Differential Effect of Novel Plant Cystatins on the Adhesive Behaviour of Normal and Cancer Breast Cells

Kamelia Hristova-Panusheva[1*], Milena Keremidarska-Markova[1,2], Natalia Krasteva[1]

[1]*Department of Electroinduced and Adhesive Properties*
Institute of Biophysics and Biomedical Engineering
Bulgarian Academy of Sciences
Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria
E-mails: kameliahristova@abv.bg, nataly@bio21.bas.bg

[2]*Human and Animal Physiology, Faculty of Biology*
Sofia University "St. Kliment Ohridski"
8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria
E-mail: m_keremidarska@uni-sofia.bg

[*]*Corresponding author*

Received: November 24, 2023 **Accepted: January 31, 2024**

Published: March 31, 2024

Abstract: *In the present work, we have investigated a novel recombinant cystatin dgECP1 and its mutant form, dgECP1m1, focused on their impact on the adhesive behaviour of two breast cell lines: the cancerous, MDA-MB-231, and the normal, MCF-10A. DgECP1 cystatin is intriguing with its RGD motif, responsible for cell adhesion and typical for mammalian extracellular matrix proteins but uncommon for plant cystatins. The presence of the RGD sequence suggests the potential of the dgECP1 to influence the adhesion of cancer cells and, respectively, cancer metastasis. A mutant form of the dgECP1cystatin, dgECP1m1, where RGD is replaced with HGD tripeptide, was also investigated. We found that both phytocystatins exerted differential effects on the adhesion behaviour of normal and cancer cells. In the case of dgECP1 cystatins, the effect on cancer cell adhesion also depends on the mode of administration of the cystatin to cells. When dgECP1 is pre-adsorbed on a substrate, it improves the attachment of breast cancer cells and induces cell aggregation, which is more typical for normal breast cells, and oppositely suppressed adhesion of cancer cells when added to the medium. The mutant form, dgECP1m1, inhibited cancer cell adhesion independently on the way of administration. On the other hand, both plant cystatins only slightly reduced the adhesion of normal mammary cells pointing to the higher sensitivity of cancer cells to both cystatins. These preliminary results open the possibility of considering the plant cystatin dgECP1 for anti-cancer strategies.*

Keywords: *Phytoprotease inhibitors, Phytocystatins, RGD motif, Metastasis, Cell attachment.*

Introduction

Metastasis is the leading reason for the mortality of patients with cancer. Understanding the cellular and molecular basis of this process is of primary importance for effective prognosis and treatment. Cancer metastasis is the migration of cancer cells from their site of origin to diverse areas within the body, forming new tumors that often lead to patient death [14]. The metastasis cascade is a sequence of events that drives this dissemination. During the metastatic cascade, alterations in cell-cell and cell-matrix interactions are of key importance. Loss of cell-cell adhesion capacity allows malignant tumor cells to dissociate from the primary

tumor mass and invade the neighboring stroma [22]. Another critical step in tumor invasion and metastasis is the degradation of the basement membrane and extracellular matrix, which relies on various proteolytic enzymes [8]. Certain protease inhibitors, like cystatins, have the potential to hinder cancer invasion or metastasis, offering promise as specialized agents in anticancer therapies [19]. Cystatins are cysteine protease inhibitors presenting in mammals, birds, fish, and insects, as well as in plants and some protozoa. Many of these inhibitors, however, face challenges in their clinical application. Their limited pharmacokinetics arose from peptide segment instability while others struggled due to the inherent chemical reactivity [8].

Searching for plant-derived protease inhibitors to combat various clinical disorders like allergies and inflammatory conditions started in the early 1950s. These inhibitors potent in trypsin/chymotrypsin inhibition have demonstrated their ability to suppress multiple stages of carcinogenesis. For instance, soybean's Bowman-Birk trypsin-chymotrypsin inhibitor and pea seed protease inhibitors, such as rTI1B and rTI2B, homologous to BBI, have been found to decrease the growth of colorectal adenocarcinoma HT29 cells indicating their potential as cancer chemopreventive agent [5]. Plant-derived protease inhibitors offer distinct advantages over their mammalian counterparts. They are easily obtainable from seeds, which are rich sources of protease inhibitors and can be incorporated into a diet through rice, legumes, soybeans, etc. Thus, the prevention of many diseases, including cancer, can be achieved only by adding plant-based food, without side effects on the human body. Literature suggests that populations consuming higher amounts of seeds such as beans, maize, and rice exhibit lower rates of breast, colon, and prostate cancer rates [6]. Protease inhibitors derived from plants or bacteria can form a potent protease-PI complex that effectively inhibits proteolytic activity. Considering the significance of lysosomal cysteine proteases in the transition from benign to aggressive tumors suggests that cystatins may safeguard against tumor progression through various mechanisms [17, 21]. However, the role of cystatins is not always straightforward, and some may exhibit both tumor-promoting and suppressing activities [11].

Recent findings indicate that plant-derived cystatins, traditionally known as protease inhibitors exhibit a broader spectrum of biological activities. These plant cystatins have been found to influence processes such as apoptosis, immune response, and angiogenesis, thus hinting at substantial therapeutic possibilities [3]. In the cancer context, elucidating how cystatins impact cell adhesion could offer new insights into the metastatic cascade. Changes in cell adhesion properties play a critical role in cancer cell dissemination and the formation of secondary tumors making the exploration of cystatin's role in this context particularly promising.

Previously, a novel extracellular phytocystatin called dgECP1 was identified in *D. glomerata* L. embryogenic suspension cultures. The protein was cloned and expressed in *E. coli* following the methodology described in [18]. Notably, dgECP1 contains the arginine-glycine-aspartic acid (RGD) motif, commonly found in mammalian extracellular matrix proteins but not in plant cystatins. This suggests that dgECP1 might have an influence on cell adhesion. Thus, the combination of protease inhibition activity along with the presence of the RGD motif in dgECP1 makes it a promising candidate to fight cancer and cancer cells' metastatic abilities.

In the present study, we have investigated the potential anticancer effect of the recombinant cystatin dgECP1 and its mutant form, dgECP1m1. We evaluated the adhesive behaviour of two breast cell lines: the cancerous, MDA-MB-231 and the normal MCF-10A. It is important to note that breast cancer ranks among the most prevalent malignancies globally and managing its metastatic progression represents a significant challenge in its clinical settings. Understanding

how cystatins like dgECP1 modulate cell adhesion opens up novel avenues for potential therapeutic interventions aimed at impeding cancer metastasis.

Materials and methods

Isolation and characterization of phytocystatins dgECP1 and dgECP1m1

Purified recombinant extracellular phytocystatin dgECP1 and its mutant form, dgECP1m1 were kindly provided by Prof. Dr. Magdalena Tchorbadjieva and Dr. Goritsa Rakleova from the Biological Faculty of Sofia University "St. Kliment Ohridski".

Cell lines and cell culture

MCF-10A, a normal human mammary epithelial cell, and MDA-MB-231, a mammary adenocarcinoma cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) with a high content of glucose, supplemented with 10% (v/v) fetal bovine serum, FBS (Gibco) and 1% penicillin/streptomycin (Sigma-Aldrich). The medium for MCF-10A cells was additionally supplemented with 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, and 10 ng/ml hEGF. Regularly, every 2-3 days upon reaching confluence, the cells were passaged using a 0.5×Trypsin (Sigma-Aldrich), combined with 5 mM EDTA/PBS, for a 5-minute incubation at 37 °C. The cells were then seeded onto 24-well plates, containing cover glasses. The cover glasses were either pre-coated with fibronectin (FN) or cystatins or left uncoated as described below. The culture medium applied to the 24-well plates was standardized to a volume of 1 ml/cm^2.

Pre-coating of culture substrates

Cover glasses (CGs) were pre-coated with fibronectin (Sigma-Aldrich) or both types of studied cystatins at 20 µg/ml concentrations for 30 min at 37 °C just before cell seeding. Once the incubation period ended, the unbound proteins were removed by thorough washing with PBS (pH-7.4), and subsequently, the cells were seeded onto FN and cystatin-coated CGs

Cell adhesion assay

To conduct the adhesion efficiency assay, the cells were seeded on the pre-coated and non-coated CGs at 2×10^4 cells/cm^2 density and incubated in serum-free media for two hours. In the case of the non-coated samples, 20 µg/ml cystatin was introduced into the medium simultaneously with the cells. Fibronectin-coated glasses were used as the positive control, while plain cover glasses acted as the negative control. At the end of incubation, the non-adherent cells were removed by rinsing with phosphate-buffered saline (PBS). The remaining cells were stained with 10 µl/well fluorescein diacetate (FDA) at a concentration of 5 µg/ml for 2 min. The adherent cells were photographed using a Zeiss Axiovert 25 inverted microscope (Germany) at a 20× magnification. These micrographs were subsequently used to count the number of attached cells, using image analysis software (Image J).

Statistical analysis

All experiments were done in triplicate, representing the results as ± standard deviation. The experimental data were analyzed by T-test to assess the significance of differences among treatments. Statistical significance was accepted at a level of $p < 0.05$

Results and discussion

The main objective of the present study was to assess the impact of two newly obtained phytocystatins (DgECP1 and its mutant DgECPm1), on the adhesive behaviour of breast cells, and to understand if those cystatins could be used in the future therapy of breast cancer. Breast cancer is one of the most prevalent malignancies globally, with metastatic progression representing a significant challenge in its clinical management [12]. The adhesion of cancer cells to the extracellular matrix in distant tissues is a crucial step in metastasis influencing the subsequent invasion and colonization of secondary sites [7]. Cancer cells often display altered adhesion patterns, unlike normal cells, enabling detachment from the primary tumor and migration to distant locations [9]. The dgECP1 phytocystatin, the object of the present study, is unique with its RGD moiety, a sequence typical for mammals, but rare in plants. RGD is a recognition sequence in various adhesive ECM proteins, blood proteins, cell surface proteins, and is recognized by a significant part of integrins, key receptors involved in cell adhesion [2]. The presence of RGD in dgECP1 had been confirmed by immunoblotting assays. Conversely, the mutant form dgECP1m1 lacks RGD but presents the HGD sequence. Hence, comparing both forms of dgECP1 allows an understanding of how the presence or absence of the RGD sequence in a cystatin might affect cancer cell adhesion. To investigate this we pre-coated cover glasses with cystatins or introduced them into the culture medium during cell seeding. In the case where cystatins are pre-adsorbed, it is expected that cell adhesion will increase. Conversely, when cystatins are added to the medium, cell adhesion is hypothesized to be inhibited. This distinction aims to elucidate the specific effects of the RGD sequence on cell adhesion behaviour.

The chosen two breast cell lines for this study, the adenocarcinoma MDA-MB-231 cells, and the normal MCF-10A breast cells, differed significantly in their adhesive behaviour. MDA-MB-231 cells are recognized for their highly aggressive nature, characterized by rapid growth and potent metastatic abilities [16, 23]. These cells typically exhibit weak adhesion, forming loosely cohesive grape-like or stellate structures, consistent with their invasive phenotype *in vitro* [10]. In contrast, MCF-10A display a basal-like phenotype while sharing certain features with mesenchymal cell lines [15]. Our study's results have evidently highlighted a significant difference in initial adhesion between these two types of cells, indicating that the type of cystatin used and its method of administration – whether pre-adsorbed onto the substrate surface or added to the culture media – play a crucial role in influencing this adhesion behaviour (as depicted in Figs. 1 and 2).

Our findings suggest that when dgECP1 was added simultaneously to the culture medium with cells, it resulted in a diminished adhesion of both carcinoma and normal cells to the substrate. The FDA micrographs in Fig. 1 indicate that a significantly lower number of cells were able to attach to the substratum (CG) under these conditions, and they are notably rounded and small in size, even smaller than cancer cells attaching to the negative control. This suggests that cystatin potentially blocks the integrins on the cell surface hindering the adhesion to the substratum and positioning it as a promising candidate for integrin antagonists. On the FN-coated cover glasses (positive control), most cells displayed a spread cell morphology, typical for well-adhered cells owing to the presence of fibronectin, a primary adhesive protein in the extracellular matrix. Conversely, loosely attached cells showed a distinct characteristic: a round shape and smaller size.

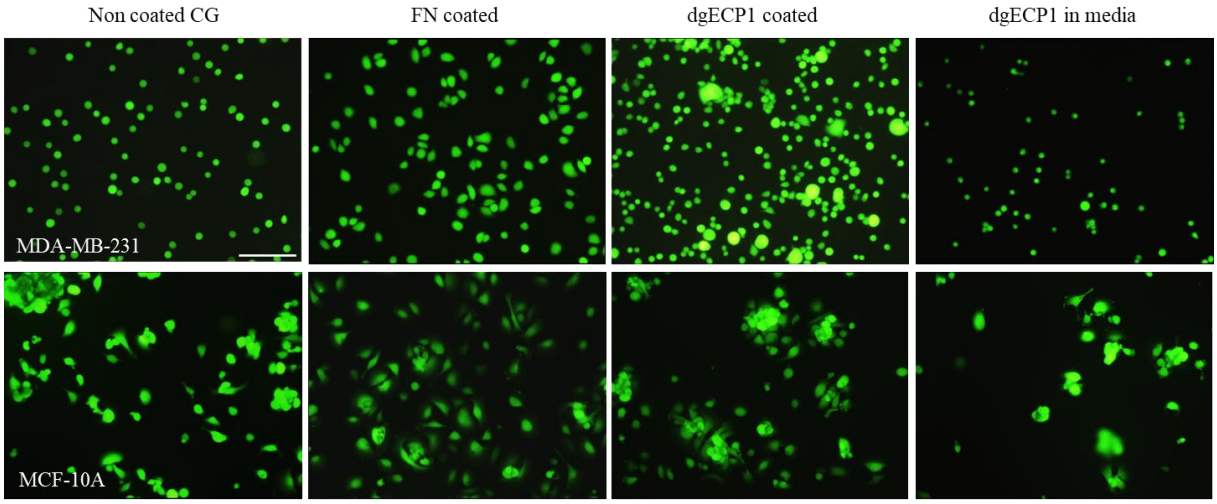


Fig. 1 The overall morphology of the cancerous MDA-MB-231 cells (upper panel) and the normal MCF-10A cells (lower panel) after adhering for 2 h on different substrates – plain coverslips, FN-coated coverslips, dgECP1 cystatin, pre-coated or added to the culture medium. FDA images are taken on an inverted fluorescent microscope. The scale bar is 100 μm.

For the normal MCF-10A breast cells, the number of attached cells was also reduced compared to the positive control. However, these cells formed aggregates similar to those observed on the dgECP1-coated surfaces and the negative control. This suggests that dgECP1 cystatin did not disrupt cell-cell interactions. This phenomenon might be attributed to the fact that normal breast cells like MCF-10A, do not typically decrease cell-cell adhesions, unlike metastatic cells [20].

Metastatic cancer cells tend to decrease E-cadherin expression while increasing interactions with the surrounding extracellular matrix (ECM) to promote migration and invasion, which often result from changes in integrin or ECM protein expression and localization [4, 20]. Interactions of integrin and ECMs are crucial for facilitating cell migration and invasion, contrasting with the role of E-cadherin in maintaining tissue integrity. Therefore, a better understanding of integrin function and searches for new integrin antagonists are of considerable interest [1]. Our observations indicate that the suppression of cell adhesion to the substratum by dgECP1 is more pronounced in cancer cells than in normal cells. These morphological observations were further supported by the assessment of the attached cells presented in Fig. 2.

The pre-coating of CG with dgECP1 cystatin appeared to stimulate the attachment of cancer cells to the substratum, as evident in Fig. 1. The quantity of adhered cells was nearly doubled in comparison to the sample coated with FN. Fig. 3 indicates a robust expression of the RGD motif that was well-recognized by the cells, in particular by their integrin receptors. Many of the cells exhibited a rounded shape, and some formed aggregates, which aligns more with the typical behaviour of normal cells. This suggests that dgECP1 alters the adhesive behaviour of the breast cancer cells leading them toward the adhesive morphology similar to that of the normal breast cells. In the case of normal MCF-10A breast cells, pre-coated dgECP1 induced aggregation, and individual cells displayed a more spread morphology. This observation suggests that dgECP1 improved both cell-ECM and cell-cell interactions in these normal cells. These findings emphasize the dual influence of dgECP1 on both cancerous and normal breast cells, indicating its potential to modify cell adhesion behaviours towards a more normalized pattern, potentially impacting both the cell-ECM interactions and the cell-cell interactions.

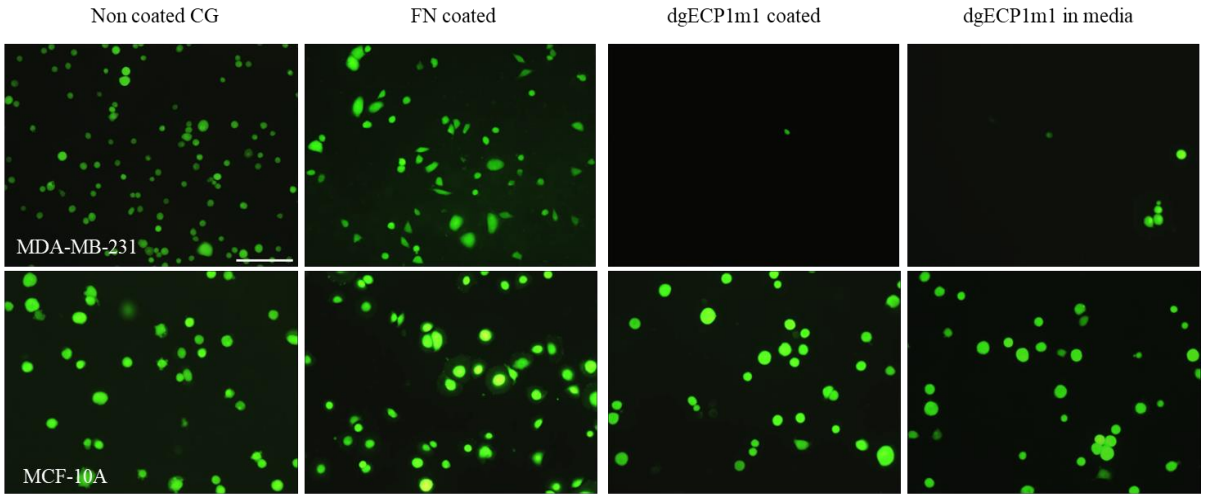


Fig. 2 Overall morphology of cancer MDA-MB-231 cells (upper panel) and normal MCF-10A cells (lower panel) adhering for 2 h on non-coated CG, FN-coated coverslips, mutant dgECP1m1 cystatin pre-coated CG or added to the culture medium. FDA images are taken on an inverted fluorescent microscope. The scale bar is 100 μm.

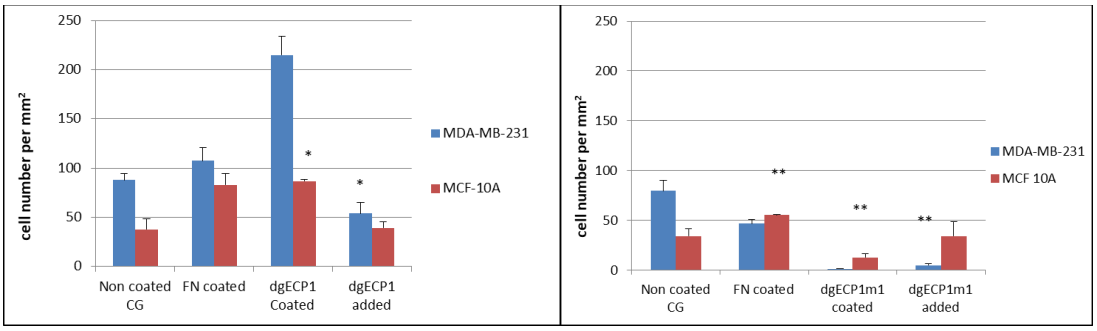


Fig. 3 Assessment of cell attachment of cancer MDA-MB-231 cells and normal MCF-10A cells after incubation with dgECP1 cystatin and its mutant form – dgECP1m1.
The number of attached cells was evaluated by Image J software.

Our findings regarding the mutant form of the dgECP1, dgECP1m1, have revealed a remarkably strong suppressing effect on the adhesion of cancer cells in both scenarios – on pre-coated samples and in samples where cystatin was added in the medium. This result contrasts with the expectation that dgECP1m1 would have no impact on cell adhesion based on the assumption that the HGD sequence would not interact with the integrin receptor. This observation is consistent with the behaviour observed in normal MCF-10A cells, where dgECP1m1 neither stimulated nor suppressed cell adhesion. The distinct and strong suppressing effect of dgECP1m1 on cancer cell adhesion suggests that its mechanism of action differs from that of dgECP1 cystatin. It appears that dgECP1m1's influence on cancer cells does not involve interactions with integrin receptors. Its impact appears linked to the malignant properties of these cells, suggesting dgECP1m1's mechanism may involve specific pathways related to cancer cell malignancy.

Conclusion

In general, our results demonstrate that RGD-containing recombinant phytocistatin dgECP1 can modulate the adhesion behaviour of breast cancer cells. The differential impact based on their introduction into cell systems is a key observation dgECP1, when preadsorbed onto the substrate, enhances attachment and induces cell aggregation in cancer cells while suppressing cell adhesion when added to the culture medium. On the other hand, dgECP1m1, featuring the HGD motif, markedly suppresses cancer cell adhesion regardless of its introduction method. In normal breast cells, both cystatins showed minimal effects on reducing adhesion.

These preliminary findings hold promise, suggesting the potential utility of the plant cystatin dgECP1 as a candidate for anti-cancer strategies due to its role in modulating cell adhesion and potentially regulating proteolytic activity. This opens up new possibilities for considering plant-derived cystatins as key regulators in altering cancer cell adhesion behaviours, offering prospects for further research into their therapeutic applications in cancer treatment strategies.

Acknowledgements

We acknowledge the contributions of Dr. Goritsa Rakleova and Prof. Magdalena Tchorbadjieva from Sofia University "St. Kliment Ohridski" for generously providing the purified recombinant phytocystatin dgECP1 and its mutant form, DgECP1m1.

References

1. Alday-Parejo B., R. Stupp, C. Rüegg (2019). Are Integrins Still Practicable Targets for Anti-Cancer Therapy? Cancers, 11, 978.
2. Bellis S. L. (2011). Advantages of RGD Peptides for Directing Cell Association with Biomaterials, Biomaterials, 32(18), 4205-4210.
3. Benchabane M., U. Schlüter, J. Vorster, M. C. Goulet, et al. (2010). Plant Cystatins, Biochimie, 92(11), 1657-1666.
4. Birchmeier W. (1995). E-cadherin as a Tumor (Invasion) Suppressor Gene, Bioessays, 17, 97-99.
5. Clemente A., J. M. Gee, I. T. Johnson, D. A. MacKenzie, et al. (2005). Pea (*Pisum sativum* L.) Protease Inhibitors from the Bowman-Birk Class Influence the Growth of Human Colorectal Adenocarcinoma HT29 Cells *in vitro*, J Agricult Food Chem, 53(23), 8979-8986.
6. Correa P. (1981). Epidemiological Correlations between Diet and Cancer Frequency, Cancer Res, 41, 3685-3690.
7. Elgundi Z., M. Papanicolaou, G. Major, T. R. Cox, et al. (2020). Cancer Metastasis: The Role of the Extracellular Matrix and the Heparan Sulfate Proteoglycan Perlecan, Front Oncol, 9, 1482.
8. Fear G., S. Komarnytsky, I. Raskin (2007). Protease Inhibitors and Their Peptidomimetic Derivatives as Potential Drugs, Pharmacol Ther, 113(2), 354-68.
9. Fife C. M., J. A. McCarroll, M. Kavallaris (2014). Movers and Shakers: Cell Cytoskeleton in Cancer Metastasis, Br J Pharmacol, 171(24), 5507-5523.
10. Holliday D. L., V. Speirs (2011). Choosing the Right Cell Line for Breast Cancer Research, Breast Cancer Res, 13, 215.
11. Keppler D. (2006). Towards Novel Anti-cancer Strategies Based on Cystatin Function, Cancer Letters, 235, 159-176.
12. Łukasiewicz S., M. Czeczelewski, A. Forma, J. Baj, et al. (2021). Breast Cancer – Epidemiology, Risk Factors, Classification, Prognostic Markers, and Current Treatment Strategies – An Updated Review, Cancers (Basel), 13(17), 4287.

13. Majumdar D. D. (2013). Recent Updates on Pharmaceutical Potential of Plant Protease Inhibitors, Int J Med Pharm Sci, 3(4), 101-120.
14. Martin T. A., L. Ye, A. J. Sanders, J. Andrew, et al. (2013). Cancer Invasion and Metastasis: Molecular and Cellular Perspective. In: Madame Curie Bioscience Database [Internet], Austin (TX): Landes Bioscience, 2000-2013, Available from https://www.ncbi.nlm.nih.gov/books/NBK164700/.
15. Neve R. M., K. Chin, J. Fridlyand, J. Yeh, et al. (2006). A Collection of Breast Cancer Cell Lines for the Study of Functionally Distinct Cancer Subtypes, Cancer Cell, 10(6), 515-527.
16. Parekh A., D. Das, S. Das, S. Dhara, et al. (2018). Bioimpedimetric Analysis in Conjunction with Growth Dynamics to Differentiate Aggressiveness of Cancer Cells, Sci Rep, 8, 783.
17. Rakashanda S., A. K. Qazi, R. Majeed, S. Rafiq, et al. (2013). Antiproliferative Activity of *Lavatera cashmeriana* – Protease Inhibitors towards Human Cancer Cells, Asian Pacific J Cancer Prev, 14(6), 3975-3978.
18. Rakleova G., A. Keightley, I. Pantchev, I. Tsacheva, et al. (2012). Identification, Molecular Cloning, and Recombinant Gene Expression of an Extracellular A-amylase from *Dactylis Glomerata* L. Embryogenic Suspension Cultures, Biotech Biotechnol Equip, 26(4), 3192-3200.
19. Rudzińska M., C. Daglioglu, L. V. Savvateeva, F. N. Kaci, et al. (2021). Current Status and Perspectives of Protease Inhibitors and Their Combination with Nanosized Drug Delivery Systems for Targeted Cancer Therapy, Drug Des Devel Ther, 15, 9-20.
20. Ruoslahti E. (1999). Fibronectin and Its Integrin Receptors in Cancer, Adv Cancer Res, 76, 1-20.
21. Teixeira E. M. G. F., D. Oliveira, R. E. Silva-López (2020). Plant Protease Inhibitors as Specific Strategies against Cancer Cells, J Appl Biotechnol Bioeng, 7(4), 169-173.
22. van Zijl F., G. Krupitza, W. Mikulits (2011). Initial Steps of Metastasis: Cell Invasion and Endothelial Transmigration, Mutat Res, 728(1-2), 23-34.
23. Wong A. T., B. M. Gumbiner, A. Sadlonova (2003). Adhesion-independent Mechanism for Suppression of Tumor Cell Invasion by E-cadherin, J Cell Biol, 161, 1191-1203.

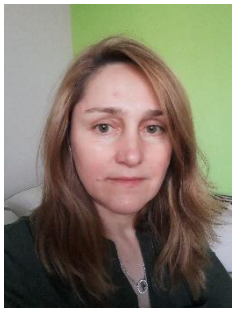
Sen. Assist. Prof. Kamelia Hristova-Panusheva, Ph.D.
E-mail: kamelia.t.hristova@gmail.com

Kamelia Hristova-Panusheva is an Assistant Professor in the Department of Electroinduced and Adhesive Properties at the Institute of Biophysics and Biomedical Engineering (IBPhBME) – Bulgarian Academy of Sciences (BAS). She defended her Ph.D. Degree in Biophysics in 2011. From 2007 to 2009, she was a research fellow at the Institute of Bioengineering of Catalonia, Barcelona, Spain. Her research interests are in the fields of cell biology, tissue engineering, materials science, and cancer.

Sen. Assist. Prof. Milena Keremidarska-Markova, PhD.
E-mail: m_keremidarska@uni-sofia.bg

Milena Keremidarska-Markova is an Assistant Professor in the Department of Animal and Human Physiology at the Faculty of Biology, Sofia University "St. Kliment Ohridski" since 2017. She defended her Ph.D. Degree in Biophysics in 2016 in the IBPhBME – BAS. She participates in research experiments in the IBPhBME and at the Faculty of Biology in the fields of animal and human physiology, cell biology, tissue engineering and nanomaterial science. Assist. Prof. Keremidarska-Markova is an author and co-author of 18 scientific publications.

Prof. Natalia Krasteva, Ph.D.
E-mail: nataly@bio21.bas.bg



Natalia Krasteva is a Professor in the Department of Electroinduced and Adhesive Properties at the IBPhBME –BAS. She defended her Ph.D. Degree in Biophysics in 2003. From 2005 to 2007, prof. Krasteva was a postdoctoral fellow at the GKSS Research Center, Institute for Polymer Research, Teltow, Germany. From the start of her scientific career until now, she has been an author and co-author of more than 50 scientific publications in peer-reviewed international journals in the fields of cell biology, tissue engineering, materials science, cancer, and genetics.