# Regulation of the urokinase-type plasminogen activator system in mammalian cell lines

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**Master's thesis / Diplomski rad**

**2020**

*Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj:* **University of Zagreb, Faculty of Food Technology and Biotechnology / Sveučilište u Zagrebu, Prehrambeno-biotehnološki fakultet**

*Permanent link / Trajna poveznica:* https://urn.nsk.hr/urn:nbn:hr:159:592804

UNIVERSITY OF ZAGREB

FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

# MASTER THESIS

Zagreb, September 2020

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1036/MB

**Université d'Orléans - Université de Zagreb**

**UFR Sciences et Techniques- Faculté de Nutrition et Biotechnologie - Faculté des Sciences**

# Master Sciences du Vivant

*Spécialité*: Biotechnologies, Biologie Moléculaire et Cellulaire

## INTERNSHIP REPORT

## Regulation of the Urokinase-Type Plasminogen Activator System in Mammalian Cell Lines

**By**

**Laura Marinić**

**(February 2019 – June 2020)**

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## TEMELJNA DOKUMENTACIJSKA KARTICA

**Diplomski rad**

**Sveučilište u Zagrebu**
**Prehrambeno-biotehnološki fakultet**

**Znanstveno područje:** Biotehničke znanosti
**Znanstveno polje:** Biotehnologija

# REGULACIJA SUSTAVA UROKINAZNOG PLAZMINOGENSKOG AKTIVATORA U STANIČNIM LINIJAMA SISAVACA

*Laura Marinić, 1036/MB*

**Sažetak:** Sustav urokinaznoga plazminogenskoga aktivatora sastoji se od urokinaznog plazminogenskog aktivatora (uPA), receptora urokinaznog plazminogenskog aktivatora (uPAR) i inhibitora plazminogenskog aktivatora-1 (PAI-1). Glavna uloga urokinaze je pretvorba plazminogena u plazmin, izvanstanične proteaze uključene u razgradnju izvanstaničnog matriksa. Uz niz fizioloških procesa, uPA sustav djeluje i kao regulator mikro-okruženja tumora te je uključen u procese metastaziranja. Cilj rada bio je usporediti aktivnost urokinaze i njezinu regulaciju u različitim staničnim linijama, pod bazalnim uvjetima i nakon tretmana s nekoliko kemoterapeutika. Stanice glioblastoma A1235 povećale su aktivnost uPA nakon tretmana alkilirajućim agensom, retinoičnom kiselinom i natrijevim salicilatom. Stanice karcinoma dojke MDA-MB-231 smanjile su aktivnost urokinaze nakon tretmana natrijevim salicilatom, a povećale nakon tretmana retinoičnom kiselinom, kao i stanična linija fibroblasta. Inhibitor dinamina inhibirao je urokinaznu aktivnost stanica A1235 i MDA-MB-231. HEK 293 stanice nisu promijenile uPA aktivnost nakon različitih tretmana. Zaključno, mehanizmi uPA sustava i njegova regulacija stanično su specifični.

**Ključne riječi:** *urokinaza, inhibitor plazminogenskog aktivatora-1, inhibitor dinamina, stanične linije sisavaca, regulacija*

**Rad sadrži:** 25 stranica, 7 slika, 55 literaturnih navoda

**Jezik izvornika**: hrvatski

**Rad je u tiskanom i elektroničkom (pdf format) obliku pohranjen u:** Knjiţnica Prehrambeno-biotehnološkog fakulteta, Kačićeva 23, Zagreb

**Mentor:** *Izv. prof. dr. sc. Maja Matulić*

**Stručno povjerenstvo za ocjenu i obranu:**
*1.* Prof.dr.sc. *Mariastefania Antica*
*2.* Prof.dr.sc. *Richard Daniellou*

**Datum obrane:** 21. rujna 2020.

## BASIC DOCUMENTATION CARD

**Graduate Thesis**

**University of Zagreb**
**Faculty of Food Technology and Biotechnology**

**Scientific area:** Biotechnical Sciences
**Scientific field:** Biotechnology

# REGULATION OF THE UROKINASE-TYPE PLASMINOGEN ACTIVATOR SYSTEM IN MAMMALIAN CELL LINES

*Laura Marinić, 1036/MB*

**Abstract:** The urokinase-type plasminogen activator system consists of urokinase plasminogen activator (uPA), urokinase type plasminogen activator receptor (uPAR) and plasminogen activator inhibitor-1 (PAI-1). The main role of urokinase is the conversion of plasminogen to plasmin, extracellular protease involved in the degradation of extracellular matrix. uPA system has a role in number of physiological processes, as well as in the metastatic processes. The aim of the study was to compare the urokinase activity and its regulation in different cell lines, under basal conditions and after treatment with several chemotherapeutical agents. Glioblastoma A1235 cells increased the uPA activity after treatment with alkylating agent, retinoic acid and sodium salicylate. Breast cancer cells MDA-MB-231 downregulated urokinase activity after sodium salicylate treatment and increased after treatment with retinoic acid, as well as fibroblast cell line. Dynamin inhibitor inhibited the urokinase activity of A1235 and MDA-MB-231 cells. HEK-293 cells did not change uPA activity after different treatments. In conclusion, the uPA system mechanisms and its regulation are cell-type specific.

**Keywords:** *urokinase, plasminogen activator inhibitor-1, dynamin inhibitor, mammalian cell lines, regulation*

**Thesis contains:** 25 pages, 7 figures, 55 references

**Original in**: Croatian

**Graduate Thesis in printed and electronic (**pdf format**) version is deposited in:** Library of the Faculty of Food Technology and Biotechnology**,** Kačićeva 23, Zagreb.

**Mentor:** *Maja Matulić, PhD, Associate Professor*

**Reviewers:**
1. PhD. *Mariastefania, Antica*, Full professor
2. PhD. *Richard, Daniellou*, Full professor

**Thesis defended:** 21 September 2020

**ABSTRACT**

The plasminogen activator (PA) system is an extracellular proteolytic enzyme system associated with various physiological and pathophysiological processes. Among the various components of the PA system, urokinase-type plasminogen activator (uPA), its receptor (uPAR), and plasminogen activator inhibitor-1 are the main components. uPA, also known as urokinase, was originally isolated from human urine, and it is also present in blood and in extracellular matrix of many tissues. The primary physiological substrate of this enzyme is plasminogen, which is an inactive form (zymogen) of the serine protease plasmin. The activation of plasmin initiates a series of proteolytic cascades to degrade the components of the extracellular matrix. This study summarizes the effect of various chemotherapeutical agents (*all-trans retinoic acid*, N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester, *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine, sodium salicylate and dynasore) on different human mammalian cell lines (glioblastoma A1235, immortalized fibroblasts MJ90 hTERT, embryonic kidney HEK-293 and breast cancer cells MDA-MB-231) in respect to urokinase activity and expression of the uPA system elements. Breast cancer cells MDA-MB-231 downregulated urokinase activity after sodium salicylate treatment and increased after treatment with retinoic acid, as well as fibroblast cell line. As recent studies have demonstrated urokinase regulation by skeletal reorganization, we also analyzed the influence of dynamin inhibitor on urokinase activity. It was found that it inhibited urokinase activity in A1235 and MDA-MB-231 cell lines. HEK-293 cells did not change uPA activity after different treatments. The protein components of the PA system analyzed also indicated cell-type-specific regulation. In order to elucidate the mechanisms of the uPA system regulation, further research is needed.

**Keywords:** urokinase, plasminogen activator inhibitor-1, dynamin inhibitor, mammalian cell lines, regulation

**RÉSUMÉ**

Le système activateur du plasminogène (PA) est un système enzymatique protéolytique extracellulaire associé à divers processus physiologiques et physiopathologiques. Parmi les divers composants du système PA, l'activateur du plasminogène de type urokinase (uPA), son récepteur (uPAR) et l'inhibiteur de l'activateur du plasminogène-1 sont les principaux composants. L'uPA, également connue sous le nom d'urokinase, a été isolée à l'origine de l'urine humaine et est également présente dans le sang et dans la matrice extracellulaire de nombreux tissus. Le substrat physiologique principal de cette enzyme est le plasminogène, qui est une forme inactive (zymogène) de la sérine protéase plasmine. L'activation de la plasmine déclenche une série de cascades protéolytiques pour dégrader les composants de la matrice extracellulaire. Cette étude résume l'effet de divers agents chimiothérapeutiques (acide tout-trans rétinoïque, N- [N- (3,5-Difluorophénacétyl-L-alanyl)] - S-phénylglycine t-butyl ester, N-méthyl-N′-nitro - N-nitrosoguanidine, salicylate de sodium et dynasore) sur différentes lignées cellulaires humaines de mammifères (glioblastome A1235, fibroblastes immortalisés MJ90 hTERT, rein embryonnaire HEK-293 et cellules cancéreuses du sein MDA-MB-231) en ce qui concerne l'activité urokinase et l'expression de l'uPA éléments du système. Les cellules cancéreuses du sein MDA-MB-231 ont diminué l'activité de l'urokinase après un traitement au salicylate de sodium et augmenté après le traitement avec de l'acide rétinoïque, ainsi que la lignée de fibroblastes. Comme des études récentes ont démontré la régulation de l'urokinase par réorganisation squelettique, nous avons également analysé l'influence de l'inhibiteur de la dynamine sur l'activité de l'urokinase. Il a été trouvé qu'il inhibait l'activité de l'urokinase dans les lignées cellulaires A1235 et MDA-MB-231. Les cellules HEK-293 n'ont pas changé l'activité de l'uPA après différents traitements. Les composants protéiques du système PA analysés ont également indiqué une régulation spécifique au type de cellule. Afin d'élucider les mécanismes de la régulation du système uPA, des recherches supplémentaires sont nécessaires.

**Mots-clés:** urokinase, inhibiteur de l'activateur du plasminogène-1, inhibiteur de la dynamine, lignées cellulaires de mammifères, régulation

**ACKNOWLEDGEMENTS**

Above all, I thank my mentor Maja Matulić, PhD, Associate Professor for her understanding, patience, transmission of knowledge and overall the effort which she invested in helping me to complete this research. The memory of your company and kindness will remain with me forever.

I thank to all of my current family members, as well as the ones who will become my family in the future. Your gentle words of encouragement have led me through tough times and enforced my faith in success.

To my parents and my brother, I am tremendously thankful for the fact that with you I feel love, happiness and security to pursue future successes. Thank you for everything that you have provided me and thank you for shaping me into the person I am today.

To my dear Martin Sven, let this be a share for our future.

*"The more I study nature, the more I stand amazed at the work of the Creator."*

Louis Pasteur

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## PRESENTATION OF THE HOST ORGANIZATION

The Department of Molecular Biology is the part of the Division of Biology, on the Faculty of Science at the University of Zagreb. The Faculty of Science is one of the leading centers of science in Croatia and has a key contribution to the scientific profile of the University. The Department of Molecular Biology was established in 1989 with the aims of advancing biological research at the molecular level and establishing a study program in molecular biology. Since 2004, the Department is located at Horvatovac. Molecular-biological research is being conducted on plant and animal cell cultures and models, and intensive research is also conducted in the field of biomedicine and bioinformatics. The Department is responsible for teaching undergraduate and graduate programs in Molecular Biology as well as for teaching general courses such as Cell Biology and Genetics in all Biology Department programs. In addition to biology department professors, scientists from various scientific institutions, primarily the Ruđer Bošković Institute, participate in the lecturing. Research at the Department of Molecular Biology is underway in a dozen of research projects.

# 1. INTRODUCTION

## 1.1 The Urokinase-Type Plasminogen Activator System

The main role of the plasminogen activator system is the processing of circulating inactive zymogen plasminogen to plasmin, a ubiquitous extracellular protease, which can be carried out by two types of PA: tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Wun *et al.*, 1982; Mahmood *et al.,* 2018).

The urokinase-type plasminogen activator (uPA) system is composed of urokinase-type plasminogen activator (uPA); urokinase-type plasminogen activator receptor (uPAR), located at the cell surface; plasminogen activator inhibitor-1 (PAI-1) and plasminogen, as the main substrate.

The urokinase-type plasminogen activator, abbreviated as uPA, or shortly known as "urokinase" is a serine proteinase with $M_r$ of around 54 kDa and is synthesized and released as glycosylated dormant single chain protein (pro-uPA). Conversion of secreted zymogen pro-uPA to two-chain form of uPA linked with disulphide bond can be catalyzed by cleaving peptide bond K158-I159, most effectively by plasmin. Resulting urokinase consists of two disulphide bridge-linked polypeptide chains and three domains: a catalytic serine protease domain (159–411 amino acids) at the C-terminus, the growth factor domain (GFD), that shares homology with the epidermal growth factor (EGF) (spanning from 1 to 49 amino acids) and a kringle domain (KD) (50–131 amino acids) that resides at the N-terminus. In between the N-terminal and C-terminal region, there is a linker region (132–158 amino acids). It was first identified in urine, in 1947 by MacFarlane and Pilling, but was named by Sobel and colleagues (1952). Studies report the presence of urokinase in plasma, seminal fluid, and the extracellular matrix (ECM) of many tissues.

Another important serine proteinase is plasmin, with $M_r$ of approximately 92 kDa. Produced mainly in liver, plasminogen is a single polypeptide chain zymogen form of plasmin, and its conversion to two disulphide bridge-linked polypeptide chains is enabled by cleavage of peptide bond R560-V562, by uPA, tPA or several bacterial proteins. At its C-terminus, plasmin contains serine proteinase domain with catalytic or inhibitor-binding activity and at N-terminus 5 kringle domains.

The urokinase-type plasminogen activator receptor (u-PAR or CD87) is a glycoprotein, a compound of 313 amino acids, rich in cysteine, lined up in a single polypeptide chain with three extracellular domains (D1, D2, and D3), which are mutually linked with two flexible linker

sequences. Cleavage in D1/D2 linker region, by proteases such as matrix metalloproteases (MMPs), plasmin, chymotrypsin, and uPA, can result in the formation of a truncated uPAR. Human uPAR is heavily glycosylated. uPAR is attached to the outer leaf of the cell membrane by a glysocylphosphatidylinositol (GPI) anchor. The uPA binds to uPAR through its growth factor domain. This binding strongly enhances the capacity to generate plasmin, and furthermore, produce loop between uPA and plasmin, which results in uPA activation and up-regulation of uPAR expression (Montuori *et al.*, 2000). Furthermore, cleavage at the GPI anchor site can produce a soluble form of uPAR (suPAR), which is present in low concentrations in the blood. The function of uPAR is not only limited to uPA binding, but it can also bind to vitronectin and interact with integrins and some other transmembrane molecules, such as low-density lipoprotein receptor-related protein 1 (LRP1). It can mediate intracellular signalling by focal adhesion kinase (FAK), Src, Jak–STAT, PI3K, Rac and Akt, many of them known to be involved in cell proliferation. (Andreasen *et al.*, 1997; Aguirre Ghiso, 2002; Das *et al.*, 2003; Mahmood *et al*., 2018).

The plasminogen activator inhibitors, PAI-1 and PAI-2 belong to the serpin superfamily (serine proteinase inhibitor) and can cause the neutralization of uPA, forming a stable complex with it. PAI-1 is a 50 kDa glycoprotein and is primary physiological uPA inhibitor, while the roles of PAI-2 are still under investigation. In its active form, PAI-1 is able to bind vitronectin (VN), present in the extracellular matrix, stabilizing it in its active form. The high level of transcription of PAI-1 is stimulated by TGF-β that binds to the TGF-β-responsive sequence on the PAI-1 promoter (Irigoyen *et al.*, 1999). Both inhibitors react with uPA, but not with pro-uPA (Blasi *et al*., 1987; Andreasen *et al.*, 1997; Mahmood *et al*., 2018).

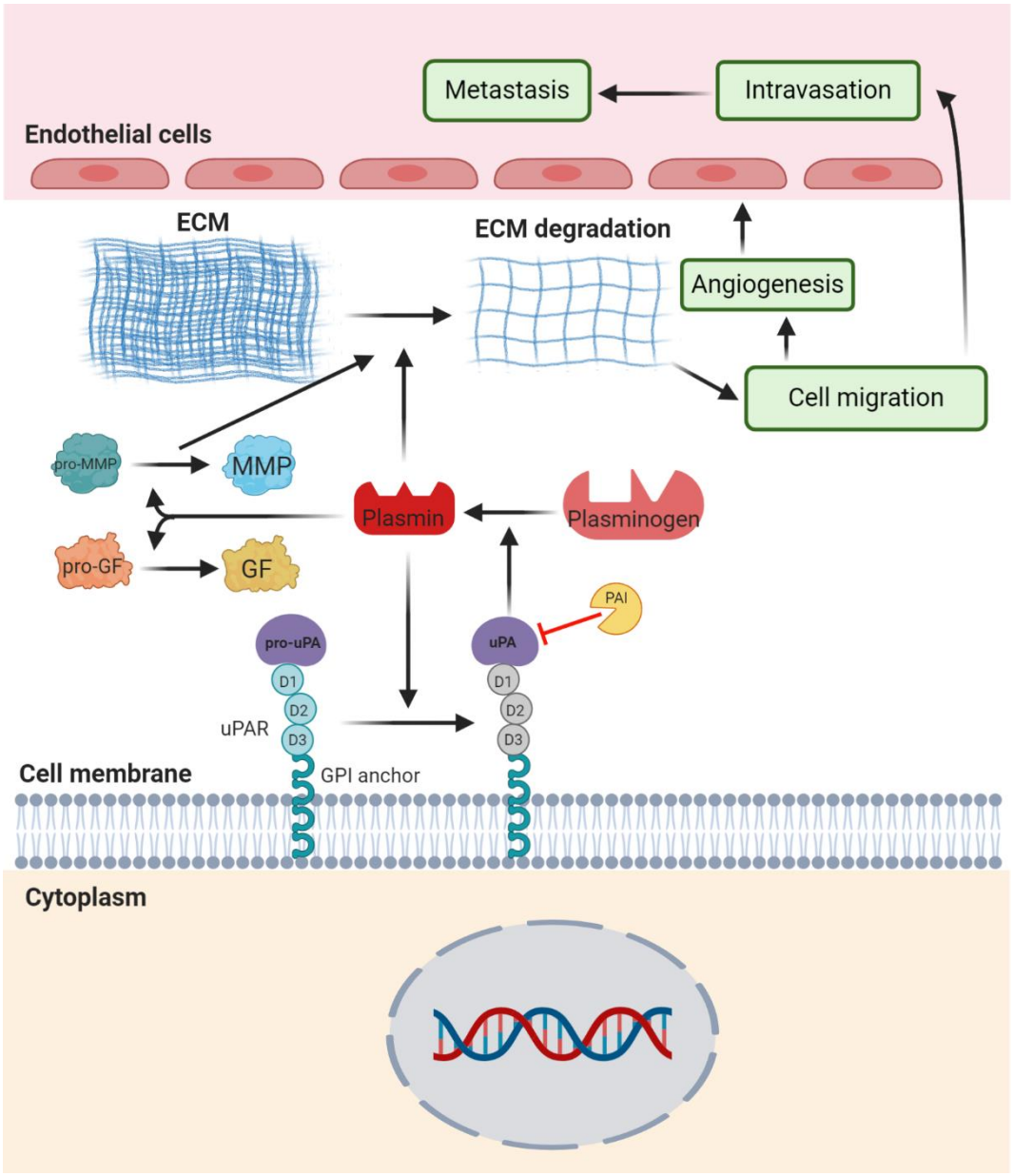


**Figure 1. uPA–urokinase-type plasminogen activator receptor (uPAR)-mediated pathways.** The GPI-anchored receptor uPAR consisting of three domains binds the zymogen pro-uPA and active uPA. The catalytically active form of uPA converts inactive plasminogen into plasmin, it can cleave and activate growth factors (GF), matrix metalloproteases (MMPs), and extracellular matrix (ECM). Plasminogen activator inhibitor-1 can inhibit the catalytic activity of uPA. *(Created with BioRender.com)*

### 1.2 Physiological Role of the uPA-uPAR System

Several important functions have been assigned to uPA–uPAR system (Figure 2). The plasminogen activator (PA) system's initial role was thought to be the degradation of thrombin clots, through activation of plasminogen or fibrinolysin, but today it is known that the plasminogen activator system has further functions such as in embryogenesis, angiogenesis, cell migration, wound healing, immune response, as well as in apoptotic cell death. Furthermore, the PA system can influence tumor growth, angiogenesis, tumor cell invasion, migration, and metastasis. The main activity of the uPA-uPAR system is the degradation of fibrin through activation of plasmin, as well as several blood clotting factors, for instance those in menstrual bleeding and extracellular matrix (ECM), thereby playing an important role in wound healing and tissue remodelling (Mahmood *et al*., 2018). Furthermore, the uPA-uPAR system also acts, directly or indirectly, as an activator of some growth factors and cytokines involved in myelopoiesis (for example TGF-β and interleukin-1). Proteolytic cleavage by urokinase at single site is required to activate hepatocyte growth factor / dispersion factor as reported by Naldini *et al.* in 1992.

The plasminogen activator system plays a role during post-lactational mammary gland involution, as well as in the proteolytic degradation of the follicle wall at the time of ovulation (Strange *et al.*, 1992; Ny *et al.*, 1997). The increased activity of uPA toward the blastocyst stage supports uPA's role in the implantation process and embryogenesis (Berg *et al.*, 1992; Aflalo *et al.*, 2004). Also, the uPA system is involved in the male reproductive system function where it improves sperm motility, stimulates acrosomal reaction and supports fertilization (Qin *et al*., 2015).

Urokinase is also used clinically as a thrombolytic agent in the treatment of severe or massive deep venous thrombosis, peripheral arterial occlusive disease, where it is administered directly to the site of the clot, pulmonary embolism, chronic kidney disease, acute myocardial infarction (AMI, heart attack), and occluded dialysis cannulas (catheter clearance) (Kunamneni *et al*., 2008; Zhang *et al.*, 2008; Horowitz *et al*., 2019).

Migration of endothelial cells occurs in vivo during the repair of vascular lesions, and in vitro, it can be stimulated by wounding the confluent monolayer of cells. This experiment was conducted by Pepper *et al.* (1987), which concluded that cells migrating from the ends of the experimental wound show high levels of uPA activity, which decreases to low levels after wound closure. The degradation and remodelling of the extracellular matrix is crucial during

tumor growth, tumor cell invasion, and metastasis. In order to escape the primary site of origin and migrate to distant tissues through the bloodstream, tumor cells break down the ECM using various proteases. These proteases include plasmin, whose activity is controlled by uPA, cathepsins, and various types of matrix metallopeptidases (MMPs) (Mahmood *et al*., 2018).

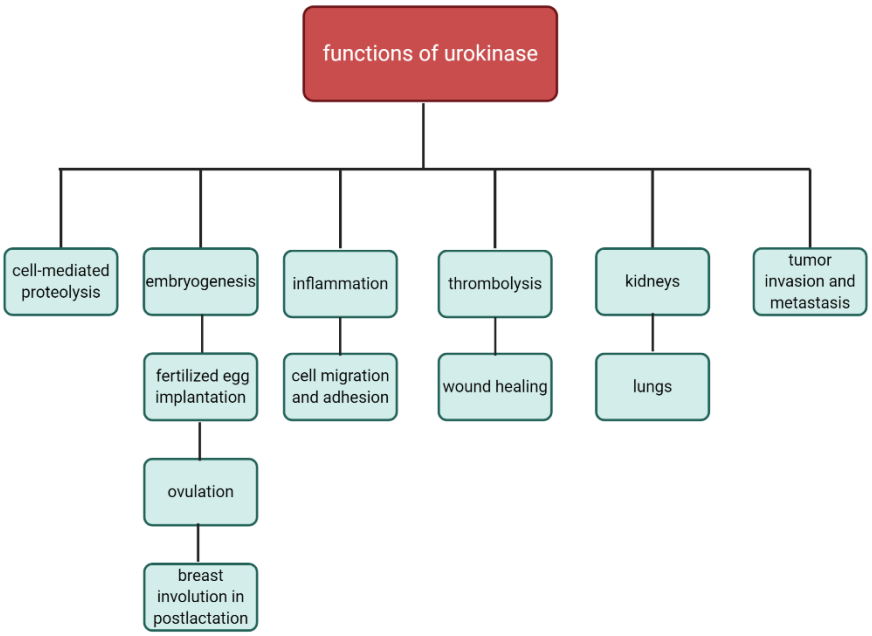


**Figure 2. Physiological and pathological roles of the urokinase-type plasminogen activator system.** In normal physiologic conditions, apart from fibrinolysis, the system takes part in different biological processes such as tissue remodelling, embryogenesis, involution of the breast in post-lactation, ovulation, implantation of a fertilized egg in the uterus; inflammation, cell migration and adhesion; thrombolysis and wound healing; in kidneys elevated levels are found in proteinuria, whereas overexpression of uPA in lungs protects against pro-fibrotic damage; it is also involved in tumor invasion and metastasis (Saleem, 2018).

### 1.3 Regulation of the uPA System

The primary function of uPA is the cleavage of plasminogen into plasmin, a protease that can degrade the ECM and activate collagenase. Since plasmin is a potent protease with a broad spectrum of substrates, its strict regulation by activation of a urokinase plasminogen activator is required. Thus, the uPA system is regulated at the level of urokinase gene expression, activity

localization, protein inhibition, and degradation (Aguirre Ghiso *et al.*, 1999; Irigoyen *et al.*, 1999). Also, urokinase inhibitors PAI-1 and PAI-2 have complex regulation at the level of transcription, activation and mRNA degradation (Nagamine *et al*., 2005). Further research shows that uPA activity can be determined by the balance between uPA and PAI-1 and regulated via master transcription factors of epithelial-mesenchymal transition (Sanchez-Tillo *et al*., 2013). The dominant regulation of the uPA gene depends on mitogen-activated protein kinases (MAPK) and phosphatidyl-inositol-3 kinase (PI3K) survival pathways. The PAI-1 inhibitor is also regulated via the signalling pathway of Transforming growth factor beta (Irigoyen *et al*., 1999).

Urokinase is encoded by the *PLAU* gene whose transcription is regulated by transcription factor Sp1, responsible for the constitutive expression of uPA in different human cell types. Transcription of Sp1 and its binding to DNA can be increased by phosphorylation via the MAPK, Erk and JNK pathways (Beneasciutti *et al.*, 2004). Beside this proximal promoter region, there are also two regulatory regions, highly conserved in the porcine, mouse, and human genome, which are activated by phorbol esters or FGF-2. Both consist of Ets (PEA3) / $AP1_A$ and $AP1_B$ binding sites. AP1 site recognizes a complex of AP1 transcription factors, composed of Jun homodimers or Jun and Fos heterodimers, which binding to DNA is induced by phosphorylation via MAPK. Promoter also contains COM mediator region, with binding sites for various proteins known as uPA expression enhancers (Verde *et al*., 1988; Nerlov *et al*., 1991; D'Orazio *et al*., 1997) and several NF-κB-like factor binding sites, as well as negative regulation elements. The complex structure of uPA promoter makes it sensitive to different signalling pathways. Among them, the dominant are those of MAP kinases and survival pathways PI3K/Akt. MAPKs can be activated by a variety of signals including growth factors (FGF-2, HGF and IGF-1), cytokines (TNF and CSF-1), and by the cytoskeleton reorganization which in turn leads to activation of the FAK/Src/Ras/Erk pathway (Nagamine *et al*., 2005). In different cell lines urokinase was also induced by cAMP, NF-κB and Wnt pathways (Besser *et al*., 1996)

### 1.4 Dynasore-Dynamin Inhibitor

Dynamin is an intracellular GTPase protein which is essential for membrane fission of clathrin-coated vesicles formed during endocytosis, as well as vesicles that bud from the trans-Golgi network. It is a multidomain protein of approximately 100 kDa and consists of a GTPase module, a lipid-binding pleckstrin-homology (PH) domain, a GTPase effector domain (GED)

essential for self-assembly, and a proline/arginine-rich C-terminal segment (PRD). In endocytosis, an invagination of the plasma membrane is formed on clathrin-coated pits and dynamin polymerizes to form a helix around the neck of budding vesicles of the plasma membrane to release a vesicle from the membrane (Preta *et al.*, 2015). Dynasore is a GTPase inhibitor that rapidly and reversibly inhibits dynamin activity. It was first identified by Macia and colleagues by screening compounds for the ability to inhibit the GTPase activity of dynamin1, and thereby to inhibit endocytosis of the transferrin receptor and low-density lipoprotein receptor (LDLR) (Macia *et al.*, 2006). In addition to its crucial role in endocytosis (Kirchhausen *et al.*, 2008), dynamin is associated with actin polymerisation. It binds directly to actin filaments and aligns them into bundles (Gu *et al.*, 2010).

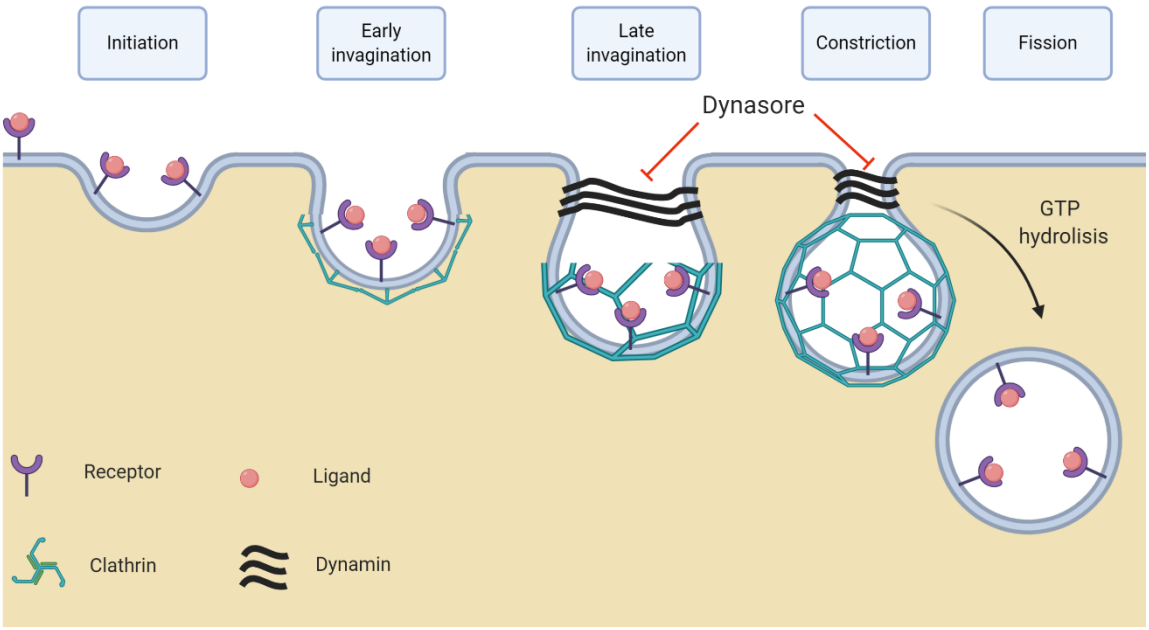


**Figure 3. Stages of clathrin-coated vesicle formation during endocytosis.** Endocytosis involves several stages: first the cargo is selected and a pit covered with clathrin is formed (initiation and early invagination). In late invagination, additional clathrin is recruited, which forms a mantle of hexagons and pentagons. In the constriction phase, dynamin at the vesicle neck forms a helical structure and induces a cut in the membrane by hydrolysis of GTP. The endocytic vesicle containing the cargo is formed in the fission phase. Dynasore inhibits the GTPase activity of dynamin, blocking the fission and endocytosis. *(Created with BioRender.com)*

### 1.5 Aim of the Study

The main aim of the thesis was to analyse how different types of mammalian cells regulate urokinase activity after treatment with various agents. Some chemotherapeutical agents used can induce DNA damage while the others were shown to be involved in a process of differentiation or influence cell cytoskeleton. For some of them it was shown on a glioblastoma cell line that they can modulate urokinase activity. Research was done on four human tumor cell lines (glioblastoma cell line A1235, breast cancer cell line MDA-MB-231, embryonic kidney cell line HEK-293 and fibroblast cell line MJ90 hTERT) and using five compounds (*all-trans retinoic acid*, *N*-[*N*-(3,5-Difluorophenacetyl-*L*-alanyl)]-*S*-phenylglycine t-butyl ester, *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine, sodium salicylate and dynasore).

## 2. MATERIALS AND METHODS

### 2.1 Cell Lines and Growth Media

Human cell lines used in the experiments were: A1235, a human glioblastoma cell line (a gift from S. A. Aaronson (Giard *et al.*, 1973)), MJ90 hTERT, an immortalized human fibroblast cell line, (a gift from I. Rubelj, PhD (Ruđer Bošković Institute, Zagreb, Croatia), HEK-293, human embryonic kidney cells and breast cancer cells (MDA-MB-231) (commercially available from the American Type Culture Collection (ATCC, USA)). Cells were grown in Petri dishes (CytoOne, Starlab, Germany) in Dulbecco's Modified Eagle Medium (DMEM, Sigma, USA) with the addition of 10% fetal bovine serum (Sigma) at 37 ° C and 5% $CO_2$ in humid incubator conditions (Hearaus, Germany).

### 2.2 Cell Growth Assessment

The crystal violet test was used for the cell growth assessment. Cells were seeded in a 96-well plate in multiplications with cell concentration of $3.5 \cdot 10^3$ cells per well. Several wells were left empty for blank. One day after seeding, the cells were treated with different agents and incubated for 24 hours. Every 24 hours one set of control and treated cells were fixed with methanol: the nutrient medium was removed, the cells were washed with phosphate-buffered saline (8 g of NaCl; 0.2 g of KCl; 1.44 g of $Na_2HPO_4$; 0.24 g of $KH_2PO_4$; HCl for adjusting pH to 7.4 and distilled water to a total volume of 1 l) and fixed with 50 µl of 100% methanol for 15 minutes. When all the cells were fixed, they were washed with PBS and 50 µl of 0.2% crystal violet solution was added to each well, including blank. After a 10 min incubation, the plate was washed with de$H_2O$ and air dried. The cells were detained in 100 µl 1% sodium dodecyl sulphate solution. Absorbance was measured at 600 nm using a microplate reading spectrophotometer (GloMax®-Multi Detection System, Promega, USA). The absorbance of the blank was subtracted from the obtained absorbance for each well and the relative value of absorbance in relation to the control was calculated.

### 2.3 Cell Treatment

Cells were treated with *all-trans retinoic acid* (ATRA) (Sigma), Dynasore (Sigma), N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine tbutyl ester (DAPT) MedChem Express, USA,

*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (a gift from B. Brdar, PhD Institute Ruđer Bošković) and sodium salicylate (Kemika, Croatia) at various concentrations. Dynasore and ATRA were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA). MNNG was dissolved in ethanol, and sodium salicylate in water.

### 2.4 Determination of Enzymatic Activity Using Radial Caseinolysis

Cells were seeded in 35 mm Petri dishes and treated with chemotherapeutics for 24 hours, when the medium was removed, the cells were washed with PBS and incubated in 500 µl of serum-free medium for 6 hours. The medium was then collected and stored at -80 ° C. Cells were scraped with a plastic scraper in 100 µl lysis buffer (137 mM NaCl; 20 mM Tris-HCl, pH 7.5; 2 mM EDTA, pH 8.0; 10% glycerol; 1% Triton X-100) and the cell lysate stored at -80 ° C. For the radial caseinolysis assay, an agarose plate containing plasminogen and casein was prepared (3% skimmed milk (Roth, Germany) 1% agarose (Sigma), 5 µg / ml human plasminogen (Sigma) and 0.1% sodium azide in PBS (Kemika)) (Matulić and Brdar, 2002). Serial dilutions of commercial uPA (Leo Pharmaceuticals, Denmark) ranging from 0.1 to 100 mU were prepared. 5 µl of the conditioned medium and serial dilutions of urokinase were put into the wells made in the agarose plate. Each sample was added in duplicates and each treatment had two biological replicas. The plate was incubated for 24 hours at 37 ° C, after that the plate was photographed and the diameters of the clear areas were measured in the ImageJ program (version 1.52a, USA). Commercial uPA was used to construct a calibration curve to determine by interpolation the uPA activity for each sample. Urokinase activity in each sample was expressed as mU urokinase/mg lysate protein and compared to corresponding control sample activity.

### 2.5 Measuring Protein Concentration

To quantify protein concentration in cell lysates, the Bradford protein assay was used. Cell lysates were diluted with $dH_2O$ with a dilution factor of 1:200 and 100 µl of Bradford reagent was added to 40 µl of each sample. Blanks were prepared by adding 100 µl of Bradford reagent to 40 µl of $dH_2O$. The Bradford reagent was prepared as described by the protocol for the Bradford analysis (Sambrook *et al.,* 1989). All measurements were made in duplicates. The absorbance of the samples was determined using a microplate reading spectrophotometer at 600 nm. The protein concentration in the samples was obtained by intrapolation from a calibrated

curve of bovine serum albumin measurements.

### 2.6 SDS-PAGE and Western Blotting

Cells were lysed in the lysing buffer in the presence of protease inhibitors (Roth, Germany), 1 µL protease inhibitor and 1 µL 100 mM PMSF in 100 µL lysis buffer. To concentrate the condition medium, 500 µl of medium was ultrafiltered using an Amicon® Ultra-0.5 device (Milipore, USA) and centrifuging at 14 000 × g for 10 minutes. 50 µg of cell lysate proteins and ultrafiltered conditioned medium were electrophoresed on a 10 % SDS-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE) using Mini PROTEAN3 Cell system (Bio-Rad Laboratories, USA) and transferred onto a PVDF membrane. The buffer for electrophoresis was composed of 25 mM Tris-HCl, pH 8.5; 120 mM glycine and 2 mM SDS. The resolving gel consisted of 2.5 ml 30 % w/v acrylamide; 1.9 ml 1.5 M Tris-HCl, pH 8.8; 75 µl 10 % SDS; 75 µl 10 % APS and 5 µl 10 % TEMED; 2.95 ml de$H_2O$. The membrane was blocked with 3% non-fat dry milk in TBST buffer (150 mM NaCl; 20 mM Tris-HCl, pH 7.5; 0.1% Tween 20), and probed with anti-uPAR (Cell Signaling Technology, Netherlands), anti-uPA (Cusabio Technology, USA), anti-PAI-1 (Becton Dickenson, USA) and β-actin antibodies (Santa Cruz Biotechnology, USA) diluted 1:1000. Secondary antibodies (anti mouse for PAI-1 and beta actin and antirabbit for uPA and uPAR) were from Sigma, diluted with 0.3% milk solution in TBST in a ratio 1: 30000. ECL method was used for protein detection (Sambrook *et al.*, 1989). After electrophoresis, the proteins from the gel were transferred onto the PDVF membrane in transfer buffer (25 mM Tris base; 192 mM glycine; 0,1% SDS; 20% methanol; pH adjusted to 8.3 using HCl), on 300-380 mA for 2 h. Secondary antibodies labelled with horseradish peroxidase were detected by a chemiluminescent reaction involving luminol, a substrate and a strong oxidizing agent (hydrogen peroxide) (reagents A and B, Bio-Rad Laboratories, USA). Strong emissions at 450 nm were captured with an X-ray film. Finally, films were densitometrically analysed using ImageJ program.

### 2.7 Statistical Analysis

Statistical analysis of obtained data was performed using Student t-test via Microsoft Excel computer program. Statistically significant values are those that differed at $p < 0.05$ level.

# 3. RESULTS

## 3.1 Urokinase Activity in Different Cell Lines after Treatment with Chemotherapeutics

As previous studies have shown that treatment with different chemotherapeutics and inhibitors can increase or inhibit urokinase activity in human glioblastoma A1235 cells, the aim of this study was to determine the effect of drugs on the urokinase-type plasminogen activator system in other human cell lines. We first analysed the urokinase activity. Human glioblastoma A1235 cells, were previously shown to increase urokinase activity after treatment with *all-trans retinoic acid* (Horvat *et al.*, 2019), sodium salicylate (Matulić and Brdar, 2001) and alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Brdar and Matulić, 1988). We compared their response with breast cancer cells MDA-MB-231, embryonic kidney cells HEK-293 and immortalized human fibroblast cells MJ90 hTERT. All cells were also treated with Notch signaling inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and dynamin inhibitor dynasore. The latter was used because the previous studies have demonstrated that cytoskeletal reorganization can influence the urokinase activity (Botteri *et al.*, 1990; Irigoyen *et al.*, 1997; Irigoyen and Nagamine, 1999).

Cells were treated with 10 μM ATRA, 25 μM DAPT, 10 μM MNNG, 15 mM sodium salicylate, and 40 μM dynasore for 24 h and the uPA activity was determined in the conditioned media by radial caseinolysis.

All four cell lines had basal uPA activity. A1235 cells increased the uPA activity after treatment with ATRA for almost 5 folds compared to untreated control cells (Figure 4A). Both treatment with MNNG and sodium salicylate increased uPA activity for ~ 2 folds comparing to control. 80% inhibition in uPA activity was observed after treatment with dynasore. DAPT did not influence the uPA activity.

MDA-MB-231 cells slightly increased uPA activity after treatment with ATRA but decreased after DAPT treatment. Sodium salicylate and dynasore treatment inhibited uPA activity. Sodium salicylate inhibited uPA activity by 80%, and dynasore by 47%. MNNG did not influence the uPA activity (Figure 4B).

HEK-293 cells did not show significant changes in uPA activity after treatment with DAPT, MNNG, sodium salicylate and dynasore (Figure 4C).

MJ90 hTERT cells are human fibroblasts immortalized by telomerase expression. While normal

fibroblasts did not show significant uPA activity (unpublished results), MJ90 hTERT expressed urokinase. These cells showed increase in urokinase activity after treatment with ATRA and DAPT for 2 and 1,5 folds, respectively, compared to control (Figure 4D). Treatment with MNNG, sodium salicylate and dynasore did not have effect on uPA activity.

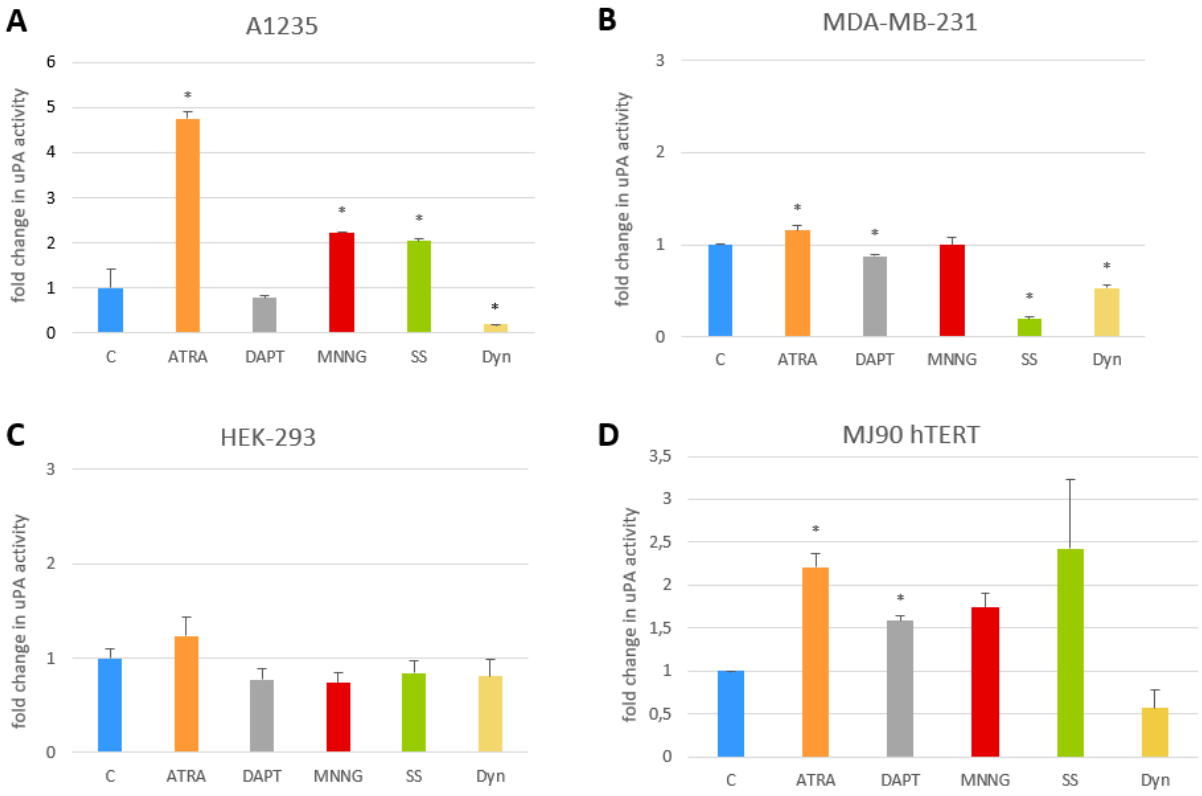


**Figure 4. Urokinase activity in different cell lines after treatment with various chemotherapeutics and inhibitors.** Cells were treated with 10 µM ATRA, 25 µM DAPT, 10 µM MNNG, 15 mM sodium salicylate and 40 µM dynasore for 24 h, and urokinase activity was determined by caseinolysis in conditioned medium and expressed as mU urokinase per µg protein. Results are presented as proportion to untreated control values. **(A)** glioblastoma cell line A1235; **(B)** human breast cancer cell line MDA-MB-231; **(C)** human embryonic kidney cell line HEK-293; **(D)** fibroblast cell line MJ90 hTERT. C: untreated cells; ATRA: all-trans retinoic acid; Dyn: dynasore, GTPase inhibitor; DAPT: N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine tbutyl ester, γ-secretase inhibitor; MNNG: N-Methyl-N′-nitro-N-nitrosoguanidine; SS: sodium salicylate. Data are expressed as mean ± SD. * The mean values were significantly different from the control ($p < 0.05$). Experiments were repeated two times, and representative results are shown.

### 3.2 The Effect of Dynasore on Inhibition of Urokinase Activity

As dynasore treatment led to inhibition of urokinase activity in A1235 cells, further studies were performed by combining treatment of cells with dynasore and those substances that led to a statistically significant increase in uPA activity. The urokinase activity was determined in serum-free conditioned media using radial caseinolysis.

Results (Figure 5) demonstrate that dynasore treatment decreased upregulated uPA activity obtained by treatment with ATRA, MNNG and sodium salicylate alone. Dynasore inhibited uPA activity for ~ 50% compared to activity in cells treated with ATRA alone but did not significantly influence the uPA activity increased by MNNG and salicilate treatment.

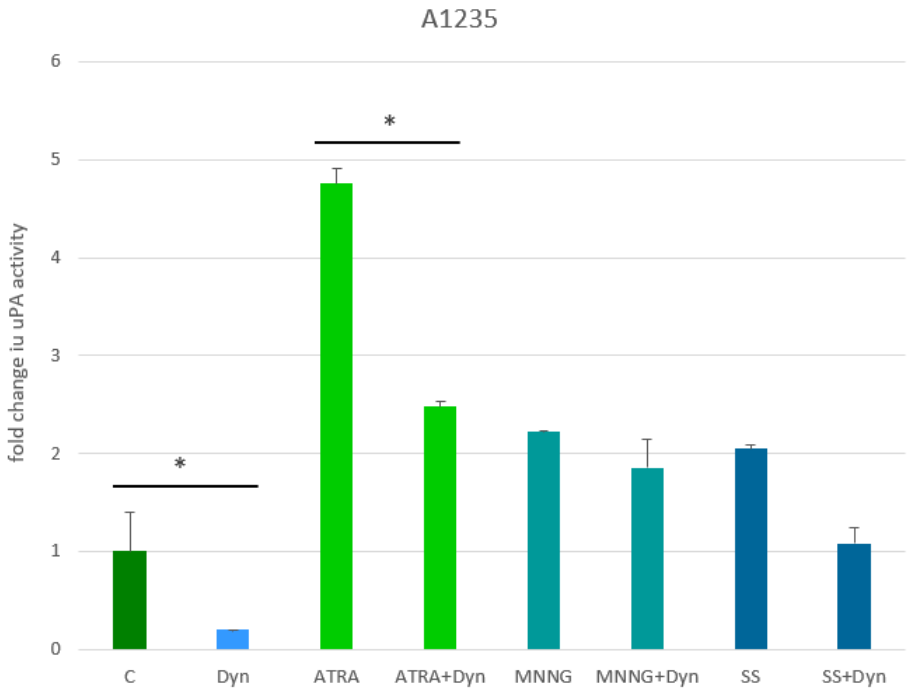


**Figure 5. Urokinase activity of A1235 cells after combined treatment with dynasore and other agents.** Cells were treated with 40 μM dynasore, 10 μM ATRA, 10 μM MNNG and 15 mM sodium salicylate Relative urokinase activity was determined by caseinolysis in conditioned medium, expressed as mU urokinase per mg protein and presented as a fold change in PA activity in comparison to untreated control cell values. C: untreated cells; ATRA: all-trans retinoic acid; Dyn: dynasore, GTPase inhibitor; MNNG: N-Methyl-N′-nitro-N-nitrosoguanidine; SS: sodium salicylate. Data are expressed as mean ± SD. * The mean values were significantly different from the control ($p < 0.05$). Experiments were repeated two times, and representative results are shown.

### 3.3 The Effect of Dynasore Inhibition on A1235 and MJ90 hTERT Cell Growth

To investigate whether inhibition of dynamin can affect the cell proliferation, two cell lines, glioblastoma A1235 and fibroblast MJ90 hTERT, were treated with dynasore and monitored for several days. The cell growth was assessed by crystal violet staining. A1235 cells treated with dynasore (Figure 6A) continued to proliferate at almost the same rate as the control cells. On the other hand, when treated with dynasore, MJ90 hTERT cells showed decreased proliferation on the last day of treatment (Figure 6B).

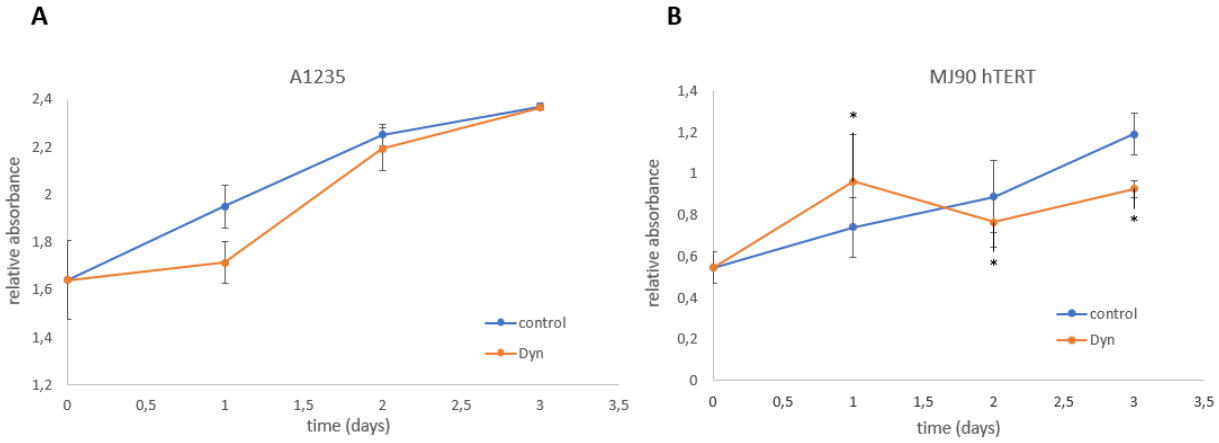


**Figure 6. Growth curve of A1235 and MJ hTERT cells treated with dynasore.** Cells were seeded on a 96-well plate and treated with 40 µM dynasore and each day one set of cells was fixed. Cells were stained with crystal violet and cell growth was determined by absorbance measurement at 600 nm. **(A)** A1235 cells; **(B)** MJ90 hTERT cells. Control: untreated cells; Dyn: dynasore. Data are expressed as mean ± SD. * The mean values were statistically significantly different from the control ($p < 0.05$).

### 3.4 The Effect of ATRA and Dynasore Inhibition on the Urokinase-Type Plasminogen Activator System Proteins

Since urokinase activity is regulated by the expression of uPA and PAI-1 inhibitor at the RNA and protein levels (Konakova *et al.*, 1998), the expression of uPA system proteins in A1235 and MJ90 hTERT cells after treatment with retinoic acid and dynasore was analysed. Cells were treated with ATRA and dynasore for 24 hours. Protein expression was analysed in cell lysates and concentrated conditioned media by Western blot.

uPA protein was detected in both cell lines, in lysates and conditioned media, after ATRA treatment. In A1235 cells' lysates, uPA expression was decreased after both ATRA and

dynasore treatment (Figure 7A). In conditioned medium, ATRA treatment increased uPA amount (Figure 7B). Considering PAI-1, it showed nearly the same expression in control and ATRA treated cells in lysates and medium. Dynasore decreased the expression of PAI-1 in the lysates. In the conditioned media from A1235 cells, uPAR was also detected. In MJ90 hTERT cells ATRA and dynasore increased the expression of uPA in lysates (Figure 7C), while in media no differences were detected. Also, ATRA and dynasore increased PAI1 expression in cell lysates, while the PAI-1 expression in the medium was on the control level (Figure 7D).

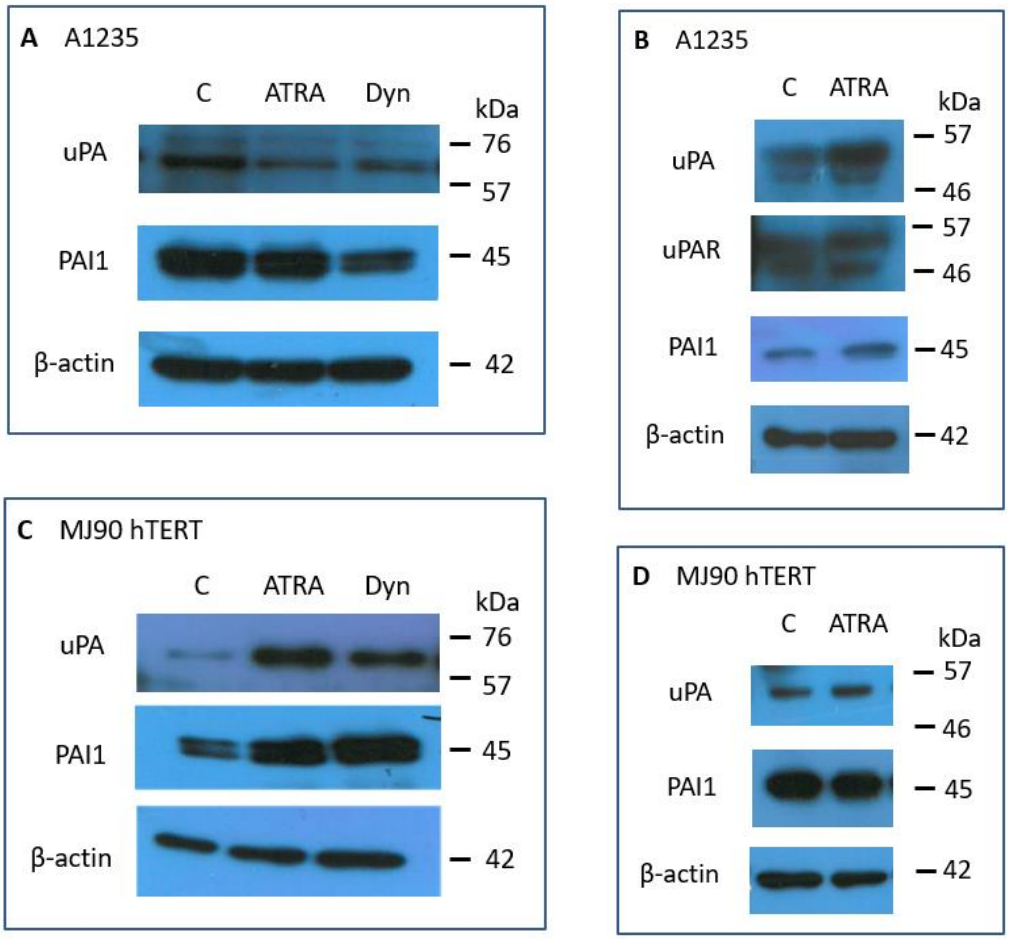


**Figure 7. Western blot analysis of uPA, PAI-1 and uPAR in A1235 and MJ hTERT cells after treatment with ATRA and dynasore.** Cells were treated with 10 μM ATRA and 40 μM dynasore for 24 h and proteins from cell lysates and conditioned media were immunoblotted against indicated antibodies. **(A)** Analysis of A1235 cell lysate; **(B)** analysis of A1235 conditioned media; **(C)** analysis of MJ90 hTERT cell lysate; **(D)** analysis of MJ90 hTERT conditioned media. C: untreated cells; ATRA: all-trans retinoic acid; Dyn: dynasore; β-actin: marker of the protein amount**.**

## 4. DISCUSSION AND CONCLUSION

The urokinase plasminogen activator (uPA) system, comprised of serine protease uPA, its receptor, uPAR, and inhibitor, plasminogen activator inhibitor-1 (PAI-1) has an important role in the break-down of extracellular matrix (ECM), fibrinolysis, inflammation, cell migration and invasion, as well as in tumor metastasis formation. On the other side, the system is also involved in signal transduction: binding of urokinase to its receptor activates a number of intracellular regulatory pathways which also include positive and negative feedback circuits and depend on the intracellular milieu in different cell types.

Since previous research has shown that treatment of tumor cells with different chemotherapeutic agents can increase or inhibit urokinase activity (Matulić and Brdar, 2001; Madunić *et al.*, 2017), the aim of the study was to analyze the influence of various agents (ATRA, DAPT, MNNG, sodium salicylate and dynasore) on the urokinase-type plasminogen activation system in different mammalian cell lines (glioblastoma A1235, breast cancer MDA-MB-231, embryonic kidney HEK-293 and fibroblast MJ90 hTERT).

All-trans retinoic acid (ATRA), a derivative of vitamin A, is a key regulator of gene expression involved in embryonic development and maintenance of epithelial tissues, therefore it is a differentiating agent. ATRA is a steroid molecule which binds to its intracellular receptor and thus regulates gene expression. Since recent research has shown an increase in urokinase activity in cells of neural origin (Horvat *et al.*, 2019), the effect on other cell lines has been investigated. ATRA treatment increased urokinase activity in A1235, MDA-MB-231 and MJ90 hTERT cell lines (Figure 4). The reason for this result could be induction of retinoic receptors (RARs) by retinoic acid which physically and functionally interact with transcription factor Sp1, resulting in uPA transcription (Suzuki *et al.*, 1999).

To analyze uPA system modification by ATRA, Western blot analysis of proteins was performed in conditioned medium and cell lysates. Namely, previous studies showed that urokinase activity after ATRA treatment in A1235 cells increased both, expression of uPA and PAI-1 mRNA, while on the protein level, there were no differences in uPA expression in lysates. The results confirmed that ATRA did not increase the protein level of uPA in lysates, but extracellular level was increased. The PAI-1 expression was not significantly changed in the lysate but was in the medium. In comparison, another neuroglioblastoma cell line H4, analysed by Horvat *et al.* increased uPA activity due to increase in uPA expression and downregulation of PAI-1, on the protein and RNA level.

MJ90 hTERT cells also increased uPA activity after ATRA treatment, but showed increase in both, uPA and PAI-1 expression on the protein level. Yet, different amounts of these proteins in extracellular medium were not detected, in comparison with untreated control. To resolve this question, additional experiments should be conducted.

N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT) is an inhibitor of the γ-secretase complex. Notch is a key target of γ-secretase, therefore DAPT inhibits the Notch pathway (Xiao *et al.*, 2014). Treatment of cells with DAPT decreased urokinase activity in MDA-MB-231 cells and increased it in MJ90 hTERT cells (Figure 4), while other cell types did not change urokinase activity. Results suggest that it is possible that urokinase regulation is a secondary response to the activation or inhibition of some other signalling pathways.

N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG), an alkylating agent, is a widespread environmental carcinogen that causes DNA lesions, leading to cell death. However, MNNG can also trigger a cell-protective response by inducing the expression of DNA repair/transcription-related genes. Brdar (1986) showed that cells without expression of enzyme that repairs MNNG induced DNA damage, $O^6$-methylguanine-DNA methyltransferase (MGMT), increase the activity of urokinase after alkylation. A1235 cells are one of these cells. Increase in urokinase activity was explained as consequence of JNK pathway activation induced by DNA damage (Parra *et al.*, 2000). In conducted experiments, other cell lines treated with low concentration of MNNG were MGMT positive, and therefore did not increase urokinase activity.

Sodium salicylate is a derivative of acetylsalicylic acid (Aspirin) which has been in long-term use as a non-steroidal anti-inflammatory drug due to its analgesic and anti-inflammatory properties. Aspirin acts as an inhibitor of cyclooxygenase, an enzyme involved in prostaglandin synthesis (Amann and Peskar, 2002). Sodium salicylate does not interact directly with cyclooxigenase and its effects are consequences of its influence on several signalling pathways. It can influence several signalling pathways possibly involved in the regulation of uPA system genes, such as MAPK and NF-κB (Schwenger *et al.*, 1997; Yin *et al.*, 1998). It was shown previously that it increases uPA activity in A1235 cells and decrease in breast cancer cells, and the same results were obtained (Matulić and Brdar, 2001; Madunić *et al.*, 2017). It was also shown that there were no changes in activity in HEK-293 embryonic kidney cells and MJ90 hTERT fibroblasts. Analysis of urokinase activity after treatment with sodium salicylate in several cell lines could indicate that the effect of sodium salicylate is cell specific.

In general, while A1235 and MDA-MB-231 cells showed modulation of the uPA system after

treatment with different agents, HEK-293 cells did not show any changes. These cells are found not to express uPAR (Wei *et al.*, 1996), indicating the importance of the whole system in response to agents.

As previous experiments indicated the possible role of cytoskeleton and endocytosis in the regulation of uPA activity, different cell lines were treated with dynasore, a dynamin inhibitor which blocks the formation of clathrin-coated vesicles and disrupts the actin cytoskeleton (Botteri *et al.*, 1990; Yamada *et al.*, 2009). The results of cell treatment with dynasore showed that it acted as an inhibitor of urokinase activity in two cell lines which otherwise showed agents-induced uPA modulation, namely A1235 and MDA-MB-231. When combined with other agents, in A1235 cells it modulated their effects and decreased the activity. Analysis on the protein level revealed that in A1235 cells dynasore decreased both, uPA and PAI-1 expression. Cell growth curve did not show dynasore effects cell proliferation (Kumazaki *et al.*, 2004; Zhong *et al.*, 2019).

HEK-293 cells and fibroblasts did not show significant changes in uPA activity, after dynasore treatment. On the other hand, analysis of uPA and PAI-1 expression showed that in fibroblasts dynasore induced upregulation of both, uPA and PAI-1 in the lysates, although their extracellular amount was the same as in untreated samples.

To conclude, treatment of different cell lines with several agents showed cell specific response in modulation of uPA activity. While glioblastoma A1235 cells increased the uPA activity after treatment with MNNG, ATRA, and sodium salicylate, and downregulated it after dynamin inhibition, MDA-MB-231 breast cancer cells downregulated activity after dynasore and sodium salicylate treatment and increased after ATRA. HEK 293 cells did not change uPA activity after different treatments. Fibroblast cell line increased uPA activity after ATRA treatment. Expression of proteins involved in uPA system showed even more complex cell specific regulation which requires further research.

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