

## Introduction

Overexpression and activation of HER-2 protein has implication in the development and progression of a variety of tumor types. ADAM10, a transmembrane protein implicated in proteolytic extracellular domain (ECD) release of the HER-2 receptor tyrosine kinase, is an important regulator of cell proliferation and survival in 25% of breast cancers. Cleavage of HER-2 ECD in HER-2 overexpressing human breast cancer cell lines like SK-BR-3, results in a constitutively activated truncated membrane bound kinase and thus in ligand independent signaling. High levels of circulating ECD of HER2 are correlated with poor patient prognosis since ECD contains the binding area for monoclonal antibody (Herceptin). Combination of monoclonal antibody that targets HER-2 in subsaturating concentration and ADAM10 inhibitors that targets photolytic activity, results in decreased proliferation of HER2 overexpressing cell lines (1). The usual standards for testing the influence of new chemical entities (NCE) on cellular functions and physiological responses present in organs are 2D cell culture testing platforms. However, a significant number of such functions are lost with conventional 2D approach, which give arise to a more frequent use of 3D cell culture systems, crucial for cellular behavior and tumorigenicity (2, 3). 3D approach overcome difference in cell morphology, proliferation and differentiation that enable more precise biological insights into cellular functions.

## Objective

The objective of this study was to explore difference in activities of herceptin and ADAM10 inhibitor (GI254023X), or their combination on proliferation of breast cancer cell line SK-BR-3, that overexpress HER2, in 2D and 3D cell culture formats. Unlike SK-BR-3, MDA-MB-231 and T-47D have normal HER2 expression level, so they are used as comparators.

## Materials and methods

Human breast cancer cell lines SK-BR-3 (HTB-30), MDA-MB-231 (HTB-26) and T-47D (HTB-133) were purchased from ATCC.

The siRNA (ADAM10 and non targeting siRNA) were purchased from Ambion. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) transfection reagent according to manufacturer's recommendation. Total RNA was isolated using RNeasy Plus Mini kit (Qiagen) 72 hours post-transfection.

ADAM10 mRNA levels were analyzed by RT-qPCR on the 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using the TaqMan method and GAPDH as a housekeeping gene.

ADAM10 protein expression level was determined by SDS-PAGE on 4-12% Bis Tris gel and subsequent Western blot analysis using nitrocellulose membrane, ADAM10 polyclonal antibody (Millipore) and actin monoclonal antibody (Sigma) as primary antibodies, alkaline phosphatase labeled secondary antibodies (Santa Cruz) and BCIP/NBT detection system.

For 2D cultures, cells were grown in 96 well Cell star polystyrene plates, while for 3D cultures 96 well Perfecta 3D hanging drop plates were used. In order to determine the influence of herceptin (trastuzumab, Roche), GI254023X (small molecule ADAM10 inhibitor, Okeanos), or combination of subsaturating herceptin concentrations and GI254023X on the cell viability in both formats, cytotoxicity studies were performed in a way that  $5 \times 10^3$  cells per well were seeded 4 hours prior to the treatment. Cell viability assay was performed according to the manufacturer's instruction, using Cell titer 96 aqueous solution, MTS kit (Promega) and Cell proliferation biotrak Elisa system (GE Healthcare). Treatment duration was 144 hours.

## References

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## Results

### Growth rate characterization and ADAM10 protein expression confirmation in three breast cancer cell lines

Exponential growth for SK-BR-3, T-47D and MDA-MB-231 cell lines was determined to be between 72 and 168 hours of cell culture time. According to the literature data all three cell lines express ADAM10, with SK-BR-3 showing the highest expression level.

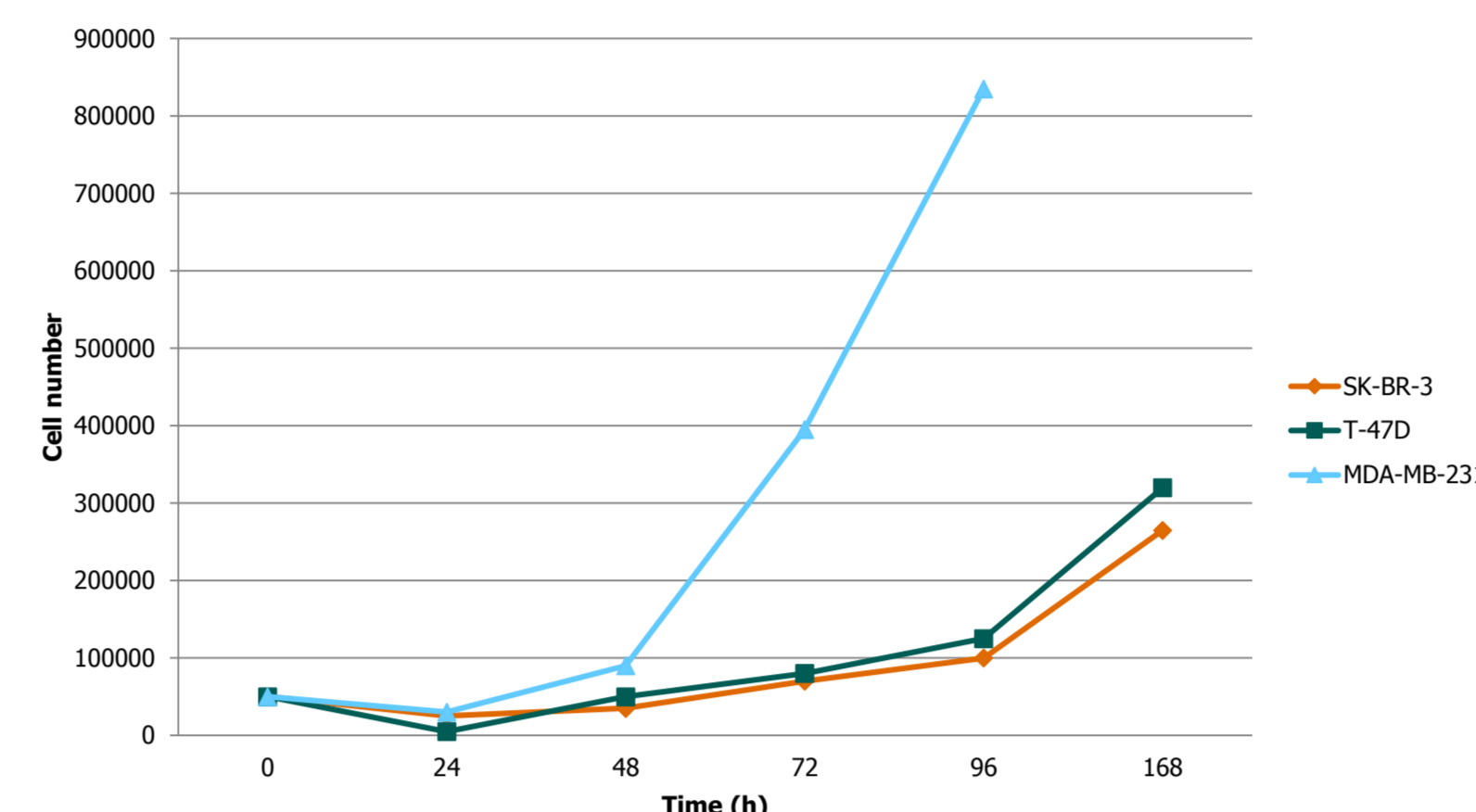


Figure 1. Growth rate curves.

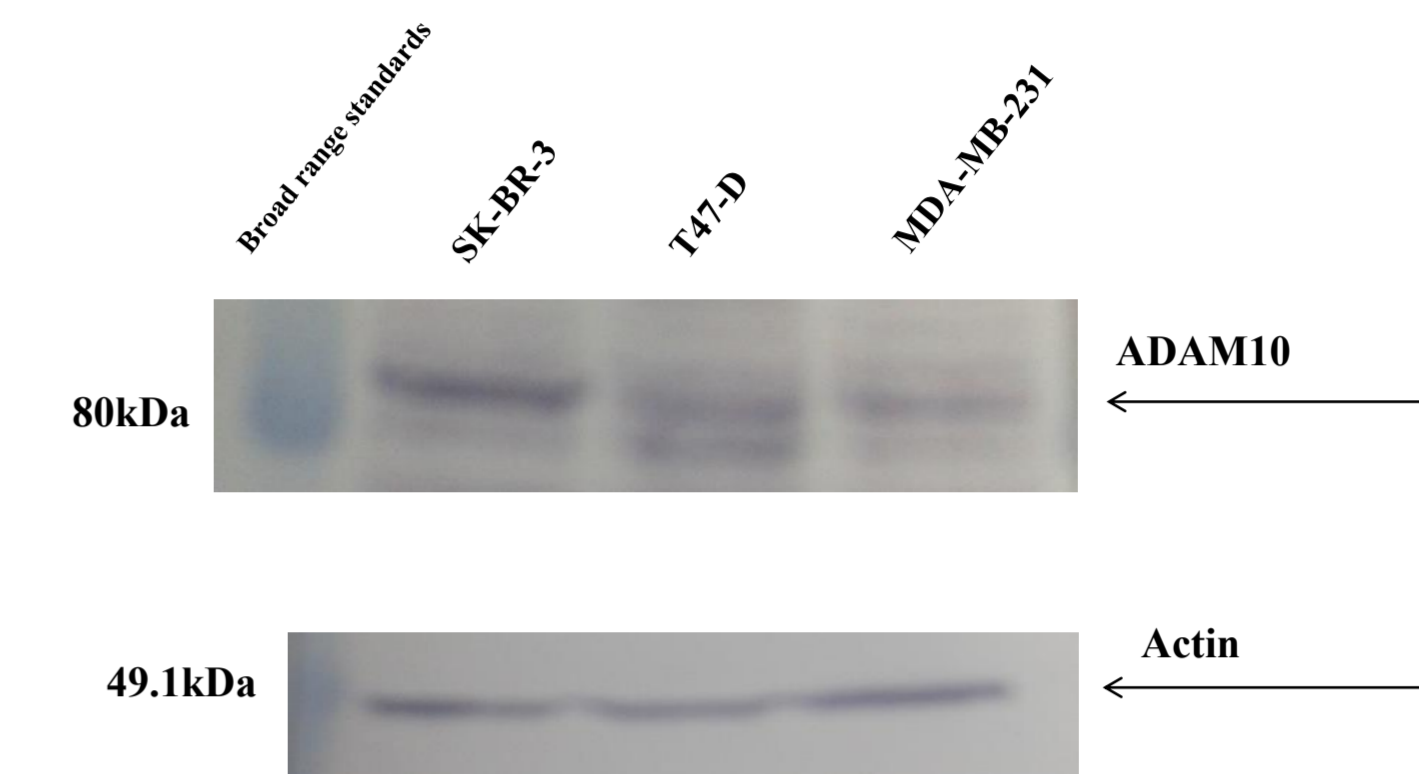


Figure 2. Western blot analysis of ADAM10 expression pattern.

### Effect of ADAM10 silencing on SK-BR-3 cell proliferation

Knock down (KD) of ADAM10 in SK-BR-3 cell line was efficient, resulting in 97% silencing. 72 hours after transfection cell proliferation was determined using MTS and BrdU assays.

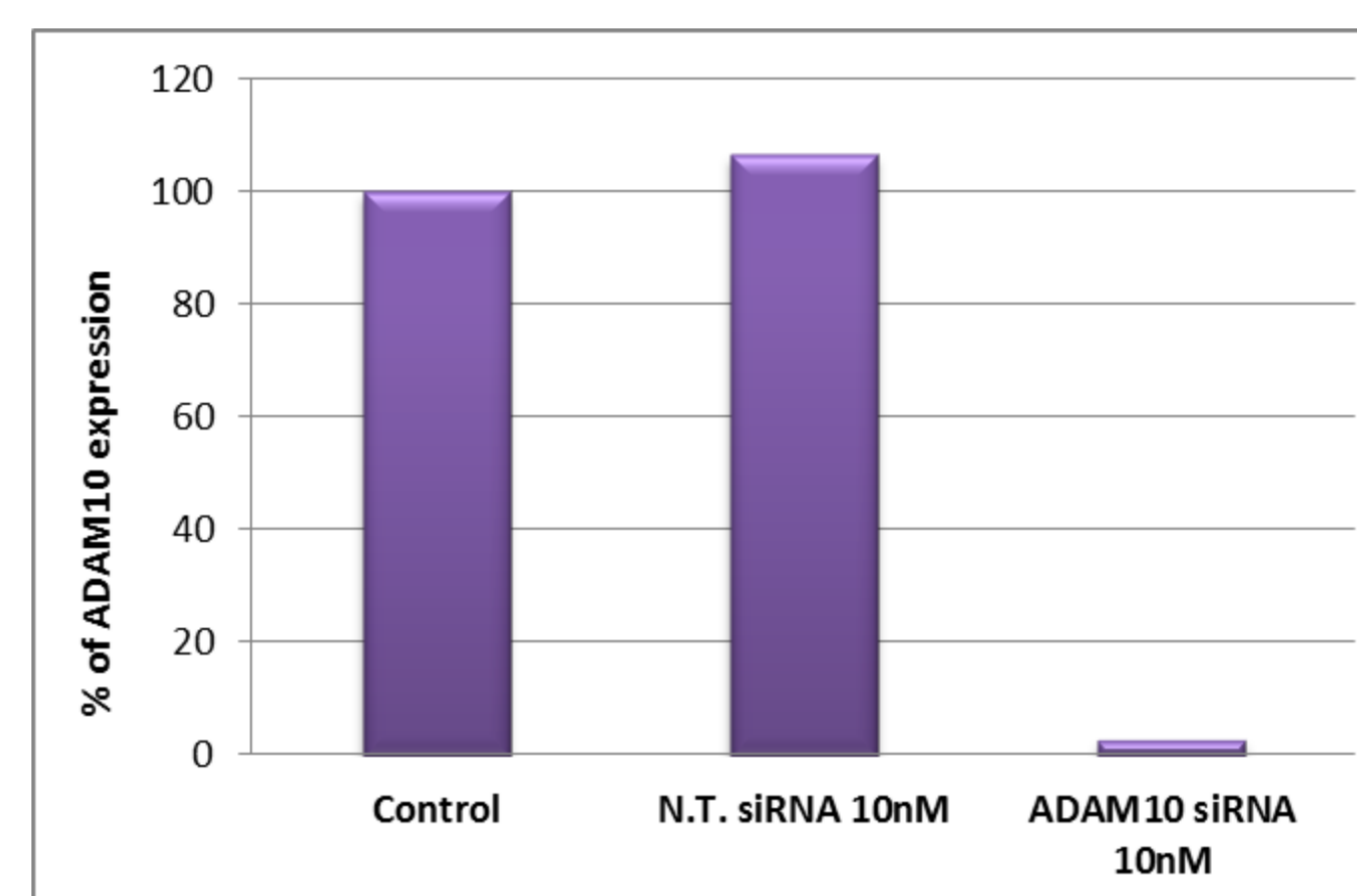


Figure 3. Transient knockdown of ADAM10

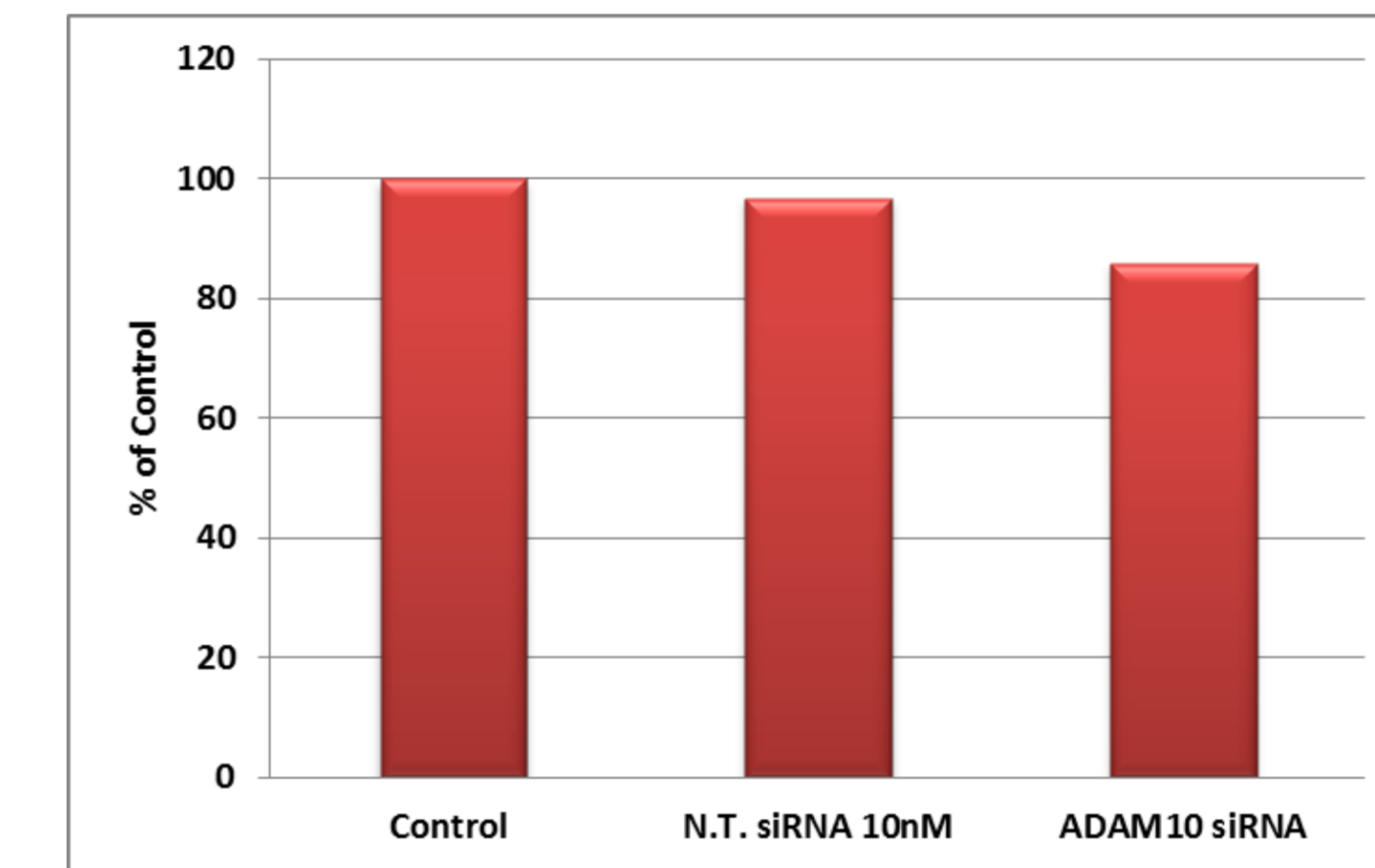


Figure 4. Influence of ADAM10 KD on proliferation, MTS assay

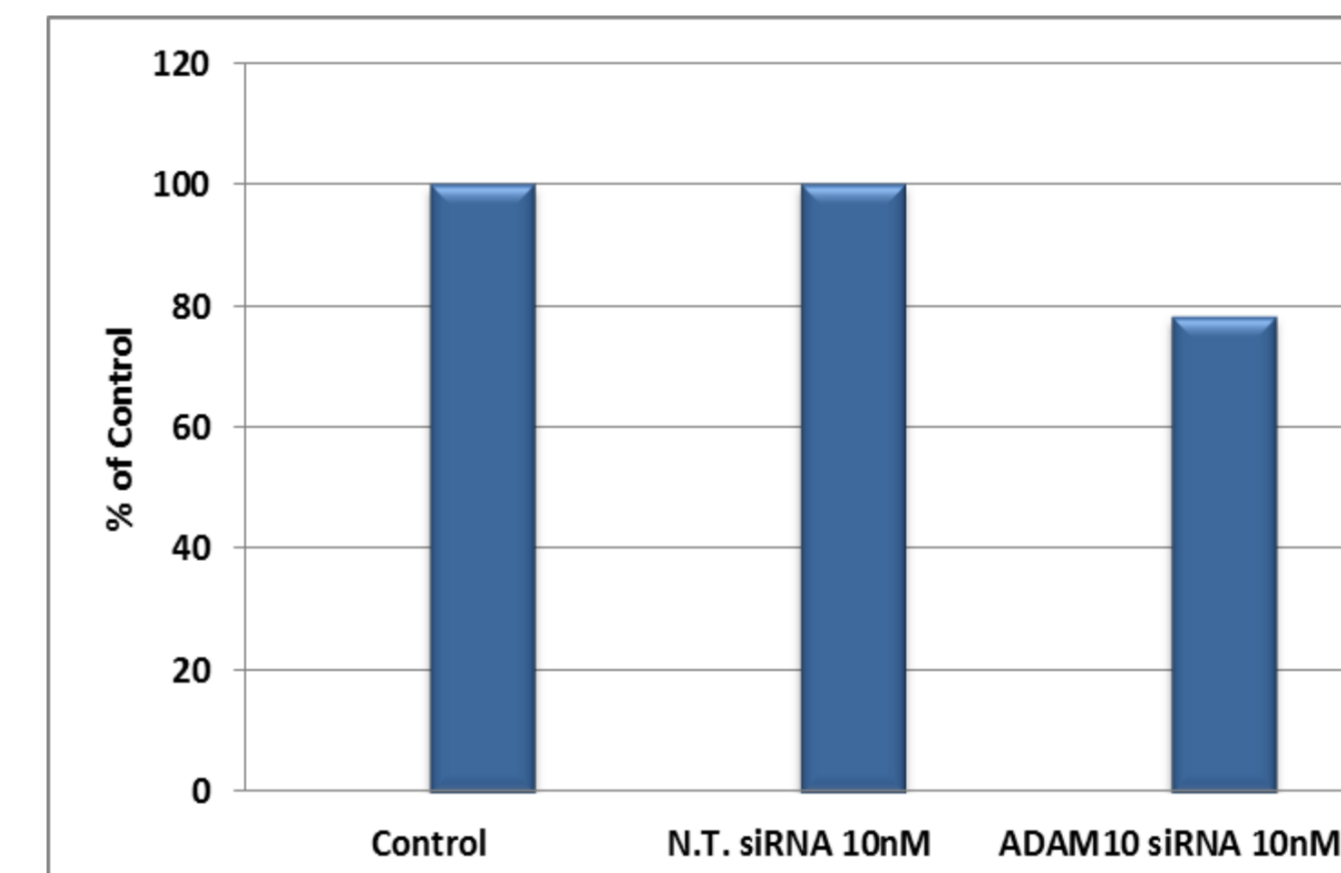


Figure 5. Influence of ADAM10 KD on proliferation, BrdU assay

### Influence of herceptin and ADAM10 inhibitor (GI254023X) on SK-BR-3 proliferation rate in 2D and 3D formats

For determination of the influence of testing substances on the cell viability and comparison of 2D and 3D culture formats, SK-BR-3 cell line was treated in 2D and 3D formats during 144 hours with herceptin, GI254023X, or their combination. Results obtained in 2D and 3D format are comparable (Table 1.). Tested concentrations were 20, 5, 0.2 and 0.02  $\mu\text{M}$  for herceptin. 1 and 10  $\mu\text{M}$  for GI254023X.

Table 1. Inhibition of SK-BR-3 cell proliferation (%). Data expressed as the mean  $\pm$  std.dev. for N=5.

	2D format	3D format
Herceptin 20 $\mu\text{g}/\text{ml}$	37	32
Herceptin 5 $\mu\text{g}/\text{ml}$	42	35
GI254023X 10 $\mu\text{M}$	0	2
GI254023X 1 $\mu\text{M}$	0	9
Herceptin 0.2 $\mu\text{g}/\text{ml}$	39	30
Herceptin 0.2 $\mu\text{g}/\text{ml}$ + GI254023X 10 $\mu\text{M}$	38	41
Herceptin 0.2 $\mu\text{g}/\text{ml}$ + GI254023X 1 $\mu\text{M}$	36	42
Herceptin 0.02 $\mu\text{g}/\text{ml}$	8	2
Herceptin 0.02 $\mu\text{g}/\text{ml}$ + GI254023X 10 $\mu\text{M}$	7	15
Herceptin 0.02 $\mu\text{g}/\text{ml}$ + GI254023X 1 $\mu\text{M}$	14	14

## Conclusions

All three breast cancer cell lines used in this study express ADAM10, although in a different extent. Common to these cell lines is that there is no effect on cell proliferation when ADAM 10 is silenced. Since only SK-BR-3 cells overexpress HER2, activity of herceptin, ADAM10 inhibitor or their combination on cell viability in 2D and 3D format were further conducted on this cell line only. Unlike data described in the literature (5) subsaturating concentration of herceptin (0.2  $\mu\text{g}/\text{ml}$ ) influenced cell viability comparable to an effect of concentration of 5  $\mu\text{g}/\text{ml}$  (40% of measured inhibition). Small molecule inhibitor of ADAM10, alone at both 1 and 10  $\mu\text{M}$ , or in combination with herceptin did not influence cell viability. Results obtained in 2D and 3D formats showed similar values. In conclusion, herceptin differs from a several other chemotherapeutic substances that are less potent inhibitors of breast cancer cell viability in 3D format compared to 2D format (4, 5).