non-working inhibition of hENT1, since the cell viability measured for cells treated with only Gem is similar to the cell viability measured for the cells treated with a combination of Gem and inhibitor. It also indicates the occurrence of Gem uptake, since the cell viability is generally lower after 48 hours than after 24, indicating that Gem has an effect on the cells.

Our hypothesis was that the reason for these inconclusive results could be due to the fact that inhibitor and Gem were added at the same time. This could mean that there was no time for the inhibitor to elicit its effect before Gem uptake occurred, meaning that the inhibition took place after Gem were taken up. From this hypothesis, we tried a Pre-Treatment instead, where the cells were treated with inhibitor before the cells were treated with a combination of Gem and the inhibitor.

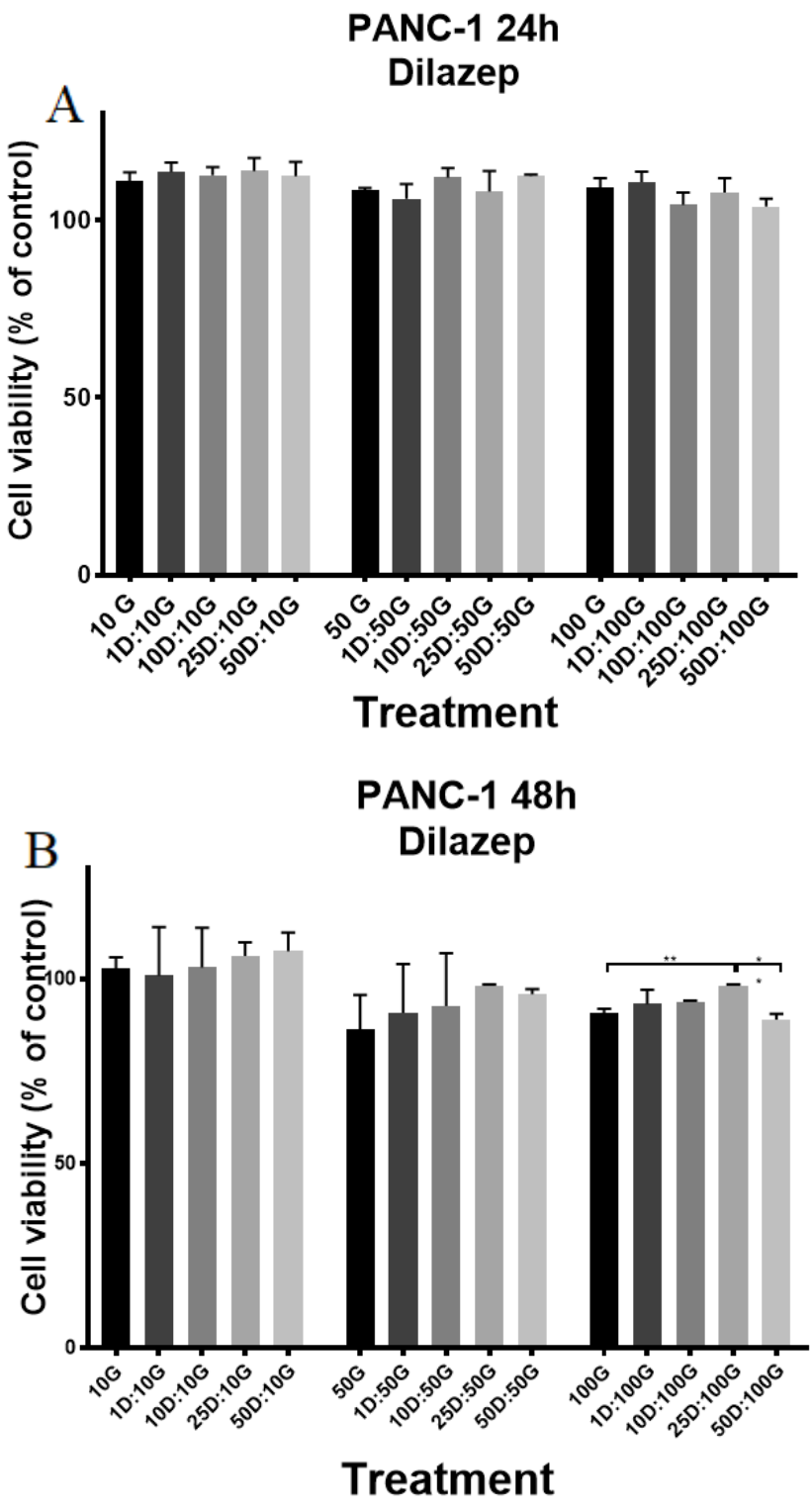

Figure 1. Co-Treatment on PANC-1 with DZ.
The results indicate an unsuccessful inhibition of hENT1 by DZ, since the cell viability for the cells with hENT1 inhibited is not higher. From left to right: Gem (black bar), 1 nM DZ + Gem, 10 nM DZ + Gem, 25 nM DZ + Gem and 50 nM DZ + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem.

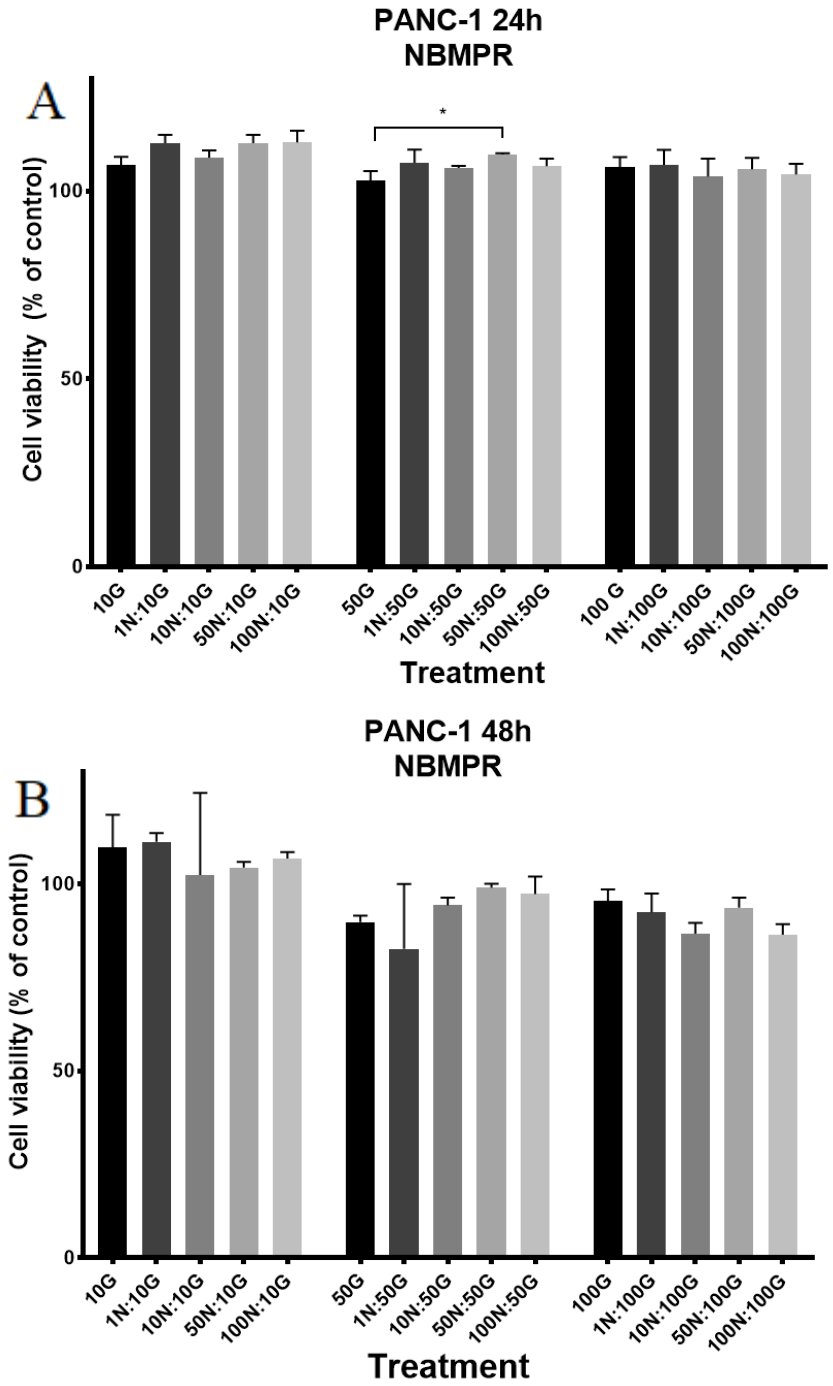

Figure 2. Co-Treatment on PANC-1 with NBMPR
The results are inconsistent, especially for the 48-hour treatment. After 24 hours, the cell viabilities are very similar to one another, a trend that is not present after 48 hours. Common to the two graphs is that both indicate the results of an unsucessful inhibition of hENT1, since cell viability does not increase for cells treated with NBMPR. From left to right: Gem (black bar), 1 nM NBMPR + Gem, 10 nM NBMPR + Gem, 50 nM NBMPR + Gem and 100 nM NBMPR + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem.

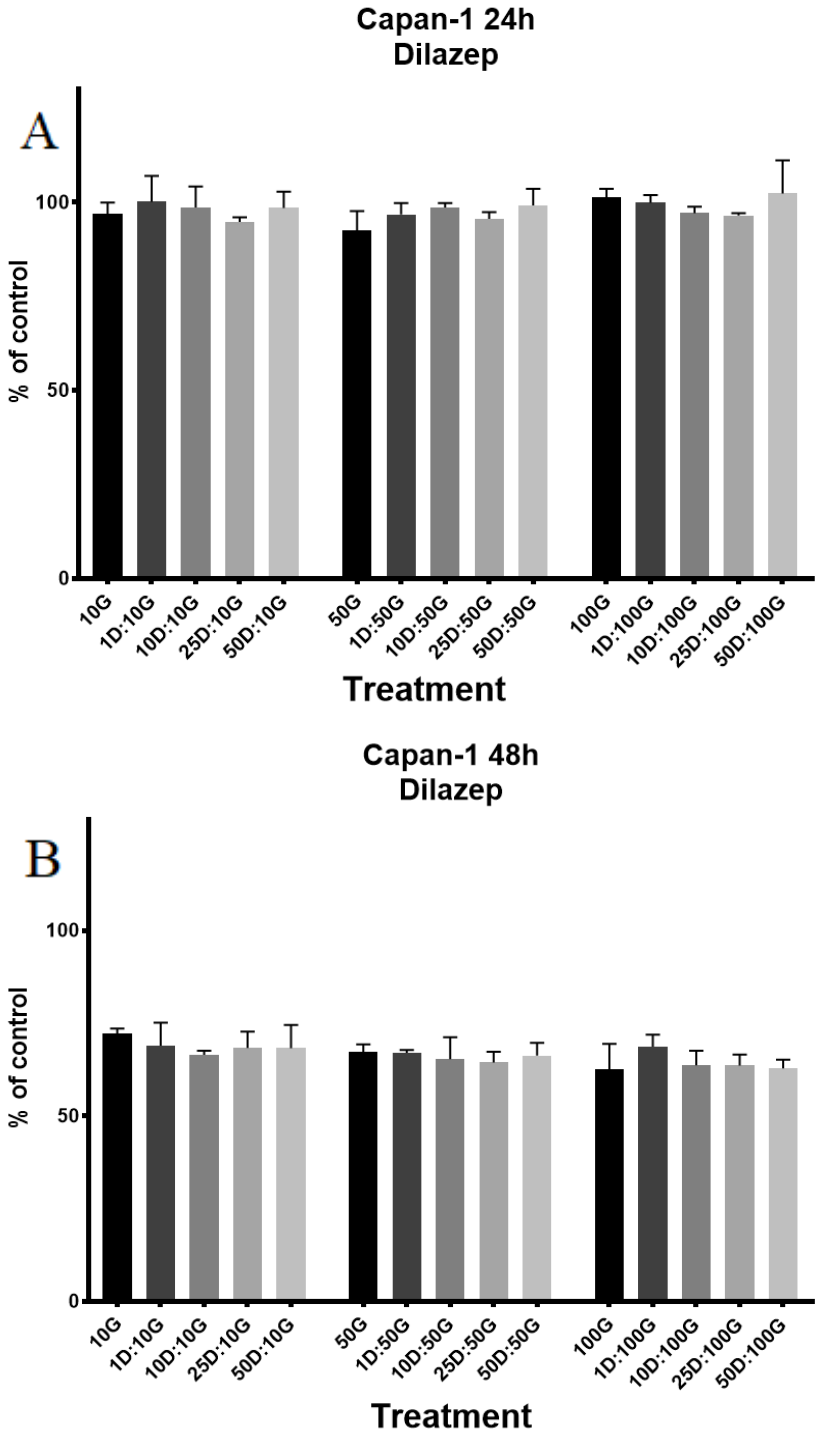

Figure 3. Co-treatment with Dilazep using Capan-1 cells.
The results do not show difference between cells treated with Gemcitabine and cells treated with a combination of Gemcitabine and Dilazep. The cell viability is not changed for the cells with hENT1 inhibited, which indicates an unsuccessful inhibition. From left to right: Gemcitabine (black bar), 1 nM Dilazep + Gemcitabine, 10 nM Dilazep + Gemcitabine, 25 nM Dilazep + Gemcitabine and 50 nM Dilazep + Gemcitabine. The first group of bars represents cells treated with 10 µM Gemcitabine, the second 50 µM Gemcitabine and the third 100 µM Gemcitabine.

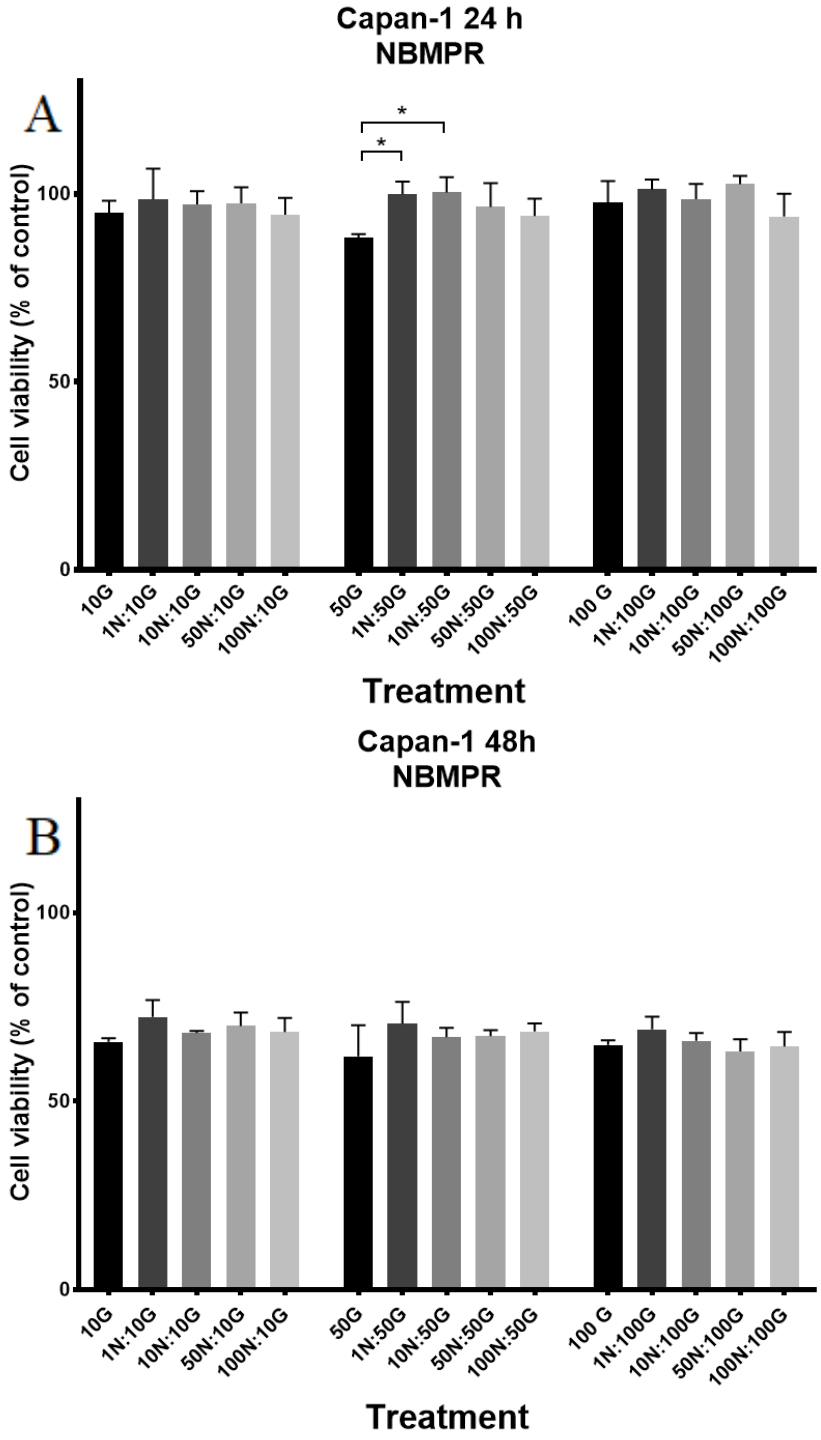

Figure 4. Co-treatment with NBMPR using Capan-1 cells.
The results from the 24 hour-treatment show two significant differences; between cells treated with 50 µM Gem and cells treated with 1 nM NBMPR + 50 µM Gem as well as cells treated with 10 nM NBMPR + 50 µM Gem. These significant differences do not recur in the 48-hour treatment. From left to right: Gem (black bar), 1 nM NBMPR + Gem, 10 nM NBMPR + Gem, 50 nM NBMPR + Gem and 100 nM NBMPR + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem.

Pre-treatment

The Pre-Treatment was initiated because of the inconclusive results from the Co-treatment. Here, the cells were incubated with the inhibitor before the cells were treated with a Co-Treatment. In other words, the cells were treated with the inhibitor before treated with a combination of the inhibitor and Gem. 2, 4 and 24 hours incubation time with inhibitor before a Co-Treatment were tested. This treatment was tested on Capan-1, since the treatments on BxPC-3 had not yet begun.

2- and 4-hour Pre-Treatment were initially tested. The 2-hour Pre-Treatment incubation time shown in Figures 5 and 6 show similar results as the Co-Treatment does. The cell viability measured for the cells treated with only Gem were very similar to the cell viability measured for the cells treated with a combination of inhibitor and Gem. Both NBMPR and DZ show this trend. Because these results were so similar to the Co-Treatment, we increased the concentrations for the inhibitor and increased the incubation times for the Pre-Treatment. Instead of nanomolar concentrations, we tried micro molar (0.5, 1, 5 and 10 µM) concentrations for both NBMPR and DZ. The incubation time for the Pre-Treatment was also increased to 4 and 24 hours before Co-Treatment.

The results for the 4 hour Pre-Treatment can be seen in Figures 7 and 8. As with previous experiments, these also indicate that the inhibition does not work properly. Both for DZ and NBMPR, the inhibition is modest both after 24 and 48 hours. Again, the results show that Gem has an effect on the cells treated with Gem alone, as well as the cells treated with a combination of inhibitor and Gem. This result also shows that Gem has an effect on the cells, but that the inhibitor does not block uptake of Gem via hENT1.

24 hour Pre-Treatment was also tested on BxPC-3, the results for which can be seen in Figures 9 and 10. These results are somewhat promising, since they show a trend of increasing cell viability with increasing inhibitor concentration. Important to bear in mind is that these results are pooled results from two experiments and the results from these two experiments differed from one another. This combined with the fact that the inhibition, although significant, still is poor laid the foundation for the decision to try a third inhibitor, DP.

The results for Capan-1 can be seen in Figures 11 and 12, and does show a similar trend as the results from BxPC-3 did, although not as prominent. This, too, supported the decision to try DP.

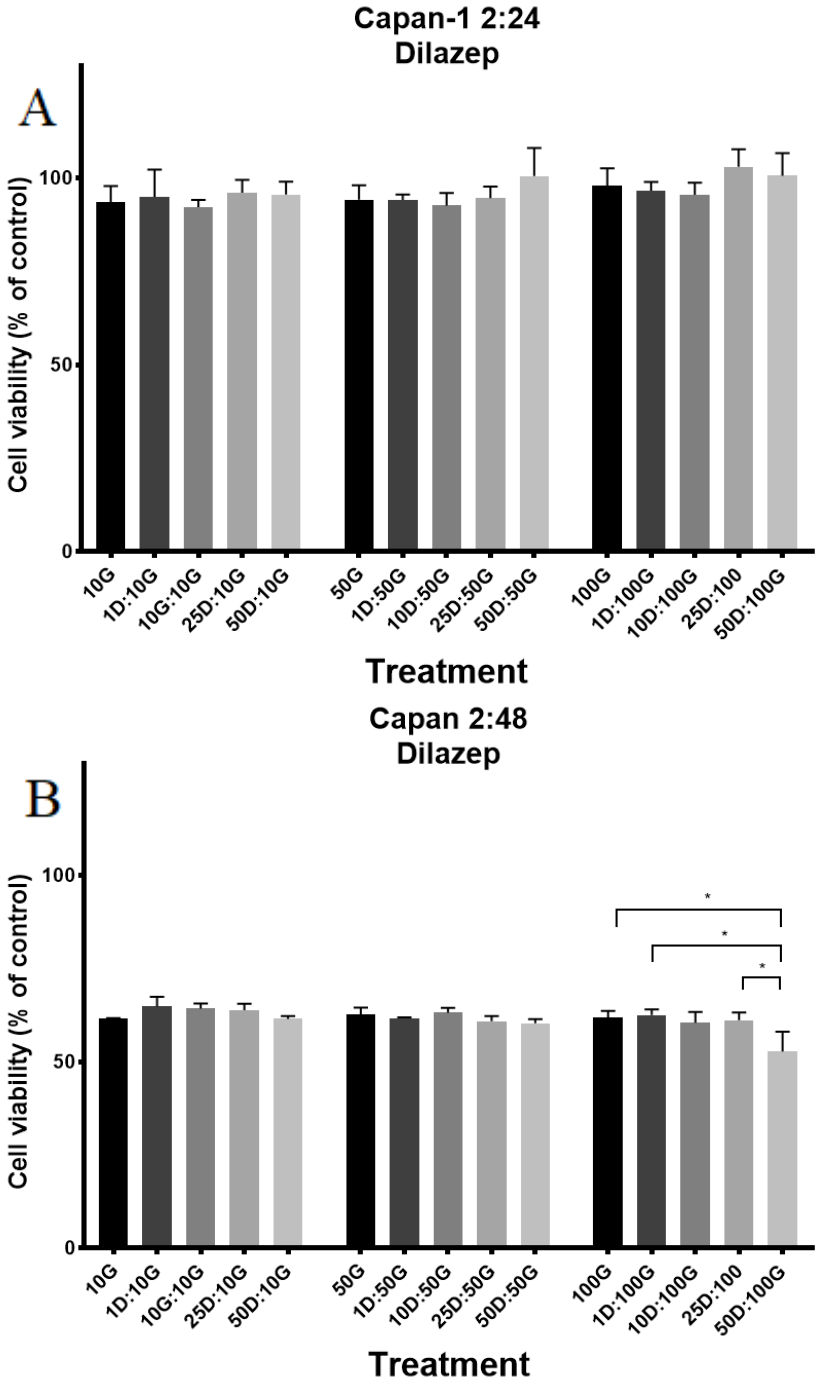

Figure 5. A 2-hour Pre-Treatment with DZ followed by a 24- or 48-hour Co-Treatment using Capan-1.
This data does not show an inhibited Gem uptake since the cell viability for the cells treated with Gem is not entirely different from the cell viability displayed by the cells treated with a combination of DZ and Gem. From left to right: Gem, 1 nM DZ + Gem, 10 nM DZ + Gem, 25 nM DZ + Gem and 50 nM DZ + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem.

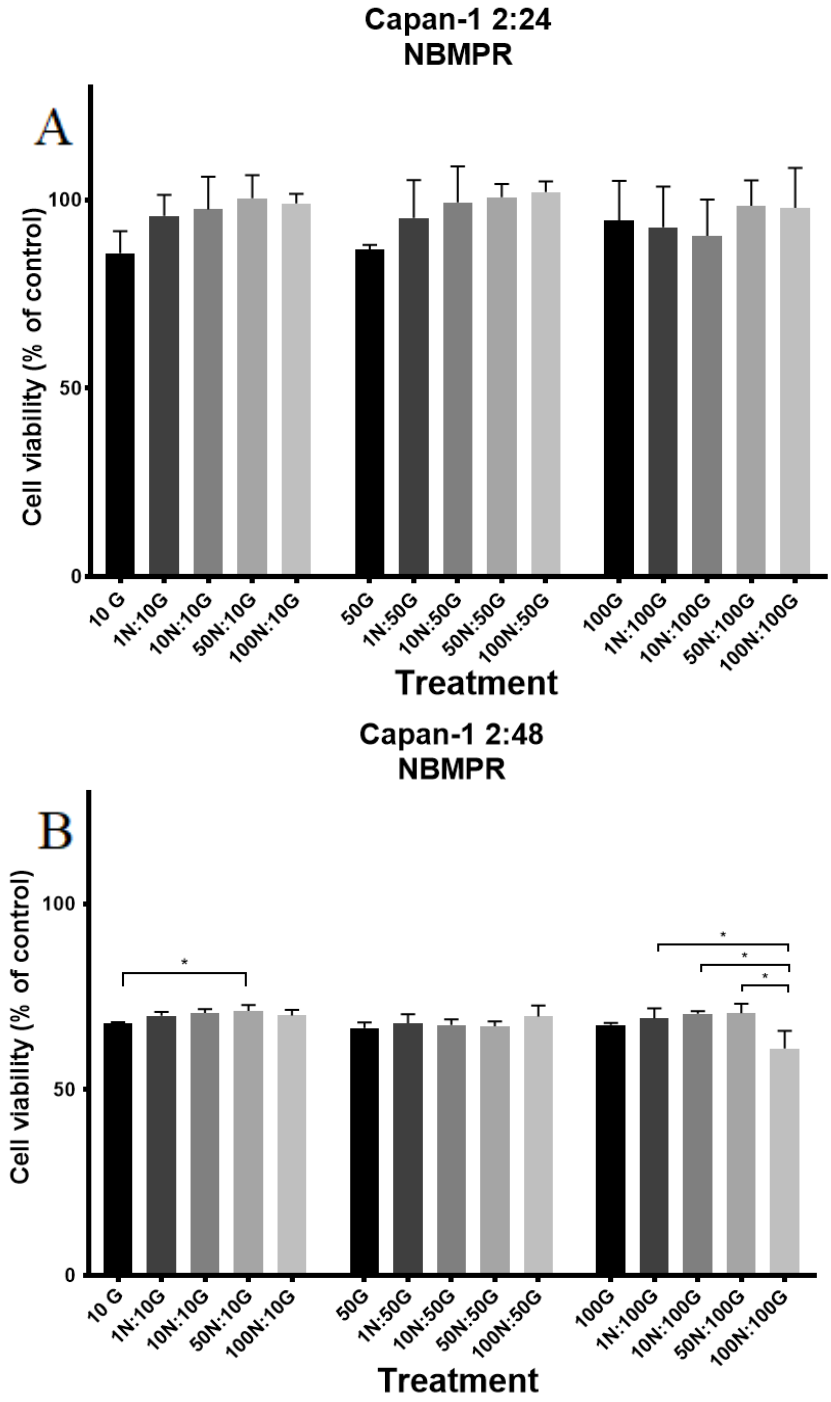

Figure 6. A 2-hour Pre-Treatment with NBMPR followed by a 24- or 48-hour Co-Treatment using Capan-1. This data show a promising trend after 24 hours, a trend which is not present after 48 hours. The 48 hour result indicates hENT1 not being inhibited, since the cell viability for the cells treated with Gem does not differ from the cell viability displayed by the cells treated with a combination of NBMPR and Gem. From left to right: Gem, 1 nM NBMPR + Gem, 10 nM NBMPR + Gem, 50 nM NBMPR + Gem and 100 nM NBMPR + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem.

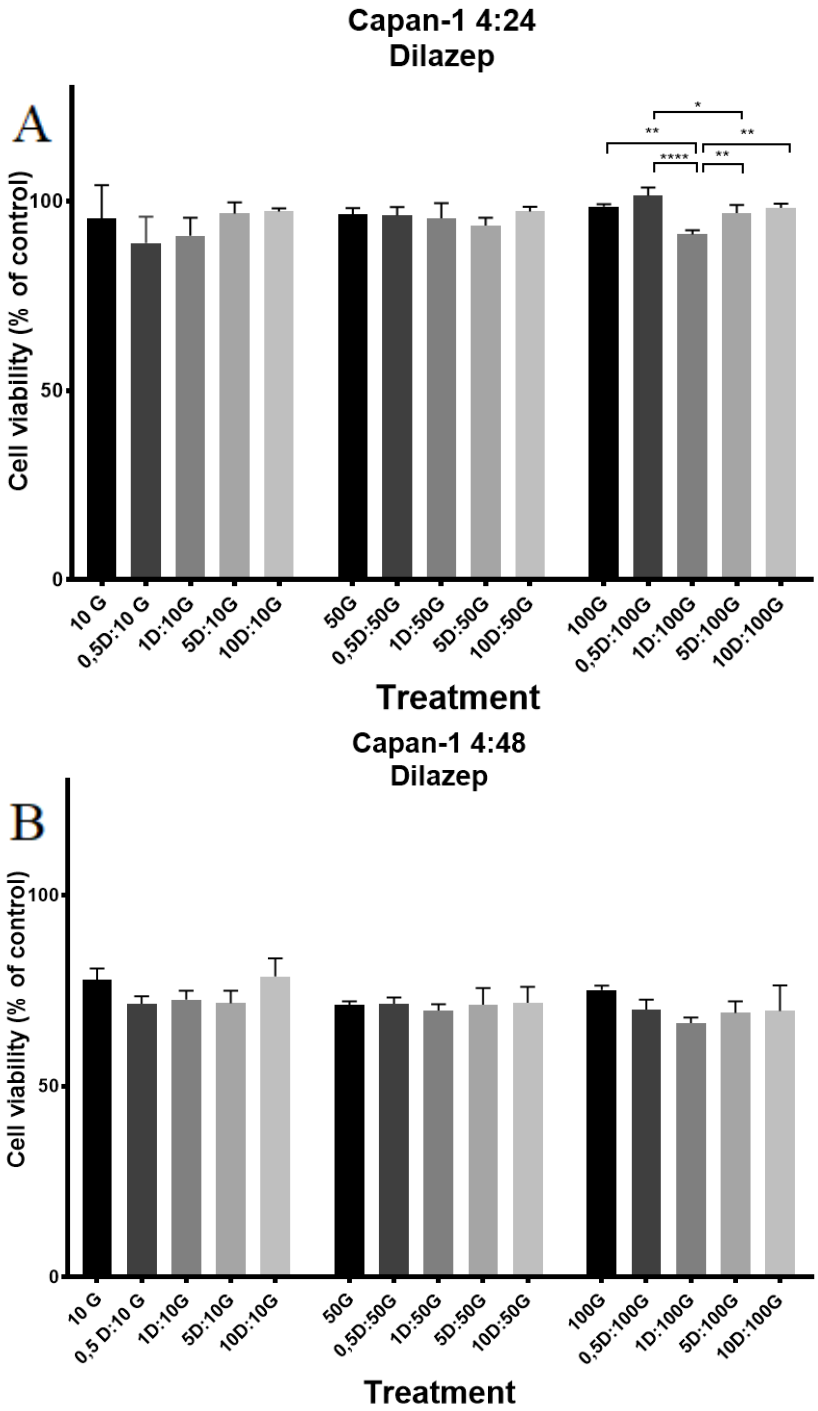

Figure 7. A 4-hour Pre-Treatment with DZ followed by a 24- or 48-hour Co-Treatment using Capan-1.
This data does not show a decreased Gem uptake. The cell viability for the cells treated with Gem does not differ from the cell viability displayed by the cells treated with a combination of DZ and Gem, indicating that the treatments do not differ from one another. From left to right: Gem, 1 nM DZ + Gem, 10 nM DZ + Gem, 50 nM DZ + Gem and 100 nM DZ + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem.

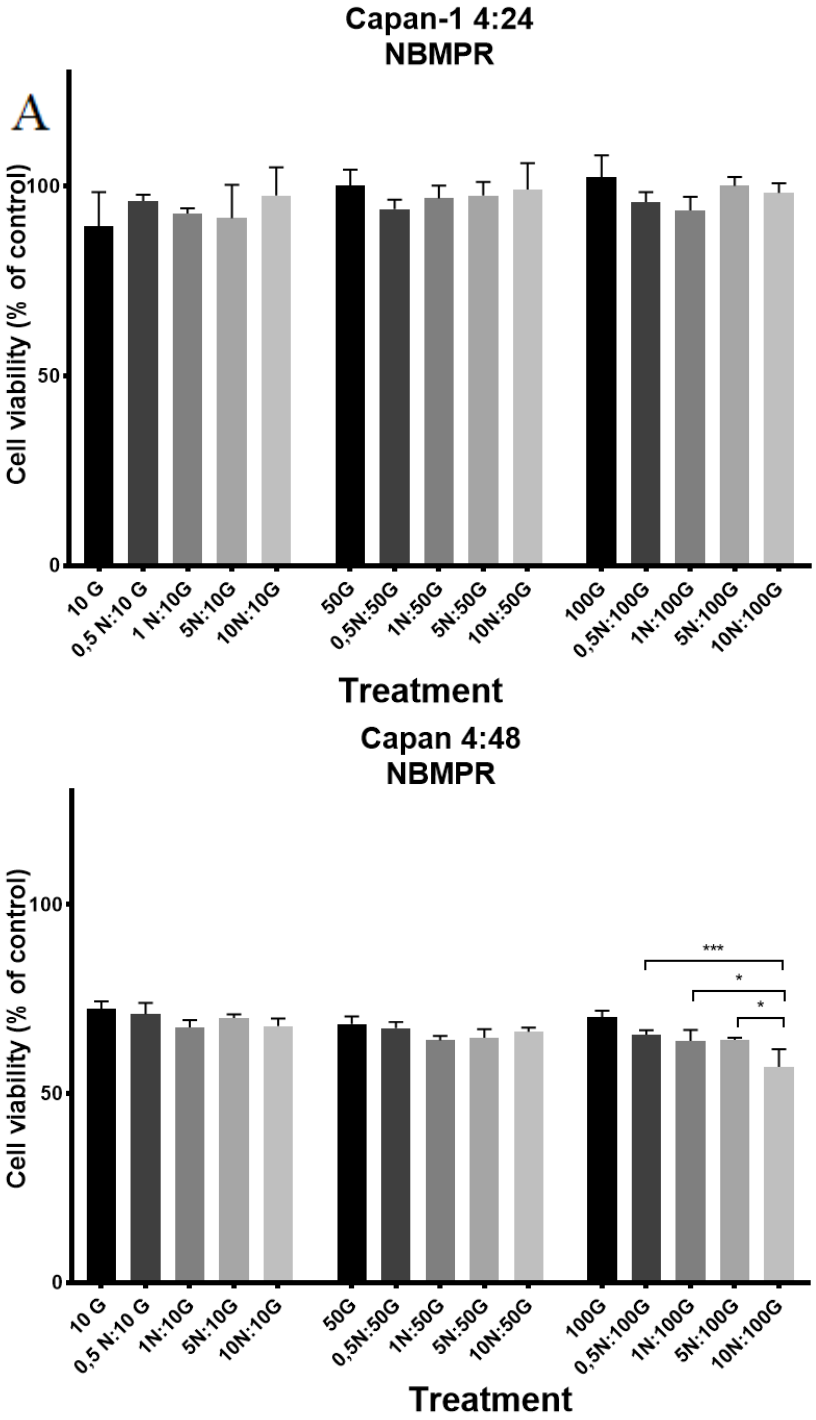

Figure 8. A 4-hour Pre-Treatment with NBMPR followed by a 24- or 48-hour Co-Treatment using Capan-1. This data does not show an inhibited Gem uptake since the cell viability for the cells treated with Gem is not higher than the cell viability displayed by the cells treated with a combination of NBMPR and Gem. This indicates a successful Gem uptake, and therefore an unsuccessful hENT1 inhibition. From left to right: Gem, 1 nM NBMPR + Gem, 10 nM NBMPR + Gem, 50 nM NBMPR + Gem and 100 nM NBMPR + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem.

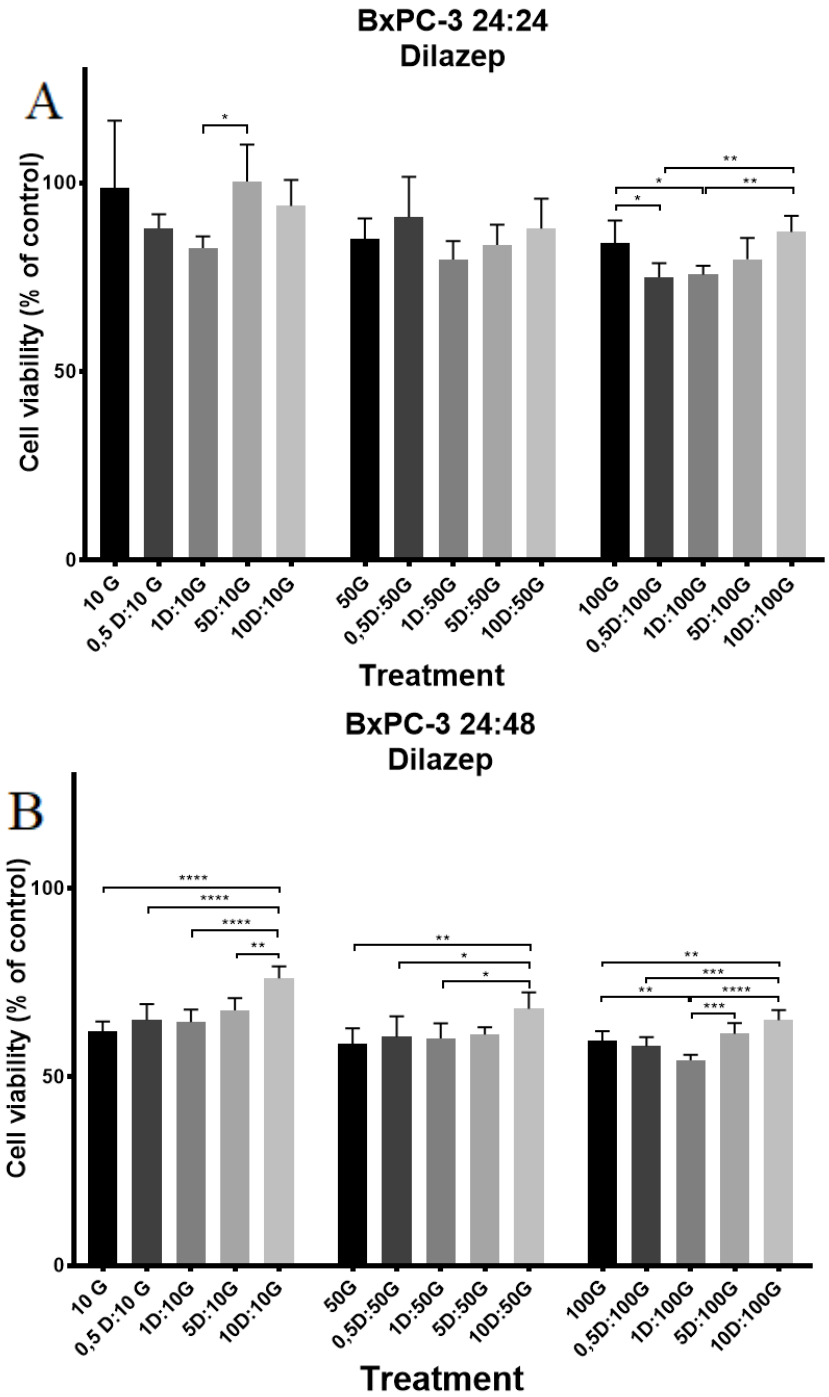

Figure 9. A 24-hour Pre-Treatment with DZ followed by a 24- or 48-hour Co-Treatment using BxPC-3.
From left to right: Gem, 1 nM DZ + Gem, 10 nM DZ + Gem, 50 nM DZ + Gem and 100 nM DZ + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem. This data does show an inhibited Gem uptake. The cell viability for the cells treated with Gem is ifferent from the cell viability displayed by the cells treated with a combination of DZ and Gem.

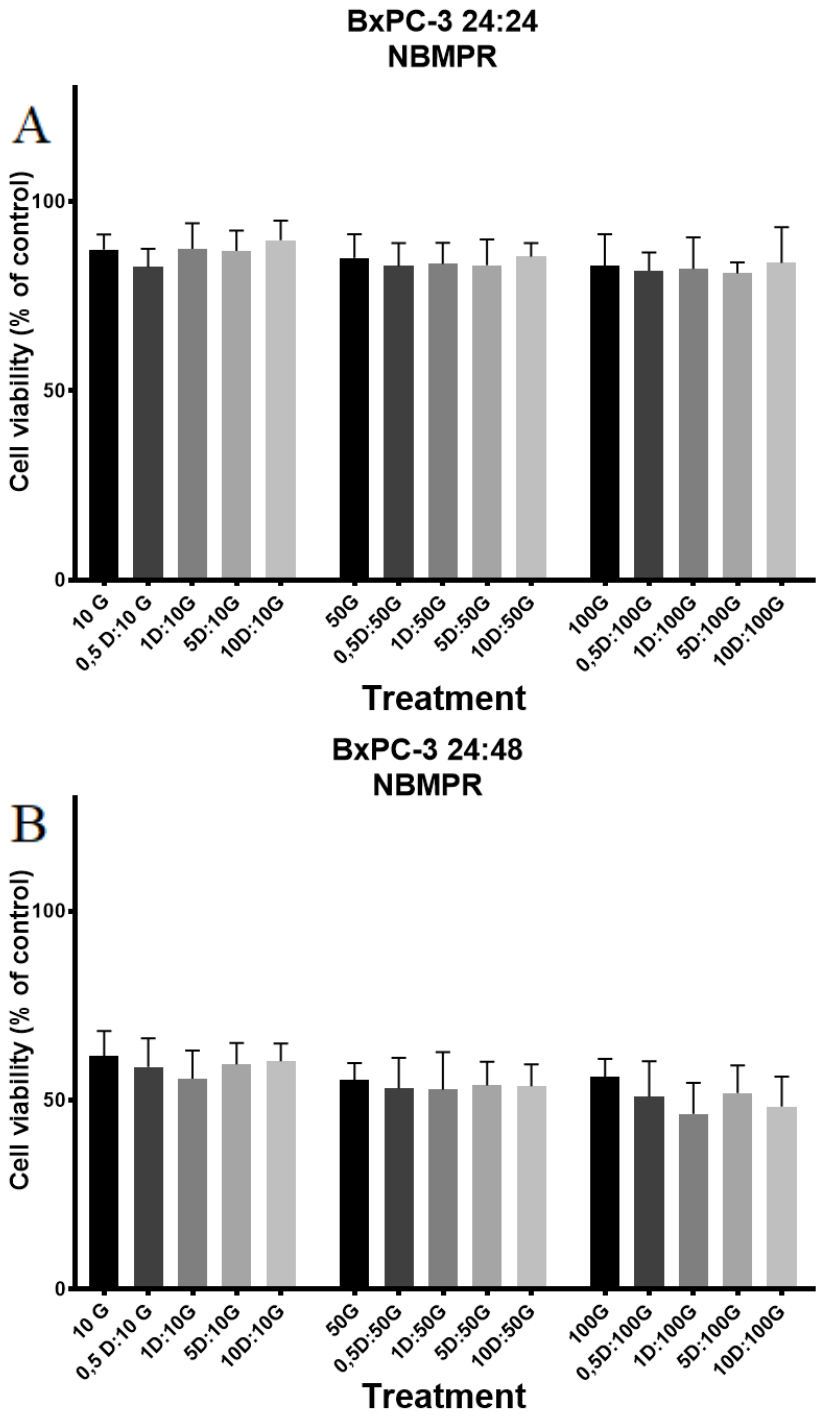

Figure 10. A 4-hour Pre-Treatment with NBMPR followed by a 24- or 48-hour Co-Treatment using BxPC-3. From left to right: Gem, 0.5 µM NBMPR + Gem, 1 µM NBMPR + Gem, 5 µM NBMPR + Gem and 10 µM NBMPR + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem. This data does not show an inhibited Gem uptake since the cell viability for the cells treated with Gem is not significantly different from the cell viability displayed by the cells treated with a combination of NBMPR and Gem.

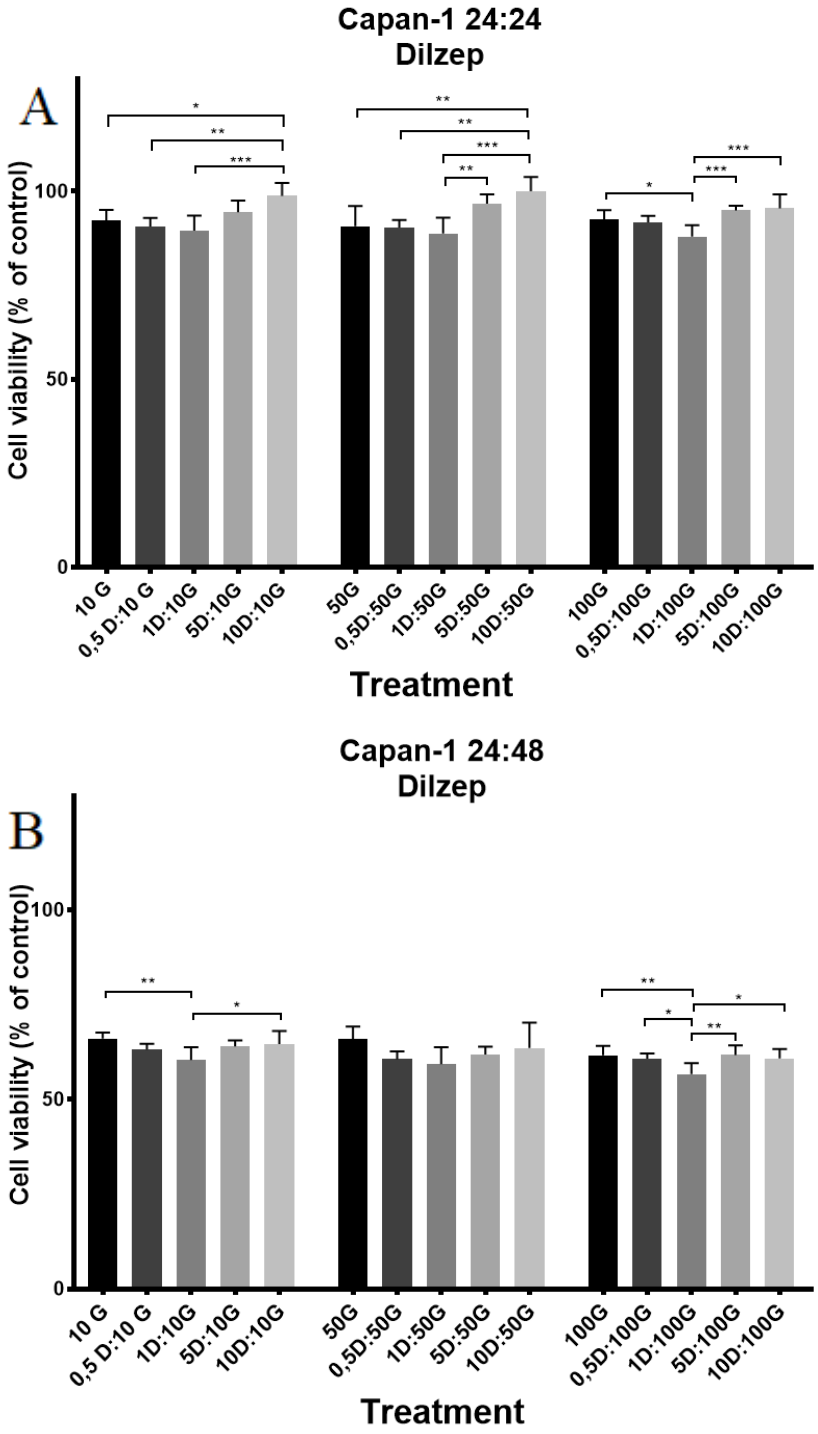

Figure 11. A 24-hour Pre-Treatment with DZ followed by a 24- or 48-hour Co-Treatment using Capan-1.
From left to right: Gem, 0.5 µM DZ + Gem, 1 µM DZ + Gem, 5 µM DZ + Gem and 10 µM DZ + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem. This data does to some extent show an inhibited Gem uptake. After 24 hour Co-Treatment (left graph), there is a trend showing increased cell viability with increasing DZ concentration. This trend is not present after 48 hour Co-Treatment. Important to note it that the results showed here are pooled from two separate experiments. Data from each of the experiments can be seen in table E, Appendix 2.

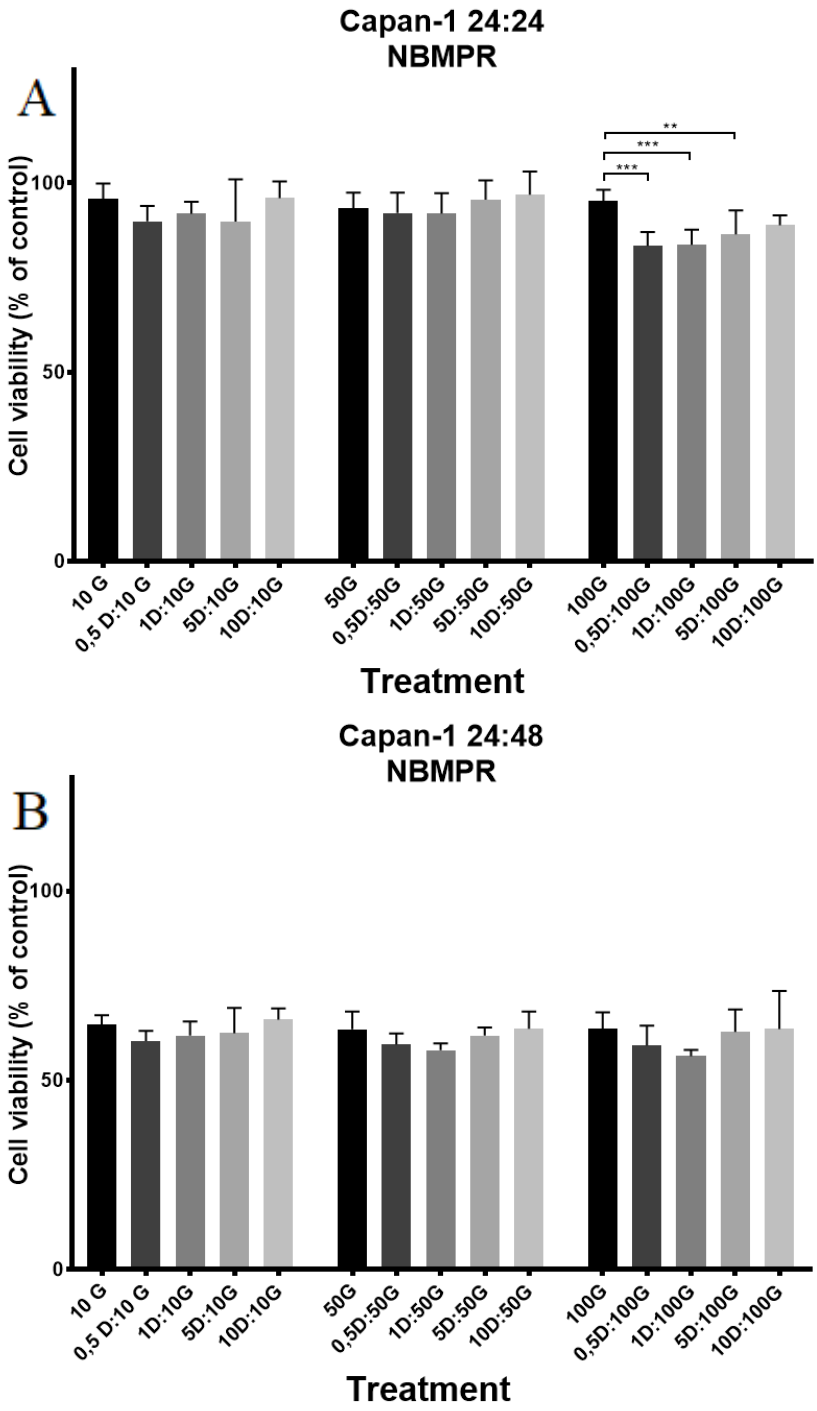

Figure 12. A 4-hour Pre-Treatment with NBMPR followed by a 24- or 48-hour Co-Treatment using Capan-1. From left to right: Gem, 0.5 µM NBMPR + Gem, 1 µM NBMPR + Gem, 5 µM NBMPR + Gem and 10 µM NBMPR + Gem. The first group of bars represents cells treated with 10 µM Gem, the second 50 µM Gem and the third 100 µM Gem. This data does not show an inhibited Gem uptake since the cell viability for the cells treated with Gem is not significantly different from the cell viability displayed by the cells treated with a combination of NBMPR and Gem, with the exception of the values in the left graph being significantly different from control (Gem only). This result is not visible after 48 hours.