Utjecaj klorpromazina na izražaj CD81 proteina u staničnim linijama središnjeg živčanog sustava

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Comentor: *Janja Tarčuković, PhD, MD*

SVEUČILIŠTE U RIJECI

ODJEL ZA BIOTEHNOLOGIJU

Diplomski sveučilišni studij

Biotehnologija u medicini

Vedrana Kauzlarić

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Mentor rada: *izv. prof. Kristina Grabušić, dipl. ing.*

Komentor: *dr. sc. Janja Tarčuković, dr. med.*

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2. Prof. Natalia Kučić, PhD, MD
3. Assoc. Prof. Kristina Grabušić, PhD
4. Janja Tarčuković, PhD, MD

The thesis has 34 pages, 11 figures and 49 literary citations.

Summary

This study examined the effects of chlorpromazine on the expression of CD81 in representative human cell lines of the central nervous system origin. Chlorpromazine is a neuroleptic that acts as a strong antagonist of dopamine receptors, which participate in processes like learning, memory, motivation, pleasure etc. Also, chlorpromazine affects many other membrane and cytosolic proteins, resulting in decreased communication between neurons and glial cells, especially astrocytes. Nevertheless, it has not yet been determined if chlorpromazine affects a specific form of intercellular communication that involves extracellular vesicles (EVs). EVs are membranous nanostructures that carry proteins, nucleic acids, lipids and other molecules. All types of cells release EVs and with them secretory cells can change the phenotype of recipient cells. The CD81 molecule is a member of the tetraspanin protein family ubiquitously expressed in all tissues, including the central nervous system. Next to the expression in plasma membrane, CD81 is present in intracellular membrane compartments and it is a recognized marker of EVs. In the nervous system, this protein is important for neuron-astrocyte interactions and is implicated in the response of astrocytes and microglia to trauma and disease.

In this study, the human glioblastoma cell line U-87 MG and neuroblastoma SH-SY5Y cells were treated with increasing concentrations of chlorpromazine (4, 8, 16 and 32 μg/ml) for 24 hours. Flow cytometry analysis of the CD81 expression has shown that chlorpromazine affects the examined cell lines in different ways. U-87 MG cells responded to chlorpromazine with a dose-dependent increase of CD81 protein level, while SH-SY5Y cells showed decreased CD81 levels. These findings are the first to date that associate chlorpromazine with changes in CD81 expression. Considering the role of this protein in neuron-astrocyte interactions and its presence in EVs, future studies are necessary to examine the effects of chlorpromazine treatment on processes important for intercellular communication in the nervous system.

Key words:

CD81, tetraspanin, extracellular vesicles, chlorpromazine, flow cytometry

Sažetak

U ovom radu ispitan je utjecaj antipsihotika klorpromazina na izražaj proteina CD81 u humanim stanicama porijekla iz središnjeg živčanog sustava. Klorpromazin je neuroleptik koji djeluje kao snažan antagonist dopaminskih receptora, koji sudjeluju u procesima poput učenja, pamćenja, motivacije, užitka i dr. Povrh toga, klorpromazin utječe na mnoge druge membranske i citosolne proteine što rezultira smanjenom komunikacijom između neurona i glija stanica, prvenstveno astrocita. Međutim, dosad nije opisano utječe li klorpromazin i na specifičan oblik međustanične komunikacije koji uključuje izvanstanične vezikule (IV-e). IV-e su nanostrukture omeđene membranom koje sadrže proteine, nukleinske kiseline, lipide i druge molekule. Izlučuju ih sve vrste stanica i njima sekretorne stanice mogu utjecati na fenotip primateljskih stanica. CD81 je član obitelji tetraspanina, transmembranskih proteina izraženih u svim tkivima, pa tako i u stanicama središnjeg živčanog sustava. Osim na plazmatskim membranama, CD81 prisutan je i na citosolnim membranskim odjeljcima te je prepoznat kao marker izvanstaničnih vezikula. U živčanom je sustavu CD81 uključen u interakcije neurona i astrocita te u odgovor astrocita i mikroglija-stanica na ozljedu i bolest.

U ovom radu stanične linije humanog glioblastoma (U-87 MG) i neuroblastoma (SH-SY5Y) tretirane su rastućim koncentracijama klorpromazina (4, 8, 16 i 32 μg/ml) tijekom 24 sata. Analiza protočnom citometrijom pokazala je da klorpromazin ima oprečno djelovanje na razine CD81 u ispitanim staničnim linijama. U stanicama U-87 MG dolazi do dozno-ovisnog povećanja CD81, dok kod SH-SY5Y stanica razina ovog proteina opada s porastom koncentracije klorpromazina. Ova saznanja prvi put povezuju klorpromazin i promjene izražaja CD81. Imajući na umu brojne uloge CD81 u interakcijama neurona i astrocita te prisutnost tog proteina na izvanstaničnim vezikulama, buduća istraživanja trebala bi podrobnije ispitati posljedice ovih promjena na procese međustanične komunikacije u živčanom sustavu.

Ključne riječi: klorpromazin, CD81, međustanična komunikacija, protočna citometrija

Acknowledgments

The elaboration of this thesis could not be accomplished without the guidance of my mentors, Assoc. Prof. Kristina Grabušić and Janja Tarčuković, PhD, MD for which I am sincerely grateful. I would also like to thank Ksenija Tulić for her assistance with the experimental work.

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Table of Contents

1. Introduction

1.1. Cells of the central nervous system

The human nervous system is a complex arrangement of tissues and cells specialized in communication. Anatomically, it can be divided into two main parts: the central nervous system (CNS) and the peripheral nervous system (PNS), as depicted in Figure 1 (3). The CNS consists of the brain and the spinal cord and it is protected by the skull and the spine (1). It functions as the central information processing and controlling unit of the entire body. The PNS is mainly comprised of peripheral nerves transmitting signals from all parts of the body to the brain and vice-versa. These nerves have cell bodies clustered in formations called ganglia. At the cellular level, the nervous system is composed of two major groups of neural cells: neurons and glial cells. The fundamental difference between the two lies in their electrical excitability– neurons can propagate electrical signals by generating action potentials, while glial cells are electrically non-excitable ([1]).

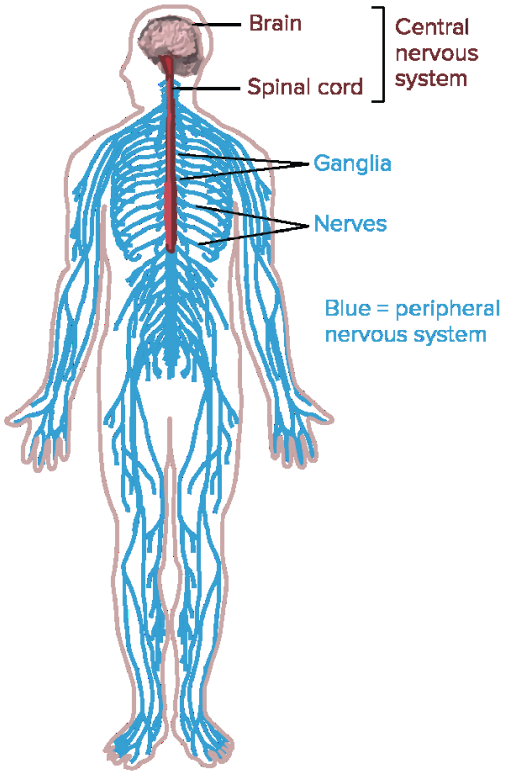


Figure 1. Overview of the human nervous system (3)

Neurons are the primary signal-processing unit of the nervous system. These highly polarized cells consist of three major parts: the cell body (soma or perikaryon), dendrites, and axons, as seen in Figure 2 ([2,3]). The cell body and dendrites are receptive domains, while axons specialize in signal transmission. Information is passed between neurons by combining chemical and electrical signals at the synapses, structures discussed in greater detail further on.

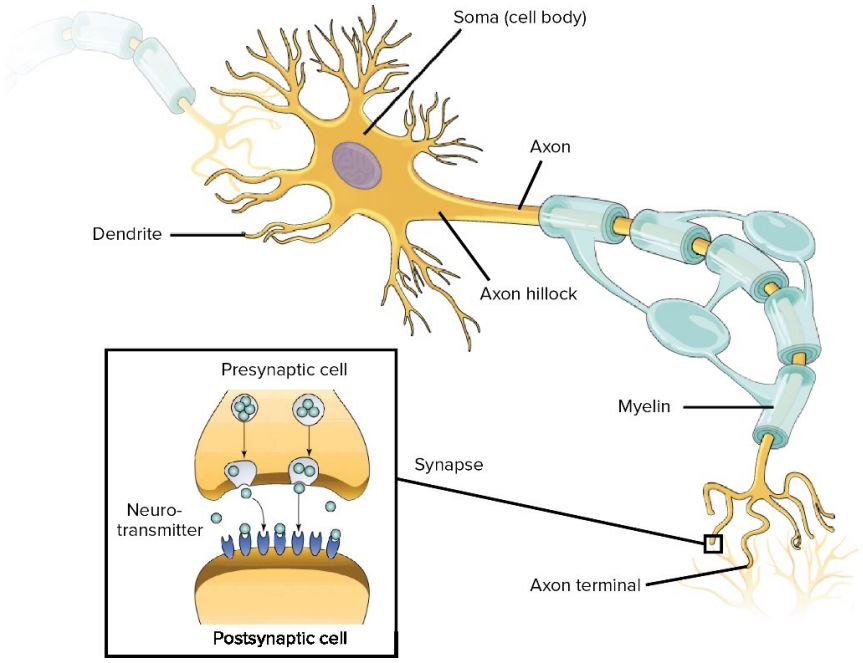


Figure 2. Structure of the neuron and the synapse (3)

There are four major types of glial cells: oligodendrocytes, astrocytes, microglia and ependymal cells (1). Oligodendrocytes produce myelin sheaths that wrap around neuronal axons and enable rapid signal conduction. Astrocytes take up 20-50% of the volume of most brain areas. They have multiple roles, the most prominent being the control of ion and neurotransmitter homeostasis and synaptic plasticity, specifically the regulation of the formation, maintenance and removal of synapses (4). Astrocytes extend from blood vessels to neurons, transport ions and other molecules, and regulate the extracellular milieu (5). They produce many

growth factors that can affect the morphology, proliferation, differentiation and survival of specific neuronal subpopulations. In addition, astrocytes can undergo a process called reactive astrocytosis in response to injury and disease, resulting in the formation of a glial scar. Microglia are the tissue macrophages of the CNS, the mediators of the immune response ([2]). They scan for signs of damage in the brain with their multiple processes and can respond quickly and dramatically. Peripheral immune cells are restricted from accessing the CNS due to the blood-brain barrier (BBB). This unique structure is comprised of specialized endothelial cells that tightly regulate the interchange of ions, molecules and cells between the blood and the brain (6). Such regulation is critical for normal neuronal function because signal transduction across neurons depends on the ionic composition in and around them ([2]). On the other hand, hormones from the circulatory system can also act as neurotransmitters and interfere with synaptic transmission. Astrocytes, microglia and nerve terminals are closely associated with the endothelial cells that form the BBB, and they have supporting roles in barrier function. The final group of glia cells are ependymal cells which line the ventricles in the CNS, which are cavities that produce the cerebrospinal fluid (CSF). Ependymal cells regulate the production of the CSF, and form the blood-CSF barrier (7). An illustration of the four main types of glial cells is shown in Figure 3.

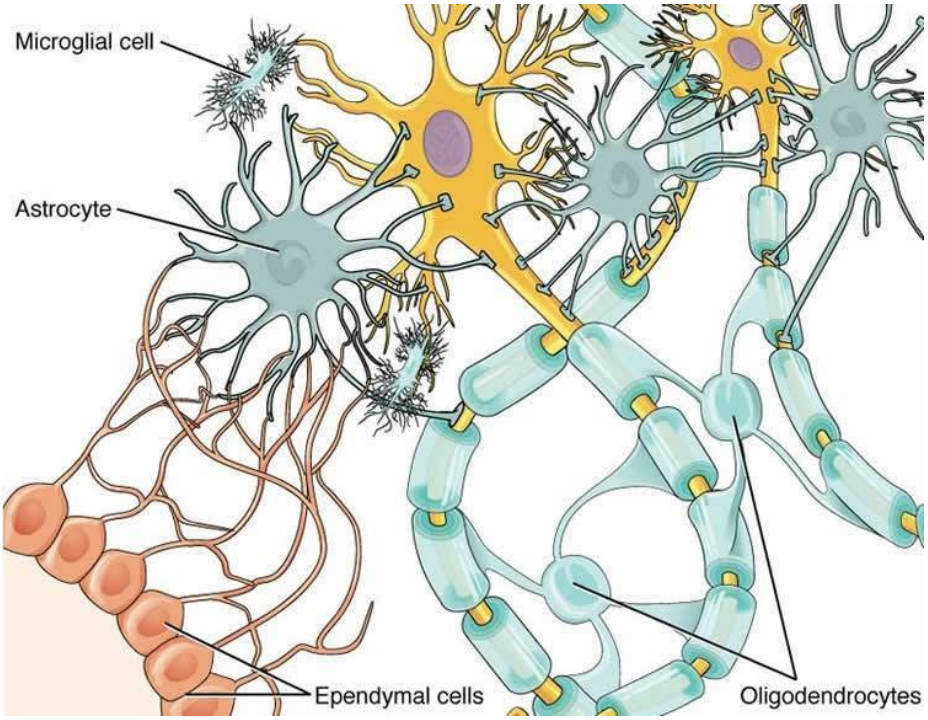


Figure 3. Illustration of different types of glial cells surrounding a neuron (8)

In summary, neurons are the main signal-processing and transmitting cells in the CNS, yet their function is highly reliant on the surrounding oligodendrocytes, astrocytes and microglia. Much remains unknown about the various roles glial cells have in physiological and pathophysiological states. A growing number of studies aims to integrate knowledge about neurons and glial cells, emphasizing the importance of their interconnectedness in the functioning of the nervous system.

1.2. Cell-to-cell communication in the CNS

1.2.1. Neuronal communication

Neuronal communication occurs at the synapse through a combination of chemical and electrical signals (9). Synapses are structures formed at the interface between axon terminals and target cells. Each synapse has a presynaptic element - the axon terminal, a synaptic cleft (approx. 20–50

μm wide), and a postsynaptic element - a dendrite, soma or another axon. Neurons receive information from other cells in the form of neurotransmitters, chemical signals that bind to specific receptors on target cells. The signal received by the postsynaptic neuron can be stimulatory or inhibitory, depending on the type of neurotransmitter released and its corresponding receptor. A neuron can simultaneously receive multiple impulses, both excitatory and inhibitory. These are processed in the cell body and cumulatively give rise to an electrical signal. In this way, the chemical information carried over by neurotransmitters translates into an electrical impulse which travels along axons. The diverse set of neurotransmitters in the CNS can be divided into: 1) small molecules such as glutamate, gamma-aminobutyric acid (GABA), glycine, acetylcholine, dopamine, serotonin, adenosine triphosphate (ATP) etc.; 2) neuropeptides such as endorphins; 3) lipid-based endocannabinoids; 4) gases such as nitric oxide, carbon monoxide etc. Glutamate and aspartate are the major excitatory neurotransmitters in the CNS, while GABA is the major inhibitory neurotransmitter in the brain.

The propagation of electric impulses along the axon depends on the electrical potential on cell membranes (10). The inside of the plasma membrane has an electrical charge of -70 to -80 mV with respect to the outside, due to selective membrane permeability to ions and different ionic concentrations in the extracellular and the intracellular space. The surface of neurons contains many ion channels which open after receiving a signal, resulting in membrane depolarization. The influx of ions briefly reverses the membrane resting potential to positive on the inside of the cell. This triggers the opening of more ion channels along the membrane and results in a depolarization wave, termed the action potential. The resulting electrical signal then travels from the initial segment of the axon (axon hillock) to the axon terminal ([2]). When the action potential reaches the axon terminal, it leads to an increase in intracellular calcium levels. This causes the fusion of neurotransmitter-filled synaptic vesicles with the plasma membrane and the

release of their content into the synaptic cleft. Once there, the neurotransmitters can cross the synaptic gap and bind to receptors on the membrane of the post-synaptic neuron, thereby completing the neuron-to-neuron signal transmission.

1.2.2. Astrocytic signalling

Glial cells have historically been considered as passive support to neurons, a position that has changed with the advancement of research methods ([1]). Based on the observed communication between astrocytes and neurons at the synapse, this view has been replaced with the notion that glial cells actively participate in information processing in the brain. A single astrocyte makes contact with a large number of neurons at a structure called the tripartite synapse. The function of these contacts is to regulate synaptic transmission by exchanging information with the synaptic neurons. Moreover, these intercellular interactions at the tripartite synapse have been implicated in the regulation of synaptic plasticity, the ability of the synapses to strengthen or weaken in accordance with neural activity (11). Astrocytes express neurotransmitter receptors and can register neuronal activity through them ([1]). They respond by increasing intracellular calcium levels and releasing gliotransmitters: excitatory and inhibitory amino acids like glutamate, aspartate, GABA and glycine; ATP, eicosanoids and prostaglandins, neuropeptides, neurotrophins and a range of cytokines. The gliotransmitters can bind to extrasynaptic receptors on both the presynaptic and postsynaptic neurons and modulate their activity (11).

1.2.3. Extracellular vesicles as mediators of intercellular communication

Cell-to-cell communication in the CNS is more diverse than the most well-known neuron-to-neuron signalling. Emerging studies are uncovering complex interactions between multiple cell types and mechanisms of information exchange in the brain. One newly proposed mechanism of cell-to-cell communication takes place via membranous vesicles they secrete (12). Extracellular vesicles are membranous nanostructures produced by almost all types of cells and present in all bodily fluids, including the cerebrospinal fluid. They encapsulate proteins, lipids and nucleic acids in a phospholipid bilayer. Initially, cells were thought to release EVs to dispose of unneeded or harmful content, but accumulating evidence points to their important role in cell-to-cell communication (13). EV cargo serves as a functional messenger involved in cellular division, survival, differentiation, response to stress and apoptosis, and can lead to a change in the phenotype of the recipient cell. As such, EVs are involved in physiological as well as pathophysiological processes and could hence serve as potential diagnostic and therapeutic targets (14).

Based on their biogenesis and size, EVs can be divided into exosomes, microvesicles and apoptotic bodies. Exosomes are reported to be between 30 nm and 100 nm in diameter, and carry specific subsets of proteins on their surface, including the tetraspanin cluster of differentiation 63 (CD63) and CD81 (15). They are generated by the endosomal system via endocytic pathways, as seen in Figure 4 (16). The intracellular precursor organelles of exosomes are called multivesicular bodies (MVBs). MVBs undergo membrane invagination resulting in the formation of intraluminal vesicles (ILVs). This MVB complex gets trafficked to the plasma membrane, and its subsequent fusion results in the secretion of ILVs in the form of exosomes. Microvesicles (100 nm to 1 μm) are reported to emerge via direct budding from the plasma membrane (13). They are more heterogenous than exosomes with respect to surface markers, and there is no known specific

marker defining them. Finally, apoptotic bodies or blebs are produced by cells undergoing apoptosis and range from 1 μm to > 2 μm in diameter. They can be identified via DNA and histone detection. Although the terms "exosomes" and "microvesicles" are commonly used throughout literature, it is now commonly accepted that there is much greater heterogeneity within these subgroups with no clear markers to distinguish them. The umbrella term "extracellular vesicles" (EVs) is recommended to avoid confusion.

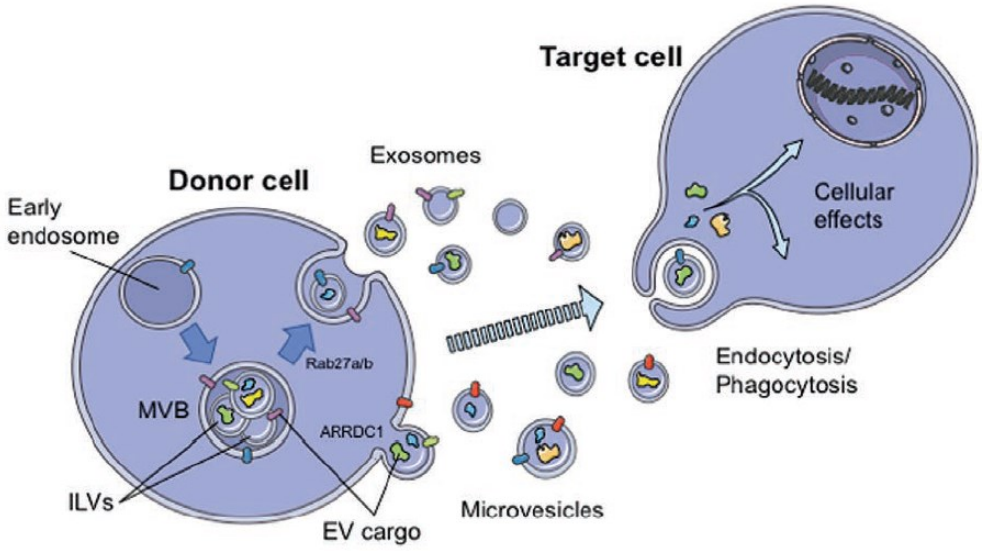


Figure 4. Illustration of EV formation, secretion and uptake. MVB = multivesicular body; ILVs = intraluminal vesicles (16)

The basis of intercellular communication via EVs is accomplished by two main mechanisms: 1) specific ligand-receptor interactions, and 2) vesicle cargo uptake into target cells through membrane fusion or vesicle internalization (18). Multiple studies point to endocytosis as the primary internalization mechanism, based on reduced EV uptake when cells are treated with endocytosis inhibitors. Nevertheless, conflicting evidence might suggest that EV uptake happens through multiple mechanisms, depending on the cell of origin, type of EVs and characteristics of the recipient cells.

1.2.4. Extracellular vesicles in the nervous system

Almost all cell types in the brain release EVs: neural stem/progenitor cells, neurons, astrocytes, oligodendrocytes and microglia (12). EVs are implicated in processes ranging from neural development, synaptic strength, plasticity and nerve regeneration to inflammation, neurodegeneration and other pathological processes. Recent studies cite the biomarker potential of EVs in a number of brain disorders, such as Alzheimer's disease or glioblastoma (12,16).

Exosome release from neurons reportedly depends on synaptic activity as they are shed upon depolarization and selectively bind to other neurons, suggesting involvement in neuron-to-neuron communication (12). Neuronal EVs are enriched with proteins related to synaptic plasticity and with activity-related miRNAs. EVs also facilitate the elimination of inappropriate synaptic connections which is important for the reorganization of neuronal circuits in the brain in physiological, as well as injury-related states. EVs in the brain are implicated in deleterious processes such as various neurodegenerative diseases, glioblastoma and inflammation. During brain injury, astrocytes in the injured tissue release ATP (excitatory neurotransmitter), and high levels of extracellular ATP induce microglia to release EVs carrying interleukin (IL)-1β, a key initiator of the acute inflammatory response (14). Astrocytes of the blood-brain barrier also respond to neuronal injury by releasing IL-1β enriched EVs, exacerbating tissue deterioration.

1.3. Tetraspanin proteins

1.3.1. General characteristics of tetraspanins

Tetraspanins are a superfamily of proteins also known as the transmembrane 4 superfamily (TM4SF), due to their characteristic four

hydrophobic domains that traverse cellular membranes (19). These proteins have two extracellular loops, one smaller (SEL or EC1) and one larger (LEL or EC2), with two short intracellular N- and C-terminal domains, as represented in Figure 5 on the example of CD81. They participate in various cellular functions, including adhesion, migration, signalling and pathogen infection (20). The tetraspanins are thought to be implicated in multi-molecular signal transduction complexes on the cell membrane.

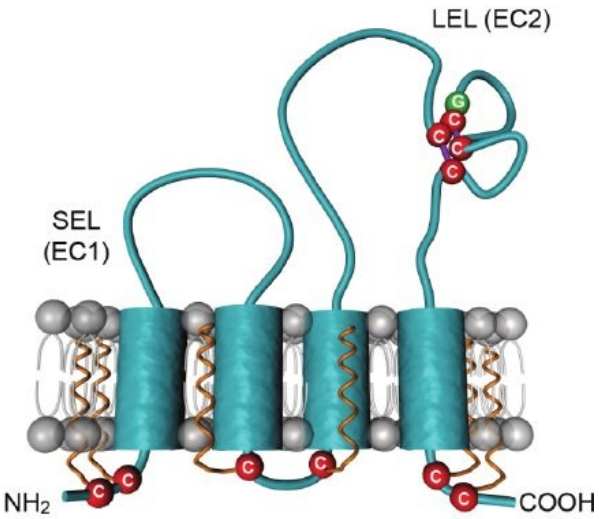


Figure 5. Schematic representation of CD81. Four transmembrane domains are characteristic for the tetraspanin family of proteins. CD81 has one small extracellular loop (SEL) and one large extracellular loop (LEL) (21)

Tetraspanins are abundantly expressed in the nervous system, and recent studies focus on their roles at the synapse (19). The tetraspanin interactions in the CNS currently being studied include the regulation of glutamatergic and dopaminergic signalling, receptor trafficking and other processes important in synaptic transmission and plasticity. For example, Tspan7 was found to form a part of a complex with the D2 dopamine receptor in the soma and along the neurites of neurons, and its silencing has led to upregulation of the surface D2 receptor (22).

Tetraspanins have been shown to associate with different partner proteins to form tetraspanin-enriched microdomains (TEMs) (19). One variant of

such partner proteins are integrins, cell adhesion molecules important in cell-cell or cell-extracellular matrix interactions, which points to the implication of tetraspanins in intercellular contacts and communication. Tetraspanin microdomains seem to participate in vesicular and cellular fusion, including EV-cell binding. The tetraspanins CD63, CD9 and CD81 are highly abundant on EVs. CD63 appears to be important for the biogenesis of lysosome-related organelles (23). CD9 knockout mice leads to defective exosome secretion in bone marrow dendritic cells (24), and treating dendritic cells with antibodies against CD81 or CD9 has led to a reduction in EV uptake (20). These and other studies point to the possible involvement of tetraspanins in EV trafficking.

1.3.2. The tetraspanin CD81

CD81 is also known as Target of the Antiproliferative Antibody 1 (TAPA-1) (25). This protein was found to be involved in a wide range of biological activities including cellular activation, proliferation, differentiation, cell-cell fusion, and adhesion). It is also implicated in infection by many pathogens. Its expression in humans is ubiquitous in many cell types, including neuronal and glial cells. CD81 is recognized as a marker of exosomes and is enriched on MVBs, their precursor organelles (26). The crystal structure of CD81 was recently published, revealing a specific cholesterol-binding site also observed in vitro (27). When cholesterol is not bound to CD81, this protein can more readily adopt an "open" conformation, and possibly favour binding to partner proteins. These speculations suggest a modulating role of cholesterol in CD81 function.

CD81 plays an important mediating role in neuron-astrocyte interactions and astrocyte reactivity. Astrocytes can re-enter the cell cycle, especially in response to trauma and disease. In homeostasis, the number of astrocytes remains mostly unchanged throughout life. This was proven to result from their direct contact with neurons (28). Specifically, CD81 was shown to be

a critical regulator of astrocytic proliferative arrest induced by neurons. This proliferative arrest is a process in which cells permanently leave the cell cycle and reach terminal differentiation. It is crucial for establishing and maintaining a proper ratio of cell types in the central nervous system. In a separate study, it was demonstrated that CD81-null mice have significantly enlarged brains compared to wildtypes (30% increase) as a result of an increased number of astrocytes and microglia, but not neurons (29). Also, when targeted with anti-CD81 antibodies, cultured rat astrocyte proliferation was strongly inhibited (25). Studies in rats have shown that after traumatic injury to the CNS, CD81 expression is increased in reactive astrocytes and microglia (30). These and other studies support the hypothesis that CD81 participates in cell-to-cell communication in the CNS and that its expression undergoes changes upon injury.

1.4. Antipsychotics

Antipsychotic drugs are generally classified into two major groups: the typical and atypical antipsychotics (31). Chlorpromazine and its derivates belong to a group of first generation or typical antipsychotics that exert their action mainly by targeting dopamine D_2 receptors. Many other antipsychotics and antidepressants were developed owing to that major discovery. Furthermore, typical antipsychotics were shown to inhibit EV uptake in vitro (32*)*. The second generation, atypical antipsychotics are derived from clozapine and act as strong D_2 and serotonin 2_A receptor antagonists. Some atypical antipsychotic drugs affect tetraspanin expression in vivo (33). Both typical and atypical antipsychotics have been shown to disrupt the intracellular cholesterol trafficking, resulting in the accumulation of lipids in the late endosome/lysosome compartment (34).

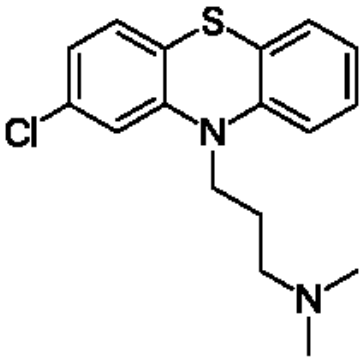


Figure 6. Structure of chlorpromazine. Created with ChemDoodle 8.0.0.3b and Adobe Illustrator CC 2015

Chlorpromazine is a dimethylamine derivative of phenothiazine – 10-(3-dimethylaminopropyl)-2-chlorphenothiazine, shown in Figure 6 (35). It is indicated for the treatment of schizophrenia, the manic manifestations of manic-depressive disorder, as pre-surgery relief for restlessness and anxiety, to control nausea and vomiting, for acute intermittent porphyria, for relief of intractable hiccups and as an adjunct in the treatment of tetanus. Its success in the therapy of schizophrenia is attributed to antagonistic action on the dopamine receptors of neurons. It blocks postsynaptic D_1, D_2, D_3 and D_4 subtypes of dopaminergic receptors (36). It also acts on serotonin receptors 5-HT1 and 5-HT2 with anxiolytic and anti-depressive effects. The combined effect of blocking D_2, histamine H_1 and muscarinic M_1 receptors result in reduced nausea and vomiting. Multiple studies show that chlorpromazine binds to several other proteins like calmodulin (CaM – intracellular Ca^{2+} receptor implicated in Ca^{2+}-mediated regulation of gene expression), certain channel proteins, DNA topoisomerase, K-Ras and other membrane proteins (37). Among other effects, it has been shown that chlorpromazine can affect endocytosis and inhibit internalisation of exosomes (38).

Chlorpromazine has varied effects in neuronal and glial cells. It was shown to exert cytotoxic effects in neuroblastoma cell lines IMR-32 and SH-SY5Y (39). It was also demonstrated to negatively affect the viability of

glioblastoma cells as well, specifically the U-87 MG cell line (37). Treatment with this antipsychotic reduced intercellular communication via gap junctions in mammalian neurons and astrocytes in vitro (40). Furthermore, a study in rats treated with chlorpromazine demonstrated a significant decrease in axosomatic synapses on neurons in the hypothalamus accompanied by an increase in astrocytic processes (41). These studies imply that chlorpromazine has a currently unexplained influence on basic aspects of cell-to-cell communication in the CNS. Despite almost 70 years of research into chlorpromazine mechanisms of action, much remains unknown and deserves more attention, especially considering the variety of molecular targets it affects.

2. Aim of the study

The aim of this study is to examine possible changes of CD81 expression in neural cells upon chlorpromazine treatment. The tetraspanin CD81 is present on plasma and intracellular membranes of cells and enriched on multivesicular bodies that give rise to exosomes. This protein is implicated in neuron-astrocyte interactions and upregulated in glial cells in response to injury. These findings suggest CD81 involvement in intercellular communication in the CNS. Chlorpromazine has a cytotoxic effect on human cell lines representative for neurons and glia, and it affects cell-to-cell communication with poorly understood underlying mechanisms. No research to date has described the effect of chlorpromazine on tetraspanin expression.

The chlorpromazine effect will be studied on two neural cell lines: U-87 MG cell line as a representation of glial cells and SH-SY5Y as a model for neurons. The specific objectives of this study are:

1) to determine cell viability of neural cell lines after exposure to increasing concentrations of chlorpromazine for 24 hours;

2) to examine the effect of different concentrations of chlorpromazine on CD81 protein levels in neural cells by flow cytometry;

3) to compare cellular responses to chlorpromazine and identify cells in which CD81 might be involved in intercellular communication.

3. Materials and Methods

3.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), *PAN-Biotech*, Aidenbach, Germany

Fetal calf serum (FCS), *PAN-Biotech*, Aidenbach, Germany

Saponin, *Carl Roth*, Karlsruhe, Germany

Chlorpromazine hydrochloride, *Sigma Aldrich*, St. Louis, USA

Ethanol, *Carl Roth*, Karlsruhe, Germany

Newborn calf serum (NBC) *PAN-Biotech*, Aidenbach, Germany

Potassium chloride (KCl), *Carl Roth*, Karlsruhe, Germany

Potassium dihydrogen phosphate (KH2PO4), *Carl Roth*, Karlsruhe, Germany

Sodium chloride (NaCl), *Carl Roth*, Karlsruhe, Germany

Disodium phosphate (Na_2HPO_4), *Carl Roth,* Karlsruhe, Germany

Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), *Carl Roth*, Karlsruhe, Germany

Sodium azide (NaN_3), *Carl Roth*, Karlsruhe, Germany

3.2. Solutions and buffers

BD Cytofix/Cytoperm™ Fixation and Permeabilization solution, *BD Biosciences*, Franklin Lake, USA

Chlorpromazine hydrochloride in ethanol, 1 mg/ml

Complete 10% cell culture medium DMEM: DMEM, FCS 10% (w/v), 2mM L-glutamine, penicillin-streptomycin 1000 i.u./ml, 0.11 mg/ml pyruvate

Gibco™ MEM Non-Essential Amino Acids 100x, *Thermo Fisher Scientific*, Waltham, USA

L-Glutamine, Powder, *PAN-Biotech*, Aidenbach, Germany

Penicillin-Streptomycin 10,000 U/ml Penicillin, 10 mg/ml Streptomycin, *PAN-Biotech*, Aidenbach, Germany

Phosphate-buffered saline (PBS), *PAN-Biotech*, Aidenbach, Germany

Saponin buffer: 1% saponin, 0,95 mM sodium azide (NaN_3), 1,9% NBC, 1x PBS

Sodium Pyruvate 100 mM, *PAN-Biotech*, Aidenbach, Germany

Trypsin-EDTA, *Sigma-Aldrich*, St. Louis, USA

Trypan Blue Solution 0,4% in PBS, *Mediatech, Inc*, Manassas, USA

Permeabilization Buffer 10X, eBioscience™ *Thermo Fisher Scientific*, Waltham, Massachusetts, USA

Flow cytometry buffer 1x: 0.15 M NaCl, 2 mM KH_2PO_4, 17 mM Na_2HPO_4, 4 mM KCl, 1 mM Na_2EDTA, 2 mM NaN_3

3.3. Antibodies

Primary antibody: polyclonal rabbit anti-human CD81, *Biorbyt*, San Francisco, USA

Secondary antibody: anti-rabbit Alexa Fluor-488, Molecular Probes, *Thermo Fisher*, Waltham, Massachusetts, USA

3.4. Cell lines

The U-87 MG glioblastoma cell line (ATCC HTB-14) was kindly provided by Hrvoje Šimić, PhD (Department of Neurosurgery, Faculty of Medicine,

University of Rijeka, Croatia). U-87 MG cells are epithelial-like adherent cells derived from brain tissue of a human male glioblastoma patient.

The SH-SY5Y neuroblastoma cells (ATCC CRL-2266) were kindly provided by Željko Svedružić, PhD (Department of Biotechnology, University of Rijeka, Croatia). This cell line is a subline of the neuroblastoma SK-N-SH cell line, established in 1970 from human bone marrow of a female patient with metastatic bone tumour. It is comprised of epithelial-like adherent and suspended cells.

3.5. Methods

3.5.1. Cell maintenance

U-87 MG cells were cultured in cell culture dishes (100 mm in diameter, Greiner, Frichenhausen, Germany) in a complete 10% DMEM medium and 0.1 µg/ml of non-essential amino acids (*Thermo Fisher Scientific*, Waltham, USA). SH-SY5Y were cultured in plastic flasks (surface area 25 cm^2, *Greiner*, Frichenhausen, Germany) in a complete 10% DMEM cell culture medium. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2.

The U-87 MG and SH-SY5Y cells were detached from the culturing dish/flask with trypsin-EDTA pre-warmed to 37°C, applied for 3 minutes or until visibly detached. The action of trypsin was blocked with pre-warmed complete 10% DMEM and the resuspended cells were transferred into FACS tubes (*BD Biosciences*, Franklin Lakes, New Jersey, USA).

3.5.2. Chlorpromazine treatment of U-87MG and SH-SY5Y cells

Cells were detached from the culturing dish/flask by trypsin-EDTA as described above. After centrifugation at 1800 rpm for 5 min at 20°C, cells were resuspended in 5 ml of fresh complete 10% DMEM. Cells were counted in the Neubauer chamber (*Marienfeld*, Lauda-Königshofen, Germany) as

described in 3.5.3. and transferred to 6-well tissue culture plates (*Greiner*, Frichenhausen, Germany) at concentration of 500,000 cells per well. The cells were incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2.

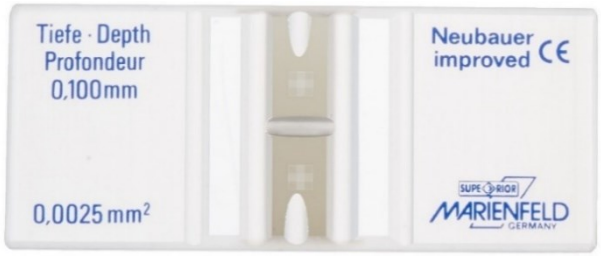
Prior to chlorpromazine treatment, the cell culture media was aspirated from the cells cultured on 6-well plates. The first set of cells was treated in duplicate with 16 and 32 µg/ml of chlorpromazine in fresh 10% DMEM. Non-essential amino acids were added to the media of U-87 MG cell line (0.1 µg/ml). The second set of cells was treated with lower concentrations of chlorpromazine (4, 8 and 16 µg/ml) for 24 hours in triplicate. This experiment was performed with approx. 500,000 cells per well for U-87 MG and 330,000 of SH-SY5Y cells per well.

3.5.3. Cell viability assessment

After 24 hours of chlorpromazine treatment, the cells were detached from the surface of the wells with trypsin, resuspended in 1 ml PBS and transferred to FACS vials (BD Biosciences, Franklin Lakes, New Jersey, USA).

Aliquots of 25 µl of cell suspensions in PBS were stained with 100 µl trypan blue. This dye can only enter dead cells which have compromised membrane integrity, while live cells remain unstained. Both live and dead cell count was performed in the Neubauer chamber (Figure 7). Their viability was estimated as the ratio of live cells to total number of cells (live and dead).

A



B

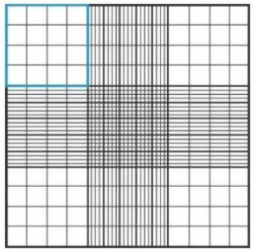


Figure 7. A) Neubauer chamber used for cell viability assessment; B) gridline indicating one of the sets of 16 squares used for counting cells[42]

The number of cells was calculated based on the following formula:

$$N = \frac{n}{4} \times f \times 10^4 \times V$$

N = calculated number of cells in a volume (*V*)

n = cells counted in 4 sets of 16 squares of the gridline, shown in Figure 7B

f = dilution factor; f = 5

3.5.4. Flow cytometry

After determining the cell count and viability in response to chlorpromazine treatment, the cells were fixed and permeabilized with Cytofix/Cytoperm Fixation and Permeabilization buffer for 20 minutes in order to examine both surface and intercellular expression of CD81. The cells were then rinsed with saponin buffer and centrifuged (2000 rpm, 2 min, 4°C). Cell pellets were resuspended in 300 µl saponin buffer, vortexed and divided into 3 FACS vials (100 µl of cell suspension per vial). This was followed by the staining procedure: negative control - no staining; control with the secondary antibody only (anti-rabbit Alexa Fluor 488, 8 µg/ml; 30 min, 4°C); double-stained - primary (anti-CD81; 1:50; 60 min, 4°C) and secondary antibody (anti-rabbit Alexa Fluor 488, 8 µg/ml; 30 min, 4°C). After each staining step, the cells were rinsed with saponin buffer and centrifuged (2000 rpm, 2 min, 4°C). Finally, cell pellets were resuspended in a 500 µl flow cytometry buffer and subjected to flow cytometry analysis (Beckton

Dickinson FACScalibur, BD Biosciences, Franklin Lakes, New Jersey, USA). Flow cytometry settings were optimized for the analysis of CD81 expression in U-87 and SH-SY5Y cells as seen in Figure 8. The analysis was performed until 10,000 cells were detected in the gated area. The FL-1 channel was used to measure the fluorescence intensity of the Alexa Fluor 488 fluorochrome, later used in the FlowJo software (*FlowJo*, Ashland, USA) to determine the mean fluorescence intensity (MFI).

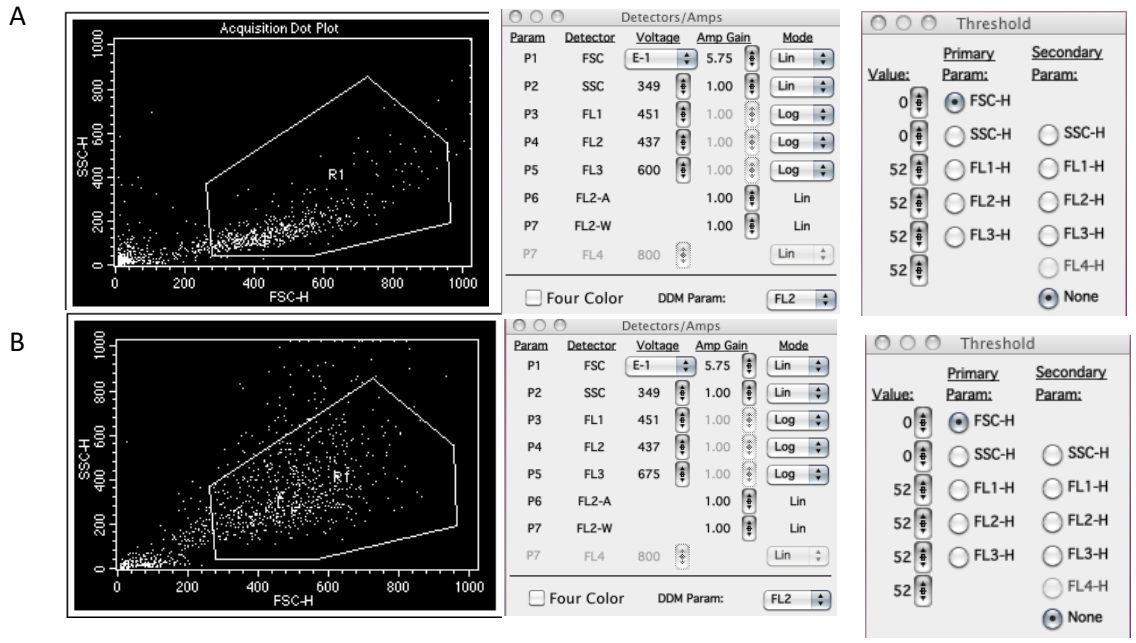


Figure 8. Flow cytometry settings used for the analysis of CD81 expression in U-87 MG (A) and SH-SY5Y cells (B) upon treatment with different concentrations of chlorpromazine.

3.5.5. Data annotation and statistical analysis

Data annotation was performed in Microsoft Excel 2019 (*Microsoft Corporation*, Redmond, USA). The results obtained by flow cytometry were checked for sample quality, graphically presented and analysed using FlowJo 10 software (*FlowJo*, Ashland, USA). MFI values of cells stained only with the secondary antibody were subtracted from the MFI of cells stained with both the primary and secondary antibody to obtain the final MFI value of anti-CD81+Alexa Fluor 488. A statistical analysis with graphical

presentation of the results was performed in GraphPad Prism 7 (*GraphPad Software, Inc*., La Jolla, USA). After testing for data distribution with the Shapiro-Wilk test, parametric or nonparametric tests were used. Data with normal distribution were further analysed with parametric tests, while data which did not show normal distribution were tested by nonparametric tests. To compare the effect of growing concentrations of chlorpromazine, ANOVA repeated measures analysis or the Friedman test was used with respective post-hoc analysis (Student's T test for dependent samples or Wilcoxon test), in accordance with data distribution. A difference of $p<0.05$ was considered significant.

4. Results

4.1. SH-SY5Y cells are more susceptible to chlorpromazine toxicity than U-87 MG cells

The aim of this study was to examine the effect of chlorpromazine on the levels of CD81 protein in U-87 MG and SH-SY5Y cells. To determine the effective concentration range, the cells were exposed to increasing concentrations of chlorpromazine (4, 8, 16 and 32 µg/ml), and their viability was assessed by trypan blue staining technique after 24 hours of chlorpromazine treatment. The results represented in Figure 9A show that the highest applied concentration (32 µg/ml) significantly decreased U-87 MG cell viability to 60 ± 14.5% in comparison to untreated cells (82 ± 23%). The viability of U-87 MG cells treated with 4, 8 and 16 µg/ml was at 89 ± 5%, 86 ± 9% and 91 ± 6%, respectively, with no statistically significant difference relative to untreated cells.

The viability of SH-SY5Y cells treated with 4, 8 and 16 µg/ml of chlorpromazine is shown in Figure 9B and it was at 62 ± 10%, 87 ± 12% and 75 ± 8%, respectively, with no statistically significant difference in comparison to untreated cells (74 ± 16%). Chlorpromazine concentration of 32 µg/ml resulted in no observed viable SH-SY5Y cells.

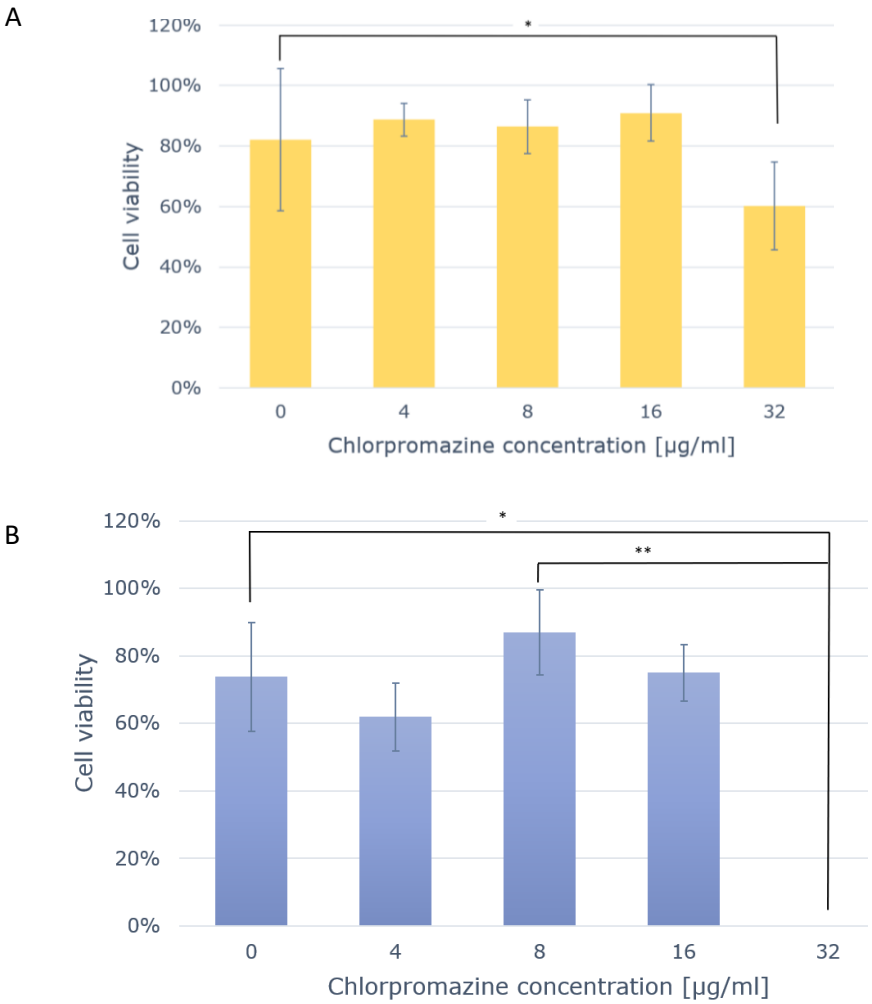


Figure 9. ***Chlorpromazine significantly affects U-87 MG and SH-SY5Y cell viability at the concentration of 32 µg/ml.*** *U-87 MG glioblastoma cells (A) and SH-SY5Y neuroblastoma cells (B) were treated with depicted concentrations of chlorpromazine for 24 hours. Cell viability was determined by trypan blue staining and expressed as the number of live cells relative to the total number of cells (live + dead). Shown are mean values of cell viability from 2 experiments performed either in duplicates or triplicates with the total number of samples as quintuplicate (0 and 16 µg/ml), triplicate (4-8 µg/ml) or duplicate (32 µg/ml). Standard deviations are depicted as vertical lines. * p≤0.05 and ** p≤0.01.*

4.2. Chlorpromazine treatment increases CD81 protein level in U-87 MG cells

Given that U-87 MG cells treated with increasing concentrations of chlorpromazine presented good viability, their CD81 protein levels were examined by flow cytometry in the following step. All flow cytometric analyses of anti-CD81 fluorescence in U-87 MG untreated and chlorpromazine treated cells were made on cells pertinent to the gate excluding cellular debris (Figure 10A). Figure 10B shows a representative dot plot of the forward scatter (FSC) parameter and fluorescence of cells treated with 32 µg/ml of chlorpromazine. A histogram depicting an overlay of fluorescence in cells treated with 0, 16 and 32 µg/ml shows that the highest applied concentration of chlorpromazine (32 µg/ml) induced a visible increase in anti-CD81 fluorescence compared to untreated cells (Figure 10C). Statistical analysis of mean MFI values shown in Figure 10D confirmed that treatment of U-87 MG cells with 32 µg/ml of chlorpromazine leads to a significant increase in anti-CD81 fluorescence relative to untreated cells (MFI_0 = 326,2 ± 53,0; MFI_{32} = 728,2 ± 18,0). Applied concentrations of 4, 8 and 16 µg/ml chlorpromazine did not produce a statistically significant change in MFI values compared to untreated cells, yet there was a significant difference of MFI at 4 compared to 32 µg/ml (MFI_4 = 336,5 ± 114,2; MFI_8 = 300,6 ± 87,9; MFI_{16} = 365,7 ± 78,6).

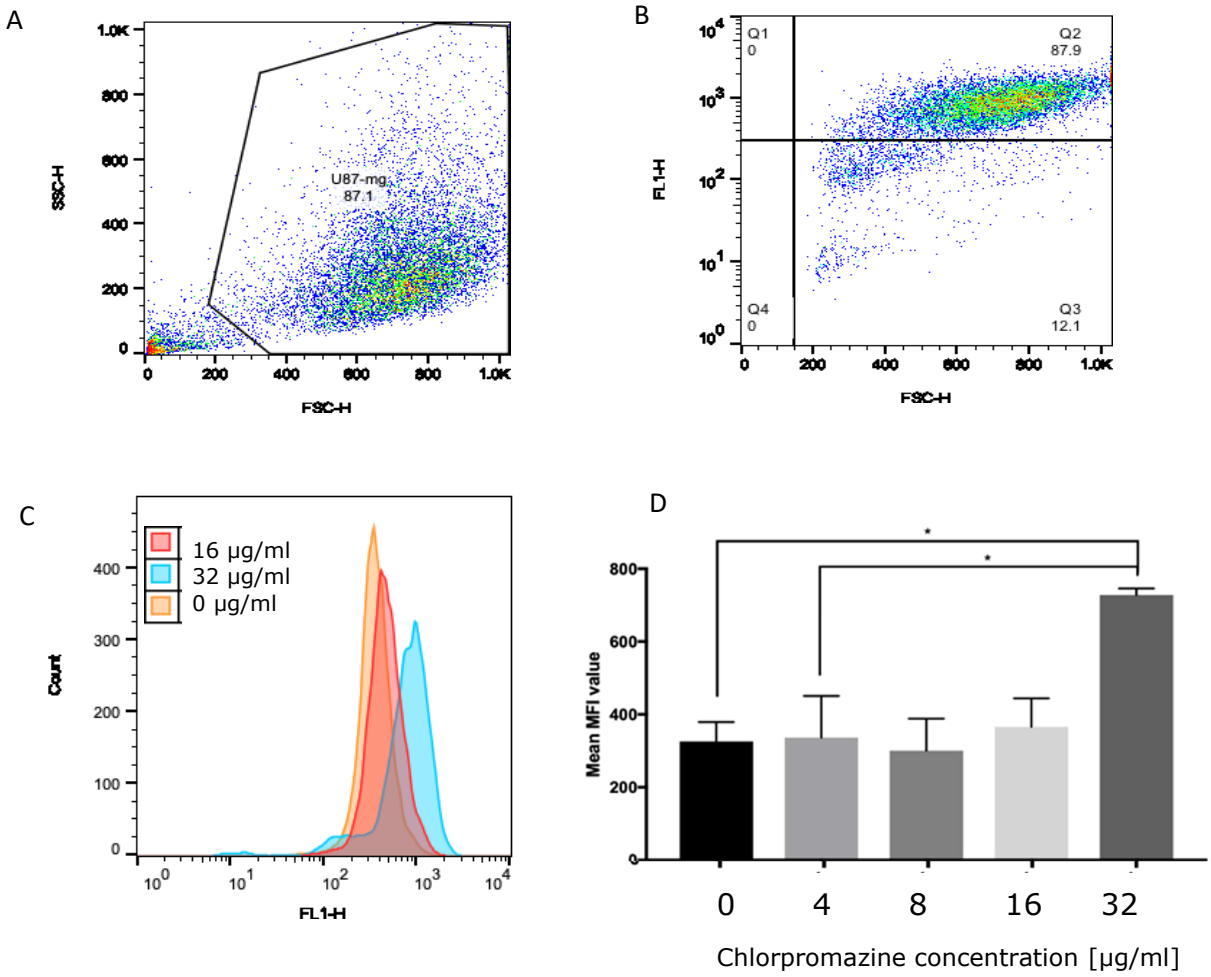


Figure 10. ***Chlorpromazine treatment increases CD81 protein level in U-87 MG cells at the concentration of 32 µg/ml.*** *Cells treated with different concentrations of chlorpromazine (0, 4, 8, 16 and 32 µg/ml) for 24 hours were stained with anti-CD81+Alexa Fluor-488 and subjected to flow cytometry; A) Representative forward (FSC) and side scatter (SSC) diagram of untreated cells used for gating; B) Representative dot plot showing FSC and fluorescence of cells treated with 32 µg/ml of chlorpromazine. C) Representative histogram showing fluorescence of U-87 MG cells treated with indicated chlorpromazine concentrations. D) Mean Fluorescence Intensity (MFI) values of anti-CD81 + Alexa-488 treated with growing concentrations of chlorpromazine (0, 4, 8, 16 and 32 µg/ml), obtained as average values of 2 experiments performed either in duplicates or triplicates with the total number of samples as quintuplicate (0 and 16 µg/ml), triplicate (4-8 µg/ml) or duplicate (32 µg/ml). Standard deviations are depicted as vertical lines. * = statistical significance p≤0.05.*

4.3. Chlorpromazine treatment decreases the expression of CD81 in SH-SY5Y cells

After assessing SH-SY5Y cell viability in response to increasing chlorpromazine concentrations (4, 8, 16 and 32 µg/ml), all samples were subjected to flow cytometry analysis of CD81 levels, even though the concentration of 32 µg/ml of chlorpromazine showed pronounced cytotoxicity in this cell type. Figure 11A shows the gate set on untreated cells and used in all further flow cytometric analysis of anti-CD81 fluorescence in SH-SY5Y cells. Figure 11B shows a representative dot plot of the forward scatter (FSC) parameter and fluorescence of cells treated with 16 µg/ml of chlorpromazine. A representative histogram overlay of anti-CD81 fluorescence illustrates a visible, yet limited decrease of fluorescence in cells treated with 4, 8 and 16 µg/ml of chlorpromazine compared do untreated control (Figure 11C). Statistical analysis of the obtained results shows a significant decrease in MFI values of CD81 in SH-SY5Y cells treated with 8 µg/ml of chlorpromazine compared to cells treated with 4 µg/ml (MFI_4 = 197,7 ± 87,9M MFI_8 = 125,4 ± 75,8) (Figure 11D). There is a distinct difference in the MFI of cells treated with 8 and 16 µg/ml of chlorpromazine relative to untreated control (MFI_0 = 191,4 ± 82,1; MFI_8 = 125,4 ± 75,8; MFI_{16} = 100,98 ± 24,8) and a pronounced decrease in the MFI of cells treated with 32 µg/ml of chlorpromazine (MFI_{32} = 12,05 ± 0,9), yet statistical significance was not met.

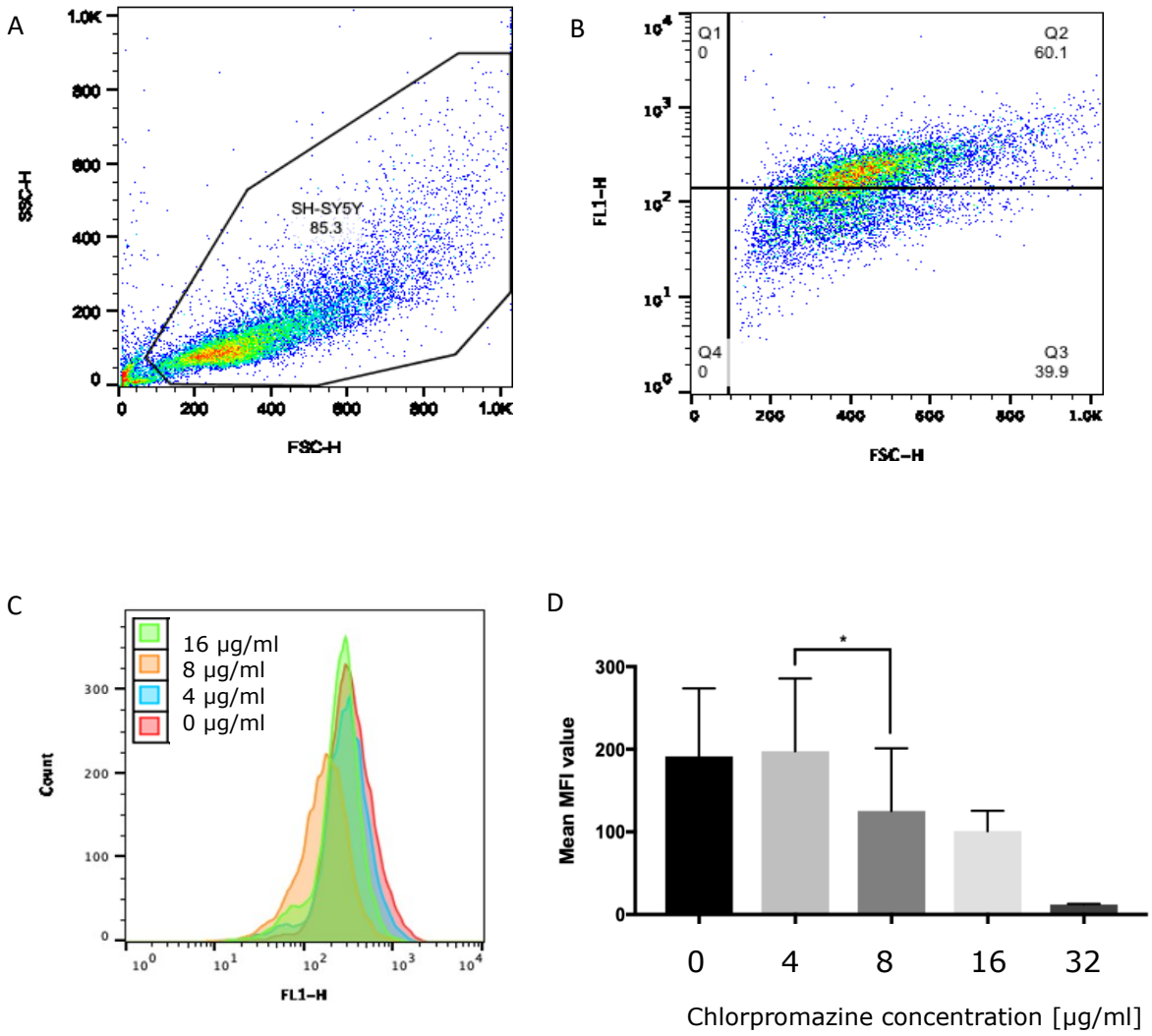


Figure 11. ***Chlorpromazine decreases CD81 expression in SH-SY5Y cells at the concentration of 32 µg/ml.*** *Cells treated with different concentrations of chlorpromazine (0, 4, 8, 16 and 32 µg/ml) for 24 hours were stained with anti-CD81+Alexa Fluor-488 and subjected to flow cytometry; A) Representative forward (FSC) and side scatter (SSC) diagram of untreated cells used for gating; B) Representative dot plot showing FSC and fluorescence of cells treated with 16 µg/ml of chlorpromazine. C) Representative histogram showing fluorescence of SH-SY5Y cells treated with indicated chlorpromazine concentrations. D) Mean Fluorescence Intensity (MFI) values of anti-CD81 + Alexa-488 treated with growing concentrations of chlorpromazine (0, 4, 8, 16 and 32 µg/ml), obtained as average values of 2 experiments performed either in duplicates or triplicates with the total number of samples as quintuplicate (0 and 16 µg/ml), triplicate (4-8 µg/ml) or duplicate (32 µg/ml). Standard deviations are depicted as vertical lines. * = statistical significance p≤0.05.*

5. Discussion

The main purpose of this study was to examine whether chlorpromazine treatment exerts an effect on the expression of tetraspanin CD81 in human neural cells, represented by the neuroblastoma SH-SY5Y and glioblastoma U-87 MG cell lines. As already introduced, intercellular communication in the CNS relies on the exchange of signals between neurons and glial cells through molecular transmitters or via extracellular vesicles. The tetraspanin family of proteins is implicated in intercellular contacts (43), while specific tetraspanins were associated with neurotransmitter signaling and receptor trafficking in neurons (22). CD81 is a tetraspanin known as a marker of exosomes, enriched on intercellular membranes (26). In neural cells CD81 is involved in neuron-astrocyte interactions, astrocyte proliferation and glial reactivity (28,30). Chlorpromazine is an antipsychotic drug with multiple indications and molecular targets. It acts as an inhibitor of dopamine, serotonin, histamine and muscarinic receptors (36). In addition, it blocks clathrin-mediated endocytosis and reduces intercellular communication via gap junctions in mammalian neurons and astrocytes (32,40). It has been associated with a decrease in synapse number accompanied by an increase in astrocytic processes (41). These findings directed our attention towards exploring the action of chlorpromazine on the tetraspanin CD81 expression in human neural cells.

Previous studies indicate that chlorpromazine has a cytotoxic effect in both U-87 MG and SH-SY5Y cells. Chlorpromazine showed significant toxicity at 32 and 320 μg/ml in SH-SY5Y cells, while lower doses did not affect cell viability (39). In the present study, as seen in Figure 9B, SH-SY5Y cell viability was significantly affected by the highest concentration of chlorpromazine that was applied (32 μg/ml), but not with lower doses, which is consistent with literature findings. In a separate study, chlorpromazine decreased U-87 MG cell viability in a dose-dependent way

after 24-hour treatment with 3.2, 6.4 and 12.8 μg/ml of this drug (37). To contrast that with our findings, the lowest concentration of chlorpromazine used on U-87 MG cells (4 μg/ml) did not cause significant cell death in this study (Figure 9A). The reasons for this discrepancy might be in the methods used for assessing cell viability. In the present study, a trypan blue dye exclusion assay with Neubauer chamber and light microscopy was used to visually examine the number of viable cells. This method is simple, inexpensive and fast, yet prone to counting errors (44). These errors can be attributed to improper filling of the counting chamber, poor cell dispersion, cell loss during dispersion, the presence of air bubbles in the chamber etc. Furthermore, trypan blue staining does not distinguish between healthy cells and ones which are still alive but losing cell functions. In the cited study, a commercial kit for WST-8 colorimetric assay was used with spectrophotometer measurement of cell viability (37). This assay has high reproducibility and avoids the possibility of counting errors (44). Due to the difference in obtained data, we propose further testing of chlorpromazine effects on U-87 MG cell viability using more sensitive methods such as the MTT test or propidium iodide staining and flow cytometry analysis. Testing in shorter time intervals might also be appropriate.

A flow cytometric analysis of anti-CD81 fluorescence was performed on U-87MG and SH-SY5Y cells treated with growing concentrations of chlorpromazine. The results obtained showed that treatment with 32 μg/ml of chlorpromazine led to a significant increase in CD81 expression in U-87 MG glioblastoma cells (Figure 10D). Lower concentrations of chlorpromazine (4, 8 and 16 μg/ml) had no statistically significant effect on CD81 expression in this cell line. Contrary to this finding, the same drug has led to the opposite effect in SH-SY5Y cells, in which treatment with 8 μg/ml of chlorpromazine caused significant decrease in CD81 expression compared to cells treated with 4 μg/ml (Figure 11D). There was a clear decrease in CD81 fluorescence in SH-SY5Y cells treated with 32 μg/ml of

chlorpromazine compared to control, but statistical significance was not met, probably due to limited number of samples. Nevertheless, there was a statistical significance decrease in CD81 fluorescence of SH-SY5Y cells treated with 32 μg/ml compared to 4 μg/ml of chlorpromazine. These results indicate that chlorpromazine downregulates CD81 in SH-SY5Y cells. However, it would be prudent to test these findings on a larger number of samples and confirm statistically significant differences between untreated controls and other treatment groups. Furthermore, the highest concentration applied caused significant cell loss and thus made it difficult to appropriately assess the CD81 expression by flow cytometry with the initial number of cells that we had. Future experiments with a greater initial cell count might provide more conclusive results in SH-SY5Y cells.

Recently, there has been a growing interest for elucidating the non-neuronal effects of chlorpromazine. It was found to increase expression levels of various proteins associated with the ubiquitination pathway in oligodendrocytes (45). CD81 is abundant on multivesicular endosomes or multivesicular bodies (MVBs) that can fuse with pre-existing lysosomes and thus deliver proteins destined for degradation (46). Our finding that CD81 is upregulated after 32 μg/ml of chlorpromazine treatment in glioblastoma U-87 MG cells also links the actions of this drug with endosomal pathways and possibly protein degradation in glial cells. The fact that chlorpromazine induces autophagy in U-87 MG cells strengthens this association (37). Other possible pathway of MVBs is fusion with the plasma membrane and the subsequent release of exosomes into the extracellular space. Knowing that CD81 is a recognized marker of exosomes, their intercellular pathways upon chlorpromazine treatment might also be affected. It is known that chlorpromazine inhibits clathrin-mediated endocytosis of exosomes, but no studies have examined its effect on EV biogenesis and secretion. The fact that antipsychotics affect cholesterol trafficking might also be associated with CD81 changes, considering the role of cholesterol in CD81 conformations (27,34).

In the context of cell-to-cell communication, one study examined the effect of chlorpromazine on gap junctions in mouse-derived neuronal cells and rat astrocytes (40). This study found a reduction of intercellular communication via gap junctions in both cell types following chlorpromazine treatment and reversible cellular retraction which reduced cell-cell contacts. A separate study described a reduction of the number of synapses in rat hypothalamic neurons, accompanied by an increase in astrocytic processes after chlorpromazine treatment (41). CD81 was found to be crucial for neuron-astrocyte interactions and the regulation of astrocyte proliferation (28). Changes in CD81 expression due to chlorpromazine treatment might participate in cited processes, considering the roles of tetraspanin proteins in cell adhesion and proliferation.

Chlorpromazine is a typical antipsychotic drug used not only in the treatment of schizophrenia and psychotic disorders, but also as an antiemetic, for relief of preoperative anxiety and intraoperative sedation, for persistent hiccups and migraines (37). Its main molecular target for antipsychotic effects appears to be the dopamine D_2 receptor, however it acts on other neuronal targets as well. Chlorpromazine was found to block voltage sensitive Na^+, Ca^{2+} and K^+ ion channels in cells derived from mouse neuroblastoma, and it blocks K^+ channels in rat sympathetic neurons (part of the peripheral nervous system) (40). Due to significant loss of neuroblastoma cells after chlorpromazine treatment in our study, it can be speculated that neurons are more susceptible to the cytotoxic effects of this drug then glial cells, possibly due to the various molecular targets to which the drug can bind. Considering the crucial roles of ion channels for signal transduction in neurons, the toxicity of chlorpromazine might be interpreted through its action on the basic communication processes. CD81 can also be implicated via its association with extracellular vesicles, and our data suggest it is downregulated by chlorpromazine in neuronal cells.

CD81 is a tetraspanin protein involved in cellular proliferation, activation and adhesion (27). It was shown to be upregulated in reactive glia upon traumatic injury to the CNS (30,47). CD81 was upregulated in reactive astrocytes in rat CNS and associated with reactive gliosis and the glial scar (48). In our study, chlorpromazine treatment of U-87 MG cells led to an increase in CD81 levels at the concentration of 32 µg/ml. In separate studies, this drug increased the levels of the anti-inflammatory cytokine interleukin (IL)-10 in the supernatant of primary rat mixed glial cell culture (49). It inhibited proton currents in microglial BV2 cells, leading the authors to speculate that the anti-inflammatory and antipsychotic actions of chlorpromazine may be partially due to its inhibition of microglial proton channels, which are known to participate in the generation of reactive oxygen species (37). Based on the observed cytotoxic effect of chlorpromazine on U87-MG cells and the increase of CD81 produced by chlorpromazine in these cells in our study, we might speculate that CD81 upregulation participates in the glial response to injury.

6. Conclusion

The results of this study suggest that chlorpromazine exerts different effects on cell viability and CD81 protein levels in U-87 MG glioblastoma and to SH-SY5Y neuroblastoma cell lines. SH-SY5Y are shown to be more sensitive to chlorpromazine toxicity in comparison with U-87 MG cells at the concentration of 32 μg/ml. Moreover, at this concentration chlorpromazine increases CD81 protein levels in U-87 MG cells, while in SH-SY5Y cells it leads to a decrease in CD81 expression. These findings provide additional information on the mechanisms of chlorpromazine action in neural cells. Further studies are required to elucidate whether the observed cellular effects are a direct or indirect result of chlorpromazine treatment due to the known impairment of endocytosis by chlorpromazine. Since this drug upregulates CD81 in U-87 MG cell line, the roles of this protein in glial cell activation should be investigated further. Additionally, here described opposite effect of chlorpromazine on CD81 protein level in model neuronal and glial cells might be relevant for intercellular communication which is especially intensive in central nervous system. Namely, CD81 is associated with extracellular vesicles known to mediate communication between cells including constituents of central nervous system. Future research should reveal whether next to intracellular levels, chlorpromazine also affects extracellular levels of CD81 protein. This data represents the first step in exploring if and how chlorpromazine changes biogenesis and secretion of extracellular vesicles in neural cells.

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8. Curriculum vitae

PERSONAL INFORMATION

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EDUCATION AND TRAINING

10/2017–Present

Master's Degree Program: Biotechnology in Medicine

Department of Biotechnology, University of Rijeka, Rijeka (Croatia)

10/2014–07/2017

Bachelor's Degree in Biotechnology and Drug Research

Department of Biotechnology, University of Rijeka, Rijeka (Croatia)

10/2011–09/2014

Master's Degree in Spanish and Portuguese Language and Literature

Faculty of Humanities and Social Sciences, University of Zagreb, Zagreb (Croatia)

12/2013–04/2014

Erasmus work placement

Clic International House, Seville, Seville (Spain)

10/2008–07/2011

Bachelor's Degree in Spanish and Portuguese Language and Literature

Faculty of Humanities and Social Sciences, Zagreb (Croatia)

WORK EXPERIENCE

10/2014–Present

Spanish and Portuguese Teacher

PAR Business School Rijeka, Omnia language center, Klub mladih Rijeka, Rijeka (Croatia)

2013–Present

Freelance translator

Global Link, Degordian, Croatian Radio 3, Trilix, Radiona.org…

PERSONAL SKILLS

Mother tongue(s) Croatian

Foreign language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C2	C2	C2	C2	C2
Spanish	C2	C2	C2	C2	C2
	University Degree				
Portuguese	C2	C2	C2	C2	C2
	University Degree				

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Communication skills
- excellent communication skills gained by working as a language teacher
- multicultural competences gained during the Erasmus work placement in Seville, Spain

Organisational / managerial skills
- organisational skills gained by editing the student magazine "Biotech - science in the society"

Job-related skills
- excellent time management skills and productivity developed by working in 3 different language teaching facilities and studying Biotechnology simultaneously

ADDITIONAL INFORMATION

Conferences International Student Congress Graz (2019), oral presentation: "Optimization of flow cytometry for the characterization of cerebrospinal fluid-derived extracellular vesicles of patients with severe traumatic brain injury"

Summer school Summer school in Pathophysiology and Public Health, Department of Biotechnology, Rijeka, Croatia (June 2019)

Courses "Communication skills in adult education", Agency for professional education and adult education, Rijeka, February 2018

Erasmus+ training: "Coaching approach to learning", Cluj-Napoca, Romania, 2015