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Drug repurposing for application in neuro-oncology using the dopamine synthesis cascade pathway

Catarina Sousa Moura

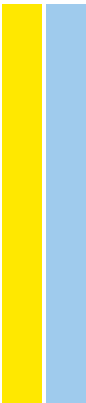
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**Drug repurposing for application in neuro-oncology using the
Dopamine synthesis cascade pathway**

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A todos vocês,

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Abstract

Neuro-oncology is a field that combines neurology and oncology, focusing primarily on the diagnosis and treatment of brain tumors. Although brain cancer is considered relatively rare, its mortality rate is quite high due to its invasive nature and difficult treatment. Dopamine (DA), a neurotransmitter crucial for regulating cognitive and emotional processes, has been associated with the cancer development, along with its receptors. Several studies suggest that DA and its receptors may play an important role in tumor growth and in the tumor microenvironment. DA is synthesized from tyrosine (Tyr) and, like other neurotransmitters and their precursors, such as tryptophan, it may be related to cancer development and progression.

In this study, we investigated the potential association of the DA synthesis cascade with brain tumors by using repurposed antipsychotic and antiemetic drugs (DA receptor antagonists) in glioblastoma (A172) and neuroblastoma (SH-SY5Y) cells. To achieve this objective, we tested the cytotoxicity of six repurposed drugs: Clozapine (CLZ), Domperidone (DOM), Droperidol (DRO), Olanzapine (OLZ), Pimozide (PIM) and Risperidone (RIS) in both cell lines. Subsequently, we evaluated the effects of the most effective drugs in each cell line in combination with Tyr (DA precursor) and hydrogen peroxide (H_2O_2) in neuronal cells. Additionally, we analyzed the effects of the most promising drug and its combinations on extracellular levels of Tyr and DA.

Among the six drugs tested, CLZ demonstrated the most significant and highest cytotoxicities in both cell lines, suggesting a significant anticancer potential, which could be attributed to its higher affinity for D4 receptors. Regarding the combinations, our results showed that CLZ combined with Tyr exhibited superior cytotoxic effects compared to its use alone in both cell lines, as confirmed by synergistic studies. The other drugs showed greater efficacy in reducing cell viability when tested alone. When combined with H_2O_2 , only CLZ showed a slight decrease in SH-SY5Y cell viability, indicating strong synergism and increased cytotoxicity under oxidative stress. In the SH-SY5Y cell line, the combination of Tyr with repurposed drugs and H_2O_2 significantly reduced cell viability, indicating greater cytotoxicity and synergism with CLZ, PIM and DOM. In contrast, the A172 cell line did not show significant changes in cell viability with this combination. After 48 hours, the extracellular concentration of Tyr combined with CLZ varied between the two cell lines, but generally, high cell viability corresponded to complete Tyr consumption, while reduced cell viability corresponded to partial Tyr consumption.

In conclusion, several antipsychotics demonstrated cytotoxic effects on neuroblastoma and glioblastoma cells, with CLZ being the most promising. Additionally, the combinations of the repurposed drugs with Tyr and H₂O₂ produced variable results, highlighting the need for further research to validate these results.

Keywords: Dopamine; Tyrosine; Drug Repurposing; SH-SY5Y; A172; Neuro-Oncology, Antipsychotic Drugs; Antiemetic Drugs; H₂O₂; oxidative stress.

Resumo

A neuro-oncologia é um campo que combina a neurologia e a oncologia, focando-se principalmente no diagnóstico e tratamento de tumores cerebrais. Embora o cancro cerebral seja considerado relativamente raro, a sua mortalidade é bastante elevada devido à sua natureza invasiva e de difícil tratamento. A dopamina (DA), um neurotransmissor com um papel crucial no cérebro ao regular uma diversidade de processos cognitivos e emocionais, tem sido associada ao desenvolvimento do cancro, juntamente com os seus recetores. Diversos estudos sugerem que a DA e os seus recetores podem desempenhar um papel importante no crescimento tumoral e no microambiente tumoral. A DA é sintetizada a partir da tirosina (Tyr) e, assim como outros neurotransmissores e os seus precursores, como o triptofano, pode estar relacionada ao desenvolvimento e progressão do cancro.

Neste trabalho, investigamos a potencial associação da cascata de síntese da DA com os tumores cerebrais, utilizando fármacos reaproveitados antipsicóticos e antieméticos (antagonistas dos recetores da DA), em células de glioblastoma (A172) e neuroblastoma (SH-SY5Y). Para atingir este objetivo, testamos a citotoxicidade de seis fármacos reaproveitados: Clozapina (CLZ), Domperidona (DOM), Droperidol (DRO), Olanzapina (OLZ), Pimozida (PIM) and Risperidona (RIS) nas duas linhas celulares. Posteriormente avaliamos os efeitos dos fármacos mais eficazes em cada linha celular em combinação com a Tyr (precursor da DA) e com o peróxido de hidrogénio (H_2O_2) nas células neuronais. Além disso, analisamos os efeitos do fármaco mais promissor e das suas combinações nos níveis extracelulares de Tyr e DA.

Entre os seis fármacos testados, a CLZ demonstrou as maiores citotoxicidades e mais significativas em ambas as linhas celulares, sugerindo um potencial anticancerígeno significativo, que poderá ser atribuído à sua maior afinidade pelos recetores D4 da DA. Relativamente às combinações, os nossos resultados mostraram que a CLZ combinada com a Tyr exibiu efeitos citotóxicos superiores em comparação ao seu uso isolado em ambas as linhas celulares, conforme confirmado pelos estudos sinérgicos. Os restantes fármacos evidenciaram maior eficácia na redução da viabilidade celular quando testados isoladamente. Quando combinados com o H_2O_2 , apenas a CLZ apresentou uma ligeira diminuição na viabilidade das células SH-SY5Y, indicando um elevado sinergismo e uma alta citotoxicidade sob o stress oxidativo. Na linha celular SH-SY5Y, a combinação da Tyr com os fármacos reaproveitados e H_2O_2 reduziu significativamente a viabilidade celular, indicando uma maior citotoxicidade e sinergismo com a CLZ, PIM e DOM. Pelo contrário,

a linha celular A172 não mostrou alterações significativas na viabilidade celular com esta combinação. Após 48 horas, a concentração extracelular de Tyr combinada com a CLZ variou entre as duas linhas celulares, mas, em geral, quando a viabilidade celular era alta, a Tyr era totalmente consumida, enquanto que com a redução da viabilidade celular, o consumo de Tyr era parcial.

Em conclusão, diversos antipsicóticos demonstraram efeitos citotóxicos nas células de neuroblastoma e glioblastoma, sendo a CLZ o mais promissor. Além disso, as combinações dos fármacos reaproveitados com a Tyr e com o H_2O_2 produziram resultados variáveis, evidenciando a necessidade de mais investigação para validar estes resultados.

Palavras-Chave: Dopamina; Tirosina; Reaproveitamento de Fármacos; SH-SY5Y; A172; Neuro-Oncologia; Fármacos antipsicóticos; Fármacos Antieméticos; H_2O_2 ; stress oxidativo.

Abbreviations

AC	Adenylate Cyclase
AML	Acute Myeloid Leukemia
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
CREB	cAMP Response Element-Binding Protein
CBP	CREB-Binding Protein
CI	Combination Index
CLZ	Clozapine
CNS	Central Nervous System
COMT	Catechol-O-Methyltransferase
CSCs	Cancer Stem Cells
DA	Dopamine
DAT	Dopamine Transporters
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DOM	Domperidone
DR	Dopamine Receptor
DRD2	Dopamine D2 receptor
DRO	Droperidol
EPS	Extrapyramidal Symptoms
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GIRK	G protein gated inwardly rectifying K ⁺ channel
GPCR	G protein-coupled receptor

GRKs	GPCR kinases
H ₂ O ₂	Hydrogen Peroxide
IC ₅₀	Half maximal inhibitory concentration
L-DOPA	Levodopa
MAO	Monoamine Oxidase
PBS	Phosphate Buffered saline
PD	Parkinson's Disease
PIM	Pimozide
PKA	Protein Kinase A
OLZ	Olanzapine
OS	Overall survival
RIS	Risperidone
Tyr	Tyrosine
VMAT2	Vesicular Monoamine Transporter 2

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Introduction

Neuro-oncology: Understanding two types of brain tumors

Neuro-oncology is a rapidly evolving field that combines neurology and cancer biology, focusing on the diagnosis, treatment, study and management of tumors that affect the central nervous system (CNS), which include the brain and spinal cord, as well as neurologic complications of cancer [1,2]. The goal of neuro-oncology is to improve the prognosis and quality of life for individuals with brain and spinal cord tumors. In the past, neuro-oncology focused mainly on the treatment of gliomas and other primary brain tumors, using methods such as surgery, radiation, and a limited range of cytotoxic chemotherapies [1]. Nevertheless, neuro-oncology has undergone rapid advances due to the introduction of safer neurosurgical methods, emerging neuroimaging technologies, new treatment options for brain metastases and the integration of molecular profiling in the diagnosis and treatment of brain tumors [1].

Brain cancer, ranked nineteenth in incidence among all cancers, is classified as a low incidence cancer. However, it ranks twelfth in mortality, indicating that brain cancer is more invasive and deadly cancer for the global population (Figure 1). While these statistics represent general data on brain cancer, it is important to note that they encompass various specific tumours types, including glioblastoma and neuroblastoma.

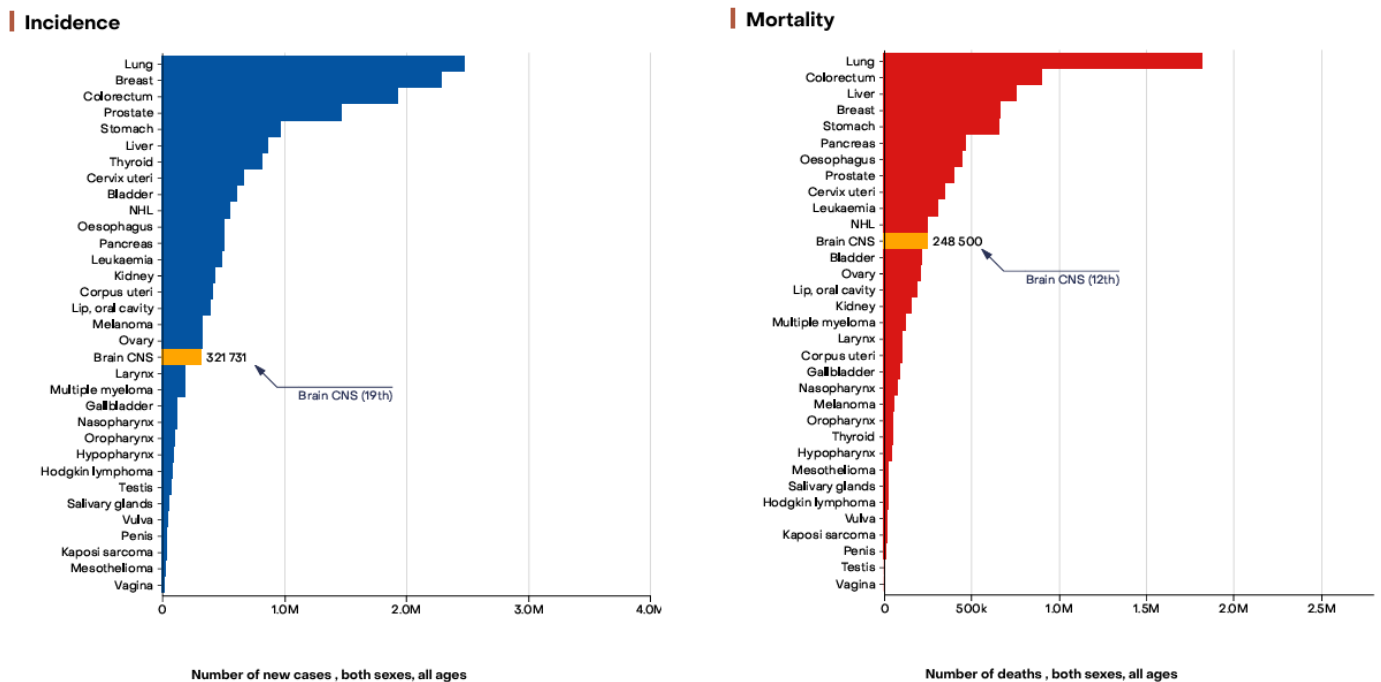


Figure 1. Estimated 2022 worldwide cancer incidence and mortality rates for both genders and all ages. Source: GLOBOCAN. Adapted from [3].

Glioblastoma is a brain tumor, classified as a World Health Organization grade IV astrocytoma, and it's the most common malignant primary tumor, accounts for 14.5% of all central nervous system tumors [4-6]. Originating from astrocytic glial cells, this tumor can occur at any age, but its incidence rises with age, typically diagnosed around 65 years, and with men displaying a higher prevalence than women [4,5]. The incidence of glioblastoma varies, ranging from 3.19 to 4.17 cases per 100,000 person-years [4], establishing it as the most prevalent and aggressive primary brain tumor in adults [7]. Although the incidence of this tumor is comparatively low when compared to cancers like lung, breast, prostate and colon cancer, glioblastoma exceeds them in terms of "average years of life" [6]. This is reflected in its aggressive nature, with a median overall survival (OS) lasting only 15 months [4]. This tumor is associated with extremely poor survival due to uncontrolled cellular proliferation, highly diffuse infiltration and angiogenesis, resistance to treatment and apoptosis, predisposition to necrosis and generalized genomic aberrations [6,8]. Moreover, the presence of tumor heterogeneity in this brain tumor increases the distinctive challenges involved in treating the disease [8]. The standard approach for newly diagnosed cases involves surgery, followed by radiotherapy and temozolomide chemotherapy [5]. This highly diffusive, invasive, and vascularized tumor type proves resistant to surgical interventions, and despite recent advancements, glioblastoma recurrence is nearly inevitable, resulting in relapse for almost all patients [6]. A definitive cure remains elusive, making treatment primarily palliative for most patients, which highlights the complex nature of this type of cancer [5,8].

Neuroblastoma is a pediatric malignancy that arises from embryonal tumors, specifically originating from the progenitor cells of the neural crest and typically occurring outside the cranial region [9]. This extracranial solid tumor is among the most prevalent and deadly in childhood, representing approximately 15% of all pediatric tumors and contributing to 15% of childhood cancer-related deaths [10,11]. With a median survival of 3 to 4 years, it ranks as the second most common tumor type, surpassed only by CNS tumors [11,12]. The diagnosis for most patients occurs before the age of 10, and there exists an inverse relationship between the age at diagnosis and survival rate. In other words, younger children tend to have a higher survival rate [13]. Furthermore, this tumor is infrequent among adults and teenagers, with a slightly greater incidence in males than in females [9,11]. Neuroblastoma is a complex tumor that exhibits distinctive characteristics, including molecular heterogeneity marked by segmental chromosomal aberrations, amplification of the MYCN oncogene, and the expression of neural growth factors [9,12]. The clinical challenge resides in the observed heterogeneity, as tumors that appear similar in phenotype and morphology can fundamentally differ in their response to therapy based on their

molecular composition [10]. The highly heterogeneous nature of this disease leads to varied outcomes, including spontaneous regression, response to therapy, therapeutic resistance, tumor recurrence, and ultimately, mortality [9]. Presently, the available therapies consist of surgery, intensive chemotherapy, radiotherapy, and stem cell transplantation [11]. Hence, there is a need to investigate novel therapeutic targets and strategies to enhance the survival rate of these cancer patients while minimizing potential side effects [9,11].

Dopamine: The brain's essential neurotransmitter

Dopamine (DA), also known as 3,4-dihydroxytyramine, is a neurotransmitter synthesized by dopaminergic neurons in the brain [14]. As one of the major catecholamine neurotransmitters in the central nervous system (CNS), DA levels in the basal ganglia are well regulated through several mechanisms involving both astrocytes and neurons [15,16]. DA acts via multiple brain pathways, with anomalies in DA and its signaling leading to various neurodegenerative, psychiatric, and immunological diseases [17]. While DA hyperactivity results in a disinhibition of specific actions, DA hypoactivity causes a broad inhibition of motor behaviors. These alterations can manifest as motor control diseases, including Tourette's syndrome, Parkinson's disease, ALS, and developmental stuttering. Consequently, this neurotransmitter plays a crucial role in numerous brain activities, including those that control movement, emotion, cognition, and reward [16,18].

For DA to perform its functions, it must attach to a specific set of receptors on the plasma membrane of target cells. These dopamine receptors (DRs) belong to the GPCR superfamily and are found in high concentrations in the brain, retina, gastrointestinal system, kidney, adrenal glands, heart, sympathetic ganglia, and blood vessels [18,19]. Five distinct subtypes of DRs have been identified: D1, D2, D3, D4, and D5. All DRs are metabotropic, meaning they produce second messengers that either activate or inhibit certain cell signaling pathways [18].

Antipsychotic medications are commonly used to treat various psychotic disorders. There are two main types of antipsychotics: first generation (typical) antipsychotics, created in the 1950s, which act as dopamine receptor antagonists, and second generation (atypical), which target other receptors, such as serotonin 2A (5-HT_{2A}) receptors [20]. While first-generation antipsychotics decrease dopaminergic neurotransmission, second-generation antipsychotics block dopamine D2 receptors (DRD2) and also antagonize serotonin receptors [20].

Dopamine synthesis cascade through Tyrosine

DA is synthesized by multiple steps, with the enzyme tyrosine hydroxylase catalyzing the rate-limiting step in this process by converting tyrosine (Tyr) to levodopa (L-DOPA) by adding a hydroxyl group to it (Figure 1). L-DOPA is in turn decarboxylated by amino acid decarboxylase to generate an early form of dopamine which will subsequently be transported by the synaptic vesicle protein VMAT2 (vesicular monoamine transporter 2) into the vesicles. This pre-dopamine transmitter is encapsulated in a vesicle along with other enzymes to continue dopamine synthesis within the transmission vesicles in the presynaptic neurons. After the neuronal activation, DA is released into the synaptic cleft where it will be able to bind to different receptors that are found on the post-synaptic cell membranes. The excess DA is either subsequently recycled back to the presynaptic neuron via dopamine transporters (DAT) or is broken down by monoamine oxidase (MAO) and/or catechol-O-methyltransferase (COMT). This synthesis–release–reabsorption process controls and regulates the physiological dopaminergic responses in response to body demands [14,16].

When found inside of the vesicles (captured by the vesicles), dopamine can also be converted into norepinephrine by the enzyme dopamine β -hydroxylase (Figure 2). And, in the adrenal medulla and in some brain regions, norepinephrine can be converted to epinephrine by the enzyme phenylethanolamine N-methyltransferase [21]. They are present in serum at low quantities and can significantly rise in response to acute stress or physical activity. Epinephrine mounts a quick and brief stress response signal in comparison to the more persistent glucocorticoids. Although both norepinephrine and epinephrine bind to adrenergic receptors, their potencies for activation differ. While epinephrine is a powerful activator of β -adrenergic receptor (β -AR), norepinephrine preferentially stimulates the α -AR [22]

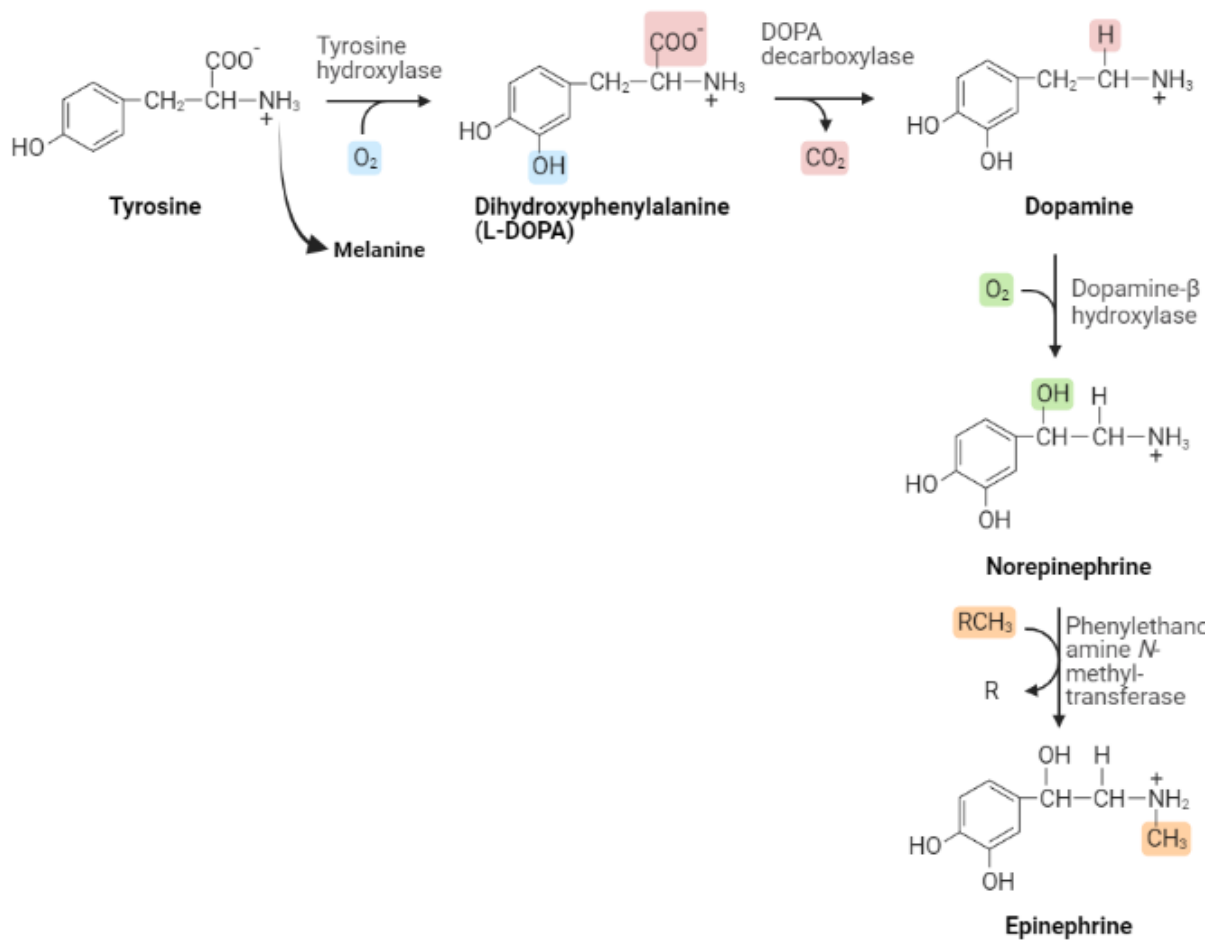


Figure 2. Pathways for biosynthesis of dopamine, a catecholamine neurotransmitter. Image adapted from Purves et al. (2018) Neuroscience. 6th Ed. Oxford University, using the BioRender on April 26, 2023.

When DA is transported to and incorporated into the vesicles, it is subsequently released into the synaptic cleft, where it can bind to the DRs (Figure 3). Through this mechanism, DA can bind to five receptor subtypes: D1–D5, each belonging to members of the G protein-coupled receptor (GPCR) family. These receptors are categorized into two main subclasses: D1R-like and D2R-like receptor families [14].

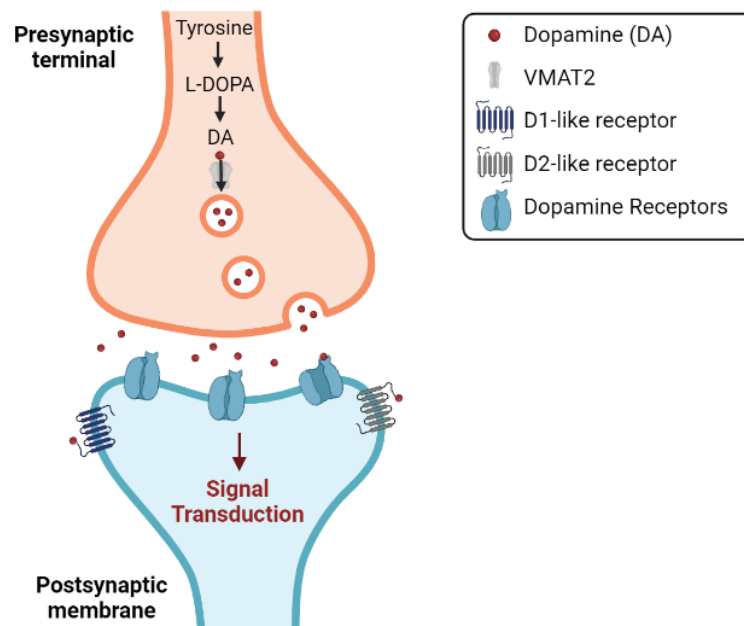


Figure 3. Synthesis of dopamine and its release into the synaptic cleft, which will allow the binding of dopamine to its different receptors, the DRs. Image adapted from Purves et al. (2018) [13], using the BioRender on 14 May 2023.

The D1R-like, including D1R and D5R, are receptors that usually bind to proteins that stimulate the enzyme adenylate cyclase (AC), which is responsible for catalyzing the hydrolysis of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), consequently increasing the production of cAMP. The cAMP will activate the protein kinase A (PKA), which in turn will phosphorylate the cAMP response element-binding protein (CREB). CREB is subsequently translocated to the nucleus to activate CREB-binding protein (CBP) which will promote the transcription of genes involved in synaptic plasticity. Accordingly, the D1Rs modulate different ion channels, including Ca^{2+} , K^+ , and Na^+ channels and a G protein gated inwardly rectifying K^+ (GIRK) channel [14].

D2R, D3R, and D4R exemplify the D2R-like receptors. They bind through coupling to proteins, leading to the suppression of the AC and PKA-dependent activation pathways as well as the activation of GIRK and the closure of voltage-activated Ca^{2+} channels. When combining with other subtypes, DA receptors can behave as monomers, dimeric, and/or oligomeric complexes. Dimeric and oligomeric complexes exhibit distinct pharmacological and functional characteristics compared to the individual receptors [14].

Furthermore, several pathways from the G protein can be activated by DA receptors. Ultimately, these pathways are final controlled by multifunctional adaptors of the arrestin protein, which recruits numerous proteins by binding to DA receptors that have been phosphorylated by GPCR kinases (GRKs), including Akt, GSK-3, MAPK, c-Src, Mdm2, and

N-ethylmaleimide-sensitive factor. The association of arrestin with phosphorylated energy receptors not only inhibits G protein activation but also facilitates receptor endocytosis [14].

As a result, depending on the receptors on the target neuron's membrane surface and how these neurons react to changes in cAMP concentration, DA can exhibit both excitatory and inhibitory effects. Because of this, it is reasonable to expect that the distinct functional roles of DA will vary depending on factors such as receptor subtype, cell type, synaptic characteristics, and interactions with other transmitters [14].

Contradictory Effect of Dopamine in Oncology

The discovery of cancer patients taking antipsychotic medications alongside their antineoplastic therapy and the observation that these patients had better clinical outcomes first supported the theory that dopamine receptors may play a key role in the progression of a tumor [23].

According to additional research, patients with Parkinson's disease (PD) or schizophrenia who take medications like ziprasidone, asenapine, quetiapine, clozapine, or aripiprazole (agonists or nonselective antagonists of DRs) or levodopa (a prodrug that results in a non-selective agonism of DRs) have a lower risk of developing a variety of cancers than people in the general population. Nevertheless, the risk of breast cancer is increased in female schizophrenia patients taking DRD2 antagonists with varying selectivity, such as haloperidol, risperidone, paliperidone, or amisulpride [23].

Epidemiological studies have found that PD and cancer may have an inverse connection that varies depending on the type of cancer. A study found a correlation between PD and a reduced risk of lung cancer, with PD patients being less likely to receive a subsequent lung cancer diagnosis. Despite the hypothesis that this could be due to PD patients' exposure to dopamine medication, researchers concluded that the observed link was likely a bias. Contrarily, other research showed a significant co-occurrence of melanoma with PD, revealing that the relative risk for melanoma in patients with PD was seven times higher than expected. The relationship between PD and melanoma, in contrast to lung cancer, is independent of the time of diagnosis. Consequently, melanoma patients have a 50% higher risk of receiving a diagnosis of PD later, and patients with PD are twice as likely to later receive a melanoma diagnosis [24].

Increasing molecular and clinical evidence suggests a direct biological link between peripheral DA signaling and cancer in terms of risk, development, and progression.

Nevertheless, epidemiological studies indicate that the relationship between DA signaling and cancer is complex and contradictory [24].

Repurposed Drugs for Oncology Using Dopamine Pathway

Drug repurposing, also known as drug repositioning, refers to the application of a drug for a different medical purpose than the one it was initially approved for [25]. This approach involves discovering novel medical applications for established drugs, encompassing approved, discontinued, shelved, and experimental medications [26,27]. It is increasingly being employed across diverse medical fields, particularly in the treatment of rare diseases, where the pharmaceutical industry's process of developing new drugs is significantly slower and more expensive (Figure 4) [26,28]. Drug repurposing is based on the concept that a single drug can affect multiple pathways or that different diseases can have molecular similarities, along with other contributing factors [27].

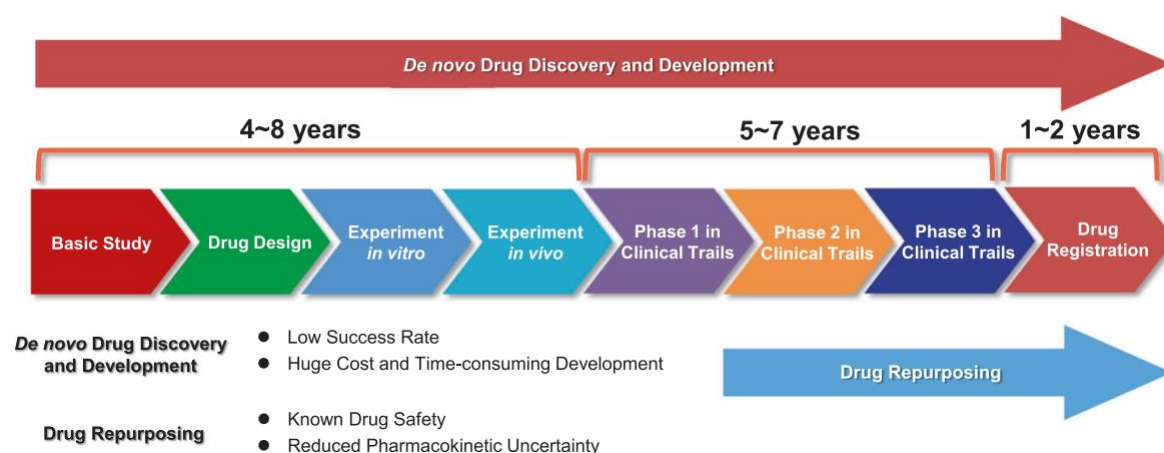


Figure 4. Comparison of De Novo Drug Discovering and Development with Drug Repurposing Approaches. Adapted from [29].

More than 40 years ago, the first demonstration of dopamine's impact on tumor cells was observed when dopamine inhibited the growth of B16 melanoma cells, but the mechanisms of this inhibition remain undiscovered [30]. Since this study, the effect of dopamine on tumor cells and on cancer treatment itself has become significant interesting, leading to numerous studies that provide molecular evidence of dopamine's mechanism in cancer.

Researchers have found that patients with schizophrenia who are treated with antipsychotics have a lower cancer incidence of certain cancer types compared to the general population [20,31]. Additionally, cancer patients who took antipsychotic medications alongside their antineoplastic treatment experienced better clinical outcomes. This

reinforced the hypothesis that DRs could play important roles in tumor growth [8], as patients receiving antipsychotics had a lower risk of developing gastrointestinal and brain malignancies, suggesting an anticancer effect of these drugs [20,23].

Numerous studies have shown that DRs are variably expressed across several types of cancers, and many investigations have demonstrated that each type of tumor has a unique pattern of DR expression. For instance, DRD3 uniquely expressed in glioblastoma at lower levels compared to a healthy brain, while DRD4 is specifically overexpressed in acute myeloid leukemia (AML). Alterations in DRs expression have been observed not only in cancer cells but also in cells linked to tumors. Manipulating the expression of DRs has been shown to affect various cancer cell functions, suggesting that these receptors play a significant role in tumor biology. In order to enhance clinical response in cancer patients, DRs were suggested as prospective therapeutic targets. However, it is currently unclear how DR-targeting medications may be used in cancer treatment [23].

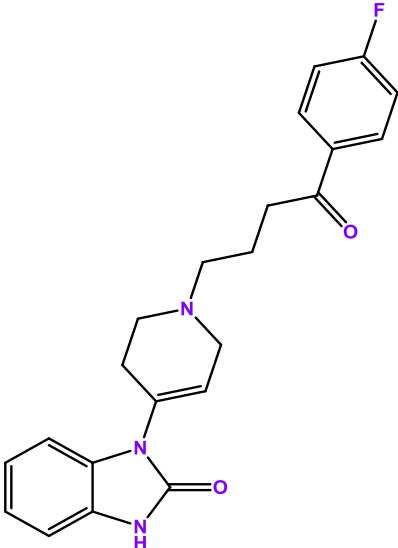
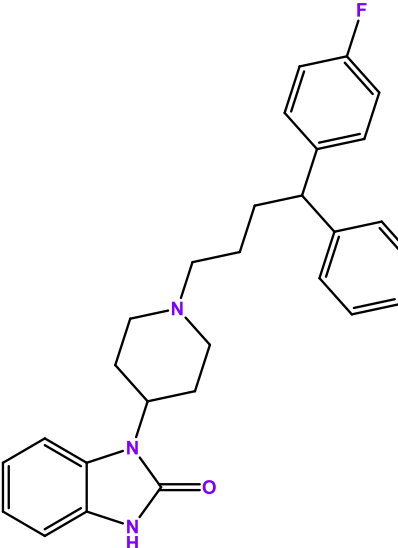
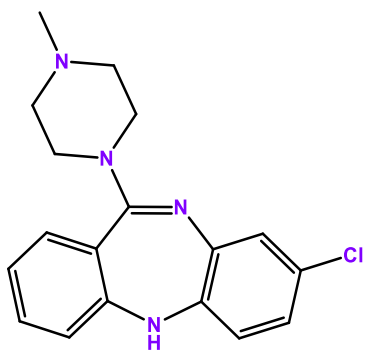
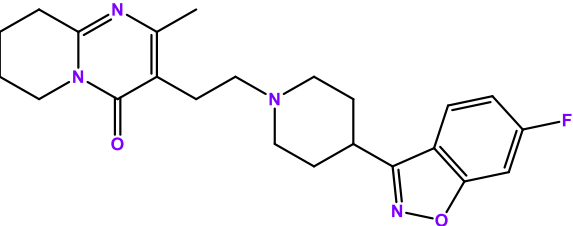
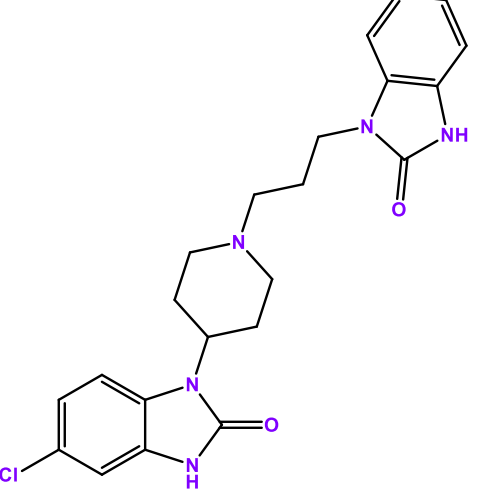
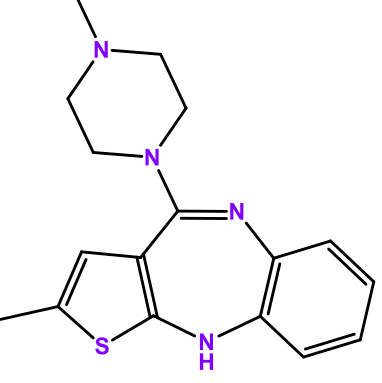
Rosas-Cruz et al. (2021) discovered that most of the medications under investigation have moderate or poor specificity, indicating that the biological effects seen may be partly due to the drugs' activity on other DRs, other GPCRs, or different cellular targets. For instance, the DRD2 antagonist pimozide, used as a second-line treatment for Tourette's disorder and shown to have anticancer properties, can also bind to alpha receptors, the serotonin receptor 5-HT7, and calmodulin. Despite their limited specificity, some DRs-targeting medications have attributes that make them appealing candidates for the development of novel anticancer therapeutics [23].

As more studies reveal that DA signaling in peripheral tissues is disrupted in cancer, this emerging field offers new insights into cancer cell vulnerabilities. It also highlights the potential of repurposing dopaminergic ligands and drugs developed for neuropharmacological purposes for cancer therapy. Given the time-consuming, costly, and frequently futile nature of medication research, repurposing dopaminergic drugs for cancer treatment could benefit both patients and drug developers [24].

The Six Promising Drugs

The six drugs that potentially offer the best opportunities for repurposing in oncology, targeting dopamine and its receptors, are listed in Table 1.

Table 1. The structures of the six oncology-repurposed drugs that use dopamine as target.

 Droperidol	 Pimozide
 Clozapine	 Risperidone
 Domperidone	 Olanzapine

Droperidol

Droperidol (DRO), a butyrophenone derivative approved by the FDA in 1970, is an antiemetic drug used in medicine for the control of psychosis and agitation, vertigo, and the treatment of benign headaches. In Europe, it is most often prescribed for the treatment of acute and chronic psychosis, while in the United States, it is predominantly used as an antiemetic [32,33]. Although droperidol was a widely used antiemetic in surgical areas for decades, the FDA increased significant about its safety in 2001. The agency issued one of its most severe warnings for an FDA-approved drug, linking DRO to QT segment prolongation and/or torsades de pointes, which, in some cases, resulted in fatal cardiac arrhythmia [34]. However, the effect of DRO antiemetic doses on cardiac arrhythmia remains uncertain, and the FDA's decision is being contested [33,34]. It is presumed that the cardiac risk associated with DRO is dosage dependent, leading to a growing trend of using the lowest effective doses of this medication, similar to the approach taken with other drugs with extremely severe side effects [34].

DRO exhibits activity at various biochemical locations, particularly as a dopamine (D2) receptor antagonist [33]. Nevertheless, no studies have yet demonstrated its anticancer activity for cancer treatment. Therefore, further research is necessary to explore droperidol's potential as a repurposed cancer drug.

Pimozide

Pimozide (PIM), a dopamine D2 receptor blocking agent, was approved by the FDA in 1984 for the treatment of Tourette's syndrome, chronic psychosis, resistant phonic, and motor tics [35,36]. By inhibiting dopaminergic, serotonergic, and other CNS receptors, PIM affects CNS neurons. And because of its affinity for the HERG channel, PIM exhibits minimal selectivity for D2 or 5-HT2A receptors, leading to several secondary changes in metabolism and central DA function that benefit resistant phonic tics and symptoms of schizophrenia and psychosis [35].

Several studies have demonstrated PIM's potential as an anticancer drug, showing promising results, especially against cancer stem cells (CSCs) responsible for chemoresistance [35,37]. PIM has already shown antiproliferative effects in various tumor cell lines, both in vitro and in vivo, including breast (MCF-7, T47D, ZR75-1B, MDAMB-231), lung (NSCLCA549, H460, HCC4006, e H1437), neuroblastoma, colorectal, pancreatic, hepatocellular carcinoma, melanoma, prostate, ovary, myeloproliferative neoplasms, osteosarcoma, and CNS tumors [35,36]. These antiproliferative effects may be due to several molecular target, such as reduced phosphorylation level of STAT5, cell cycle arrest

in G0/G1 phase and decreased levels of STAT3, decreased expression of Bcl-2, inhibition of the MAPK pathway and Ca²⁺ channels, and suppression of ERK signaling [36,38]. These mechanisms allow PIM to act effectively on tumor cells.

Clozapine

Clozapine (CLZ), a tricyclic dibenzodiazepine, is a drug that was approved by the FDA in 1983 and is still considered the most effective antipsychotic for the treatment of therapy-resistant schizophrenia [39,40]. Despite its efficacy, CLZ is still not widely administered due to its serious side effects and the need for frequent laboratory monitoring [41]. FDA as approved CLZ for two uses: treating patients with treatment-resistant schizophrenia and reducing the risk of self-harm in patients with suicidal behaviors [41].

CLZ act as a dopaminergic receptor antagonist, exhibiting high affinity for D4 receptors and low affinity for D2 receptors. It also binds with high affinity to multiple serotonin receptors [41], contributing to its antipsychotic efficacy. Despite all the above, and the fact that this drug is known to affect many neurotransmitters in the brain, its exact mechanism of action remains unclear [41]. Additionally, a study demonstrated that CLZ was able to reduce the number of AML cells after treatment, indicating potential anticancer activity in this tumor cell line [42].

Risperidone

Risperidone (RIS), a benzisoxazole derivative, was the first second-generation antipsychotic to be developed and it is a potent antagonist of the dopamine D2 and serotonin 5-HT₂ receptors found in the brain, with a significantly stronger antagonism of 5-HT_{2A} than D2 receptors [43-45]. Approved by the FDA in 1993, RIS is indicated for the acute and maintenance treatment of schizophrenia and related psychotic disorders, including acute bipolar mania [43].

The therapeutic activity of RIS is due to the combined effects of the drug and its primary metabolite, 9-hydroxy-risperidone, which shares similar receptor binding activity [43]. Extensive evidence supporting RIS's efficacy in treating both positive and negative symptoms of schizophrenia, along with a better extrapyramidal symptoms (EPS) tolerability profile than standard neuroleptics, especially at lower doses [44].

RIS has already demonstrated cytotoxic properties against proliferating gastric cancer cells and has also shown both neurotoxic and neuroprotective activities in neuroblastoma cells (SK-N-SH cell) [46,47]. Additionally, RIS has been found to decrease

the viability of colorectal cancer cells [48], highlighting its potential anticancer properties across different tumor cell lines.

Olanzapine

Olanzapine (OLZ), a thienobenzodiazepine derivative with a structure close to clozapine, was approved by the FDA in 1996 and is classified as an atypical antipsychotic [49,50]. This drug has the ability to bind to different types of dopaminergic (D1, D2, D3, D4, and D5) and serotonergic (5-HT_{2A/2C}, 5-HT₃, 5-HT₆, and 5-HT₇) receptors, with a higher affinity for 5-HT₂ receptors compared to dopamine receptors [50,51]. OLZ is used to treat manic episodes, schizophrenia, and bipolar disorder due to its antipsychotic, antimanic, and mood-stabilizing properties [50].

OLZ has shown multiple benefits in treating patients with advanced cancer by inhibiting the activity of certain GPCRs and other receptors [49]. Earlier studies have demonstrated its efficacy and safety in managing cancer-related symptoms connected to cancer, such as chemotherapy-induced nausea and vomiting, cancer pain, anorexia, and cachexia [51]. Additionally, OLZ has been shown to sensitize pancreatic CSCs to chemotherapeutic agents in vitro [23]. Moreover, the combination of OLZ and temozolomide, an alkylating agent, has been found to inhibit the growth of glioma cell lines more effectively than either treatment alone [52].

Domperidone

Domperidone (DOM) is a benzimidazole dopamine D₂ receptor antagonist that has never been approved by the FDA due to its association with adverse effects, particularly the risk of cardiac arrhythmias in cancer patients treated with high doses [53,54]. However, it has been available in several countries for several years and is commonly used to treat nausea and vomiting [55]. DOM acts on regions of the CNS that lack a blood–brain barrier, such as those that control prolactin production, but it has limited ability to penetrate the blood–brain barrier [53].

In the liver and intestinal wall, DOM is metabolized primarily and largely by cytochrome P450 3A4 (CYP3A4) [53]. DA inhibits tumor development and lung metastasis in vivo in a DRD₂-dependent manner, as these effects are reversed when DA and DOM, a DRD₂ antagonist, are given at the same time [23]. A previous study demonstrated that DOM can significantly contradict DA-mediated invasion inhibition in Hepa1-6 cells, suggesting a potential role for this drug in promoting liver carcinoma cell proliferation and invasion [56].

Table 2. Detailed summary of the six proposed drugs, including their families, targets and mechanisms of action.

Repurposed Drugs	Family	Target	Mechanism of Action
Droperidol	Antiemetic	D2 receptors	Antagonist [57]
Pimozide	First- generation antipsychotic	D2 receptors 5HT2A receptors	Antagonist [58]
Clozapine	Second-generation antipsychotic	D2 and D4 receptors serotonergic (5HT2A and 5HT2C) receptors adrenergic (α 1) receptors histamine (H1) receptors muscarinic (M1) receptors	Antagonist [59]
Risperidone	Second-generation antipsychotic	D2 receptors 5HT2 receptors	Antagonist [60]
Olanzapine	Second-generation antipsychotic	D2 and D3 receptors serotonergic (5HT2A and 5HT2B) receptors adrenergic (α 1) receptors histamine (H1) receptors muscarinic receptors	Antagonist [61]
Domperidone	Antiemetic	D2 and D3 receptors	Antagonist [62,63]

Aims

Given the importance of findings novel approaches for treating brain tumors (neuro-oncology tumors), the main aim of this master dissertation was to advance the pathway of drug repurposing in oncology using the DA synthesis cascade and propose rational lines of investigation to follow.

Thus, the study aimed to achieve the following specific objectives:

- 1) Assessing the effects of repurposed drugs, the dopamine receptors, on the cell viability of SH-SY5Y and A172 cells, within a 24, 48 and 72 hours incubation period;
- 2) Investigating the impact of the combinations of repurposed drugs with Tyrosine (Tyr) or Hydrogen Peroxide (H_2O_2) on the cell viability of SH-SY5Y and A172 cells, following 24, 48 and 72 hours of treatment incubation;
- 3) Examining the effect of drugs and drug combinations, along with Tyr and H_2O_2 , on extracellular DA and Tyr levels, after 24, 48 and hours of treatment incubation.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), and penicillin–streptomycin mixture were sourced from PAN-Biotech, while thiazolyl blue tetrazolium bromide (MTT; cat. no. M5655), tyrosine (cat. no. T3754) and hydrogen peroxide (30%; PerhydroITM; cat. no. 1.07209) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The six repurposed drugs, including Clozapine (cat. no. C6305), Domperidone (cat. no. D122), Droperidol (cat. no. D1414), Olanzapine (cat. no. O1141), Pimozide (cat. no. P1793) and Risperidone (cat. no. R3030), were also obtained from Sigma-Aldrich.

Cell Culture

The experimental work was performed with Human SH-SY5Y neuroblastoma and A172 glioblastoma cells lines (American Type Culture Collection, Manassas VA, USA). The cells were maintained at 37 °C in a humidified atmosphere with 95% air and 5% CO₂. The cells were cultured in DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin mixture (1000 U/mL; 10 mg/mL). For maintenance, cells were cultured in a monolayer and sub-cultured by trypsinization in the same medium when the cells reached a confluence of approximately 80%. The culture medium was changed every 3-4 days. Before each experiment, trypsin (0.25% trypsin-EDTA) was used to detach the cells. Afterward, cells were centrifuged at 1100 rpm for a duration of 5 minutes utilizing a Hettich centrifuge (Tuttlingen, Germany) and then seeded at a density of 4.2×10^4 cells/cm² in 96-well plates for the viability assays.

Table 3. Cells lines and their properties.

Cell Line	Disease	Tissue	Mutations
SH-SY5Y	Neuroblastoma	Bone; Marrow	MYCN; TP53; ALDH1A2; ALK; PTEN
A172	Glioblastoma	Brain	TP53; PTEN; CDKN2A; EGFR; PIK3CA; NF1; IDH1

Cell Treatment

For the treatment of the cells with the six drugs under investigation, CLZ, DOM, DRO, OLZ, PIM and RIS, the drugs were dissolved in dimethyl sulfoxide (DMSO). Each compound was prepared as a stock solution at a concentration of 200 mM for OLZ and RIS, and at a concentration of 100 mM for the remaining drugs. The concentrations tested in the cells ranged from 0.01 to 100 μ M. For the combinations, Tyr and H₂O₂ were dissolved in sterilized water (0.1% in cell culture minimum), and for these compounds a stock solution of 1000 mM for Tyr and 2 M for H₂O₂ were prepared. The amino acid Tyr was tested at concentrations of 25, 100, 250 e 500 μ M in the cell lines, while H₂O₂ was only tested at a concentration of 132 μ M.

For the combinations of the repurposed drugs with Tyr or H₂O₂, vehicles were composed of 0.1% DMSO in cell culture medium. Each treatment was tested at 24, 48, and 72 hours following the cell attachment to the plates. In all tested combinations, both agents were simultaneously introduced.

Cellular Viability Assay – MTT

Cellular viability was assessed at 24, 48, and 72 hours after the initiation of cell treatments using MTT assay. For the assay, following the removal of the culture medium, 100 μ L of MTT solution (0.5 mg/mL in PBS) was introduced into each well. Then, protected from the light, the cells were incubated at 37°C for 2 hours. Subsequently, MTT was removed and 100 μ L of DMSO was added to each well. Finally, absorbance values at 570 nm were measured in the automated microplate reader (Tecan Infinite M200, Zurich Switzerland) to evaluate the effects with the drugs alone and in combination on the cell viability of SH-SY5Y and A172 cells.

Evaluation of the Effect of Repurposed Drugs

The half of the maximum inhibitory concentration (IC₅₀) value was first determined individually for each drug in SH-SY5Y and A172 cells. The concentrations of the drugs used ranged from 0.01 to 100 μ M for single drug treatment. Subsequently, combination studies were performed by combining Tyr with the repurposed drugs and combining H₂O₂ with the repurposed drugs. Only drugs exhibiting the most promising pharmacological profile – specifically, CLZ, DOM and PIM for SH-SY5Y cells, and CLZ and RIS for A172 cells – were

further tested in combination with Tyr and H₂O₂. The concentrations of Tyr and H₂O₂ utilized were 500 µM and 132 µM, respectively.

Cell Morphology Visualization/ Assessments

After administering the drug treatment, the morphological features of SH-SY5Y and A172 cells were examined and recorded utilizing a Leica DMI 6000B Automated Microscope coupled to a Leica DFC350 FX camera (Leica Microsystems, Wetzlar, Germany). The cell-containing plate was positioned on the microscope, and the cell images were analyzed on a computer through the application of Leica Las X imaging software (v3.7.4) (Leica Microsystems, Wetzlar, Germany).

HPLC Analysis

For uHPLC procedure, samples from extracellular were subjected to the uHPLC, and the separation was carried out at a flux of 2 mL/min. Optical density of all the tested compounds was recorded at 280 nm. Quantification was performed based on standard curves for L-Tyr. Results were analyzed using the Chromera® software, version 3.2.0, Perkin Elmer (Waltham, MA, USA).

Tyr concentrations between 0.01 to 1000 µM were used to generate the calibration curve. The resulting equation ($y = 293,02x + 2141,5$; $R^2 = 0,9995$) was used to calculate Tyr concentration presented in each analyzed sample, following the recommendations of the manufacturer manual.

Data Analysis

GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) was used to create bar graphs of the cell viability and to produce concentration–response curves by nonlinear regression analysis. The viability of cells treated with each drug was normalized to the viability of control cells and cell viability fractions were plotted versus drug concentrations on a logarithmic scale.

Statistical Analysis

Statistical analysis was performed in all experiments. The results are expressed as the arithmetic mean $\pm$ standard error of the mean (SEM) from three independent cell culture

preparations, explicit in the legends of the graphs. Differences between the treated cells and the corresponding untreated control were tested using one-way ANOVA. The differences will be considered statistically significant when p value < 0.05 . All statistical analyses, graph constructions and calculations of IC₅₀ values were executed using GraphPad Prism 8 software.

Synergism Studies

Using the CompuSyn software (version 1.0; ComboSyn, Paramus, NJ, USA) and the Chou–Talalay equation, we assessed the combination index (CI) of various drug combinations using a non-fixed ratio. In this context, a CI less than 1 indicates synergism between the drugs, a CI equal to 1 indicates additivity, and a CI greater than 1 indicates antagonism.

Results

Effects of the Repurposed Drugs on Cellular Viability of SH-SY5Y and A172 cell lines

To evaluate the effects of the repurposed drugs on the viability of SH-SY5Y (Figures 5 and 6) and A172 (Figures 7 and 8) cellular lines, the cells were treated with increasing concentrations of these drugs, over an incubation period of 24, 48 and 72 hours. Cell viability percentage was evaluated using the MTT assay (Figures 5 and 7).

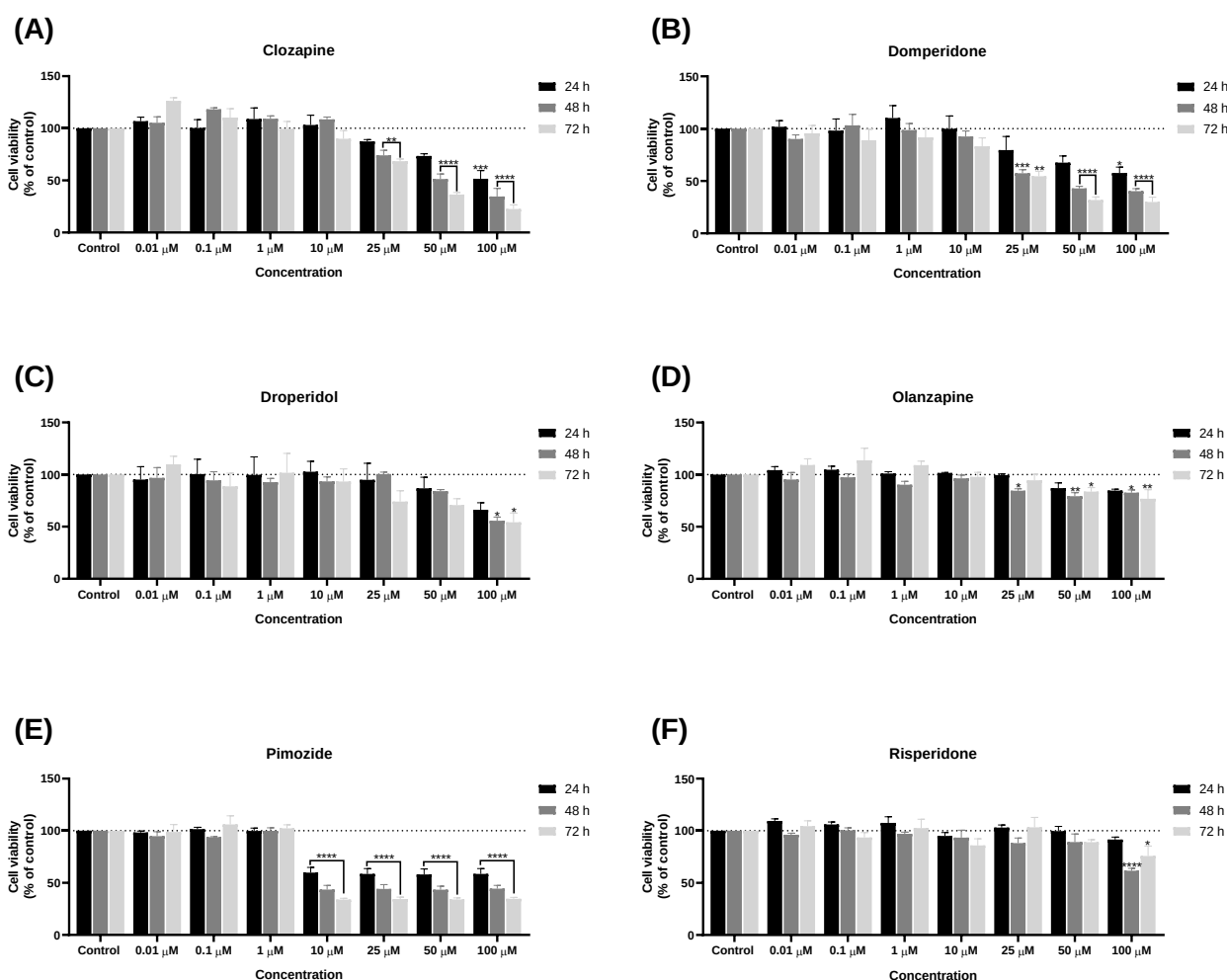
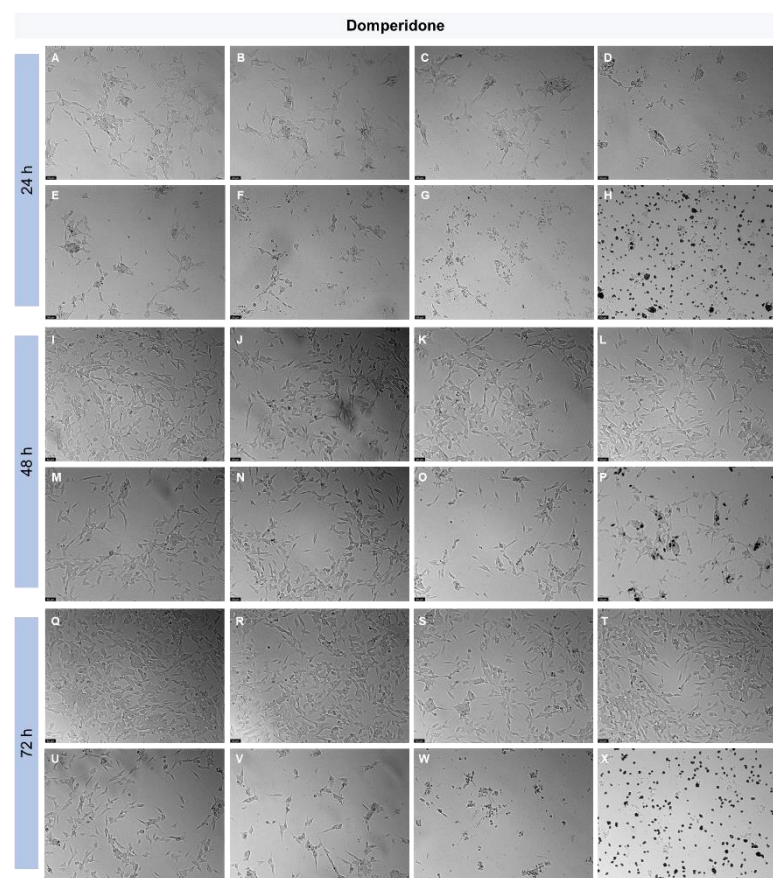
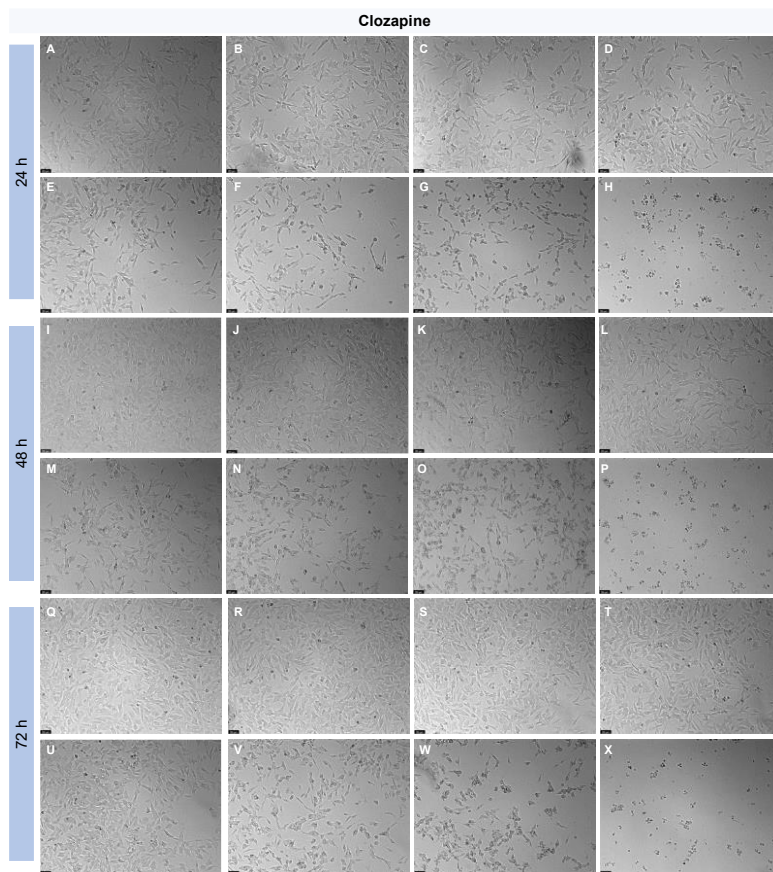
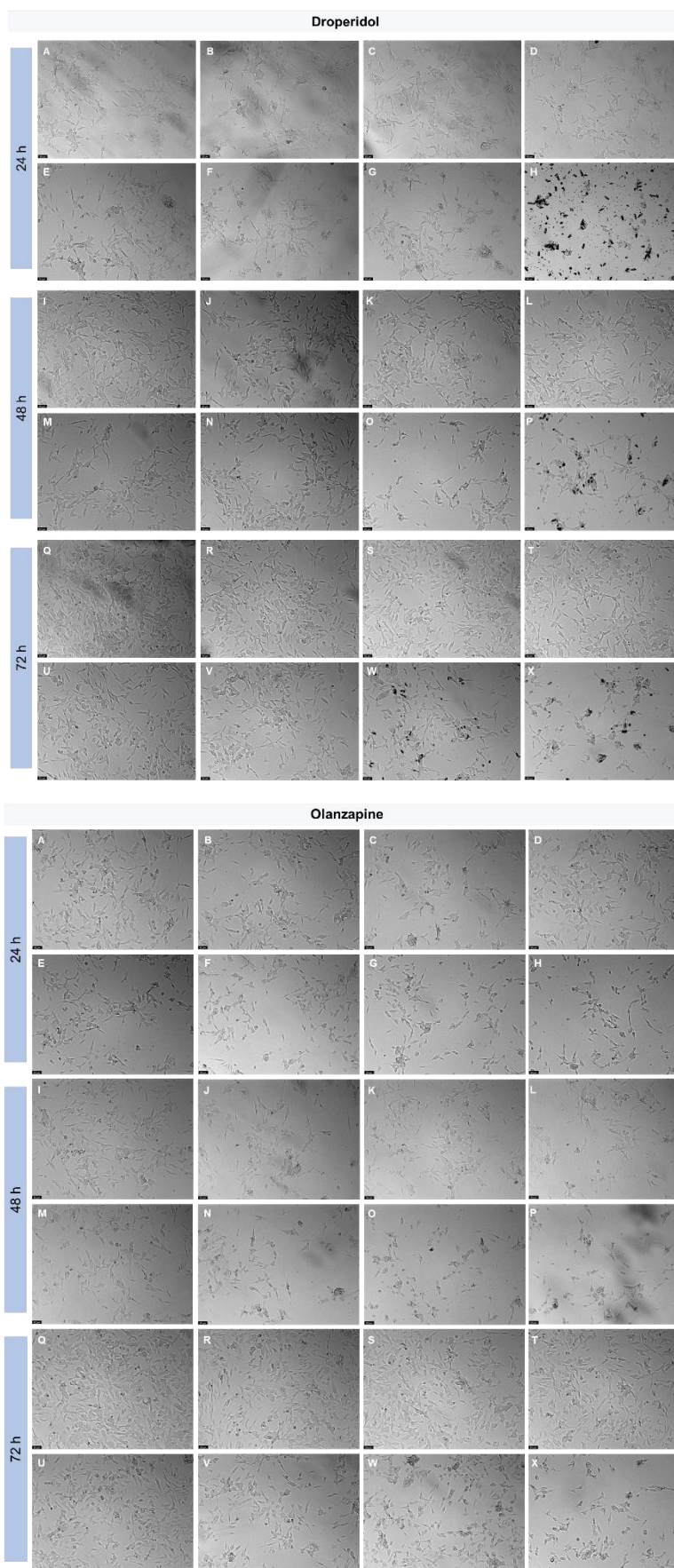


Figure 5. Effect of CLZ (A), DOM (B), DRO (C), OLZ (D), PIM (E) and RIS (F) on the viability of SH-SY5Y cells. The cells were cultured in the presence of increasing concentrations of the six drugs and treated for 24, 48 and 72 hours. Then cell viability was determined using the MTT assay. Results are expressed as percentage of the vehicle-treated control $\pm$ SEM of three separate experiments. One-way ANOVA was used as statistical test. Statistically significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. control.





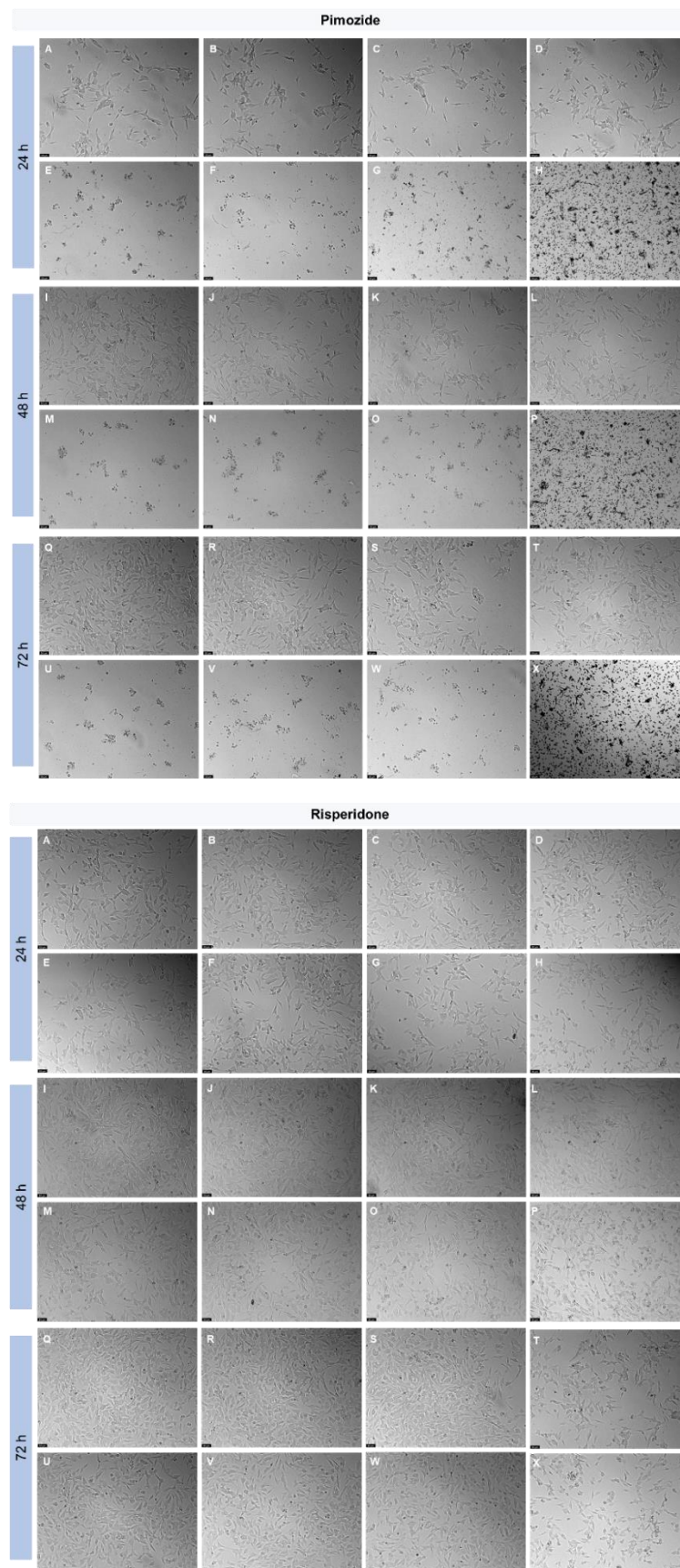


Figure 6. Microscopic visualization of the effects of CLZ, DOM, DRO, OLZ, PIM and RIS on the morphology of SH-SY5Y cells over 24, 48 and 72 hours. Cells were treated with (A,I,Q) 0.1% DMSO

(control), (**B,J,R**) 0.01 μ M, (**C,K,S**) 0.1 μ M, (**D,L,T**) 1 μ M, (**E,M,U**) 10 μ M, (**F,N,V**) 25 μ M, (**G,O,W**) 50 μ M and (**H,P,X**) 100 μ M of CLZ, DOM, DRO, OLZ, PIM and RIS. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

Analyzing the Figure 5 we verify that only three drugs of the six repurposed drugs have an inhibitory effect on the growth of the SH-SY5Y cells. DRO, OLZ and RIS showed no toxicity against neuroblastoma cells, thus demonstrating no anticancer activity in these tumor cell line. These results are further confirmed by Figure 6, which shows no morphological alterations in the cells at any concentrations and times tested for these three drugs. Regarding the other three repurposed drugs, CLZ, DOM and PIM, these drugs demonstrated statistically significant inhibitory effects on cell growth compared to the control at the highest concentrations tested.

CLZ exhibits a concentration and time dependent inhibition starting at 25 μ M, with the lowest cell viability percentage reaching approximately 23% at a concentration of 100 μ M after 72h (Figure 5 - Clozapine). Additionally, the microscopic images (Figure 6 – Clozapine) show that at the highest concentrations studied (Figures 6 - Clozapine - F, G, H), the cells changed shape, becoming rounder and smaller, with a reduced number of cells in the well. This indicates that the cells are no longer viable, demonstrating that CLZ exhibits neurotoxic effects on SH-SY5Y cells.

Regarding DOM, cell viability begins to decline at a concentration of 25 μ M and continues to decrease in a concentration and time dependent manner. The most significant reductions in the percentage of cell viability are observed at 48 and 72 hours. Although, there is some reduction at 24 hours, it is less pronounced (Figure 5 – Domperidone). The greatest decrease in the number of cells occurred at 72 hours with the 100 μ M concentration, where cell viability drops to approximately 30%, a statistically significant reduction. Additionally, the microscope images show that at higher concentrations, similar to the effects of CLZ, cells become rounder and smaller (Figures 6 -Domperidone - F, G, H).

In relation to PIM, we verified that cell viability begins to decrease at a concentration of 10 μ M and continues to decline in a concentration- and time- dependent manner, similar to the effects observed with the previous two drugs (Figure 5 - Pimozide). The drug becomes more effective over time, with 48 and 72 hours showing more significant cell death. The highest level of cell death, 66%, was visualized at 72 hours at the concentration of 10 μ M, leaving cell viability at around 34%. In the microscope images (Figures 6 -Pimozide -

F, G, H), we can also see that starting from a 10 μM concentration, the cells adopt a rounder and smaller shape.

Among the three most effective repurposed drugs, PIM demonstrated the greatest potential as an anticancer agent, highlighting the highest cytotoxic properties in the neuroblastoma cell line.

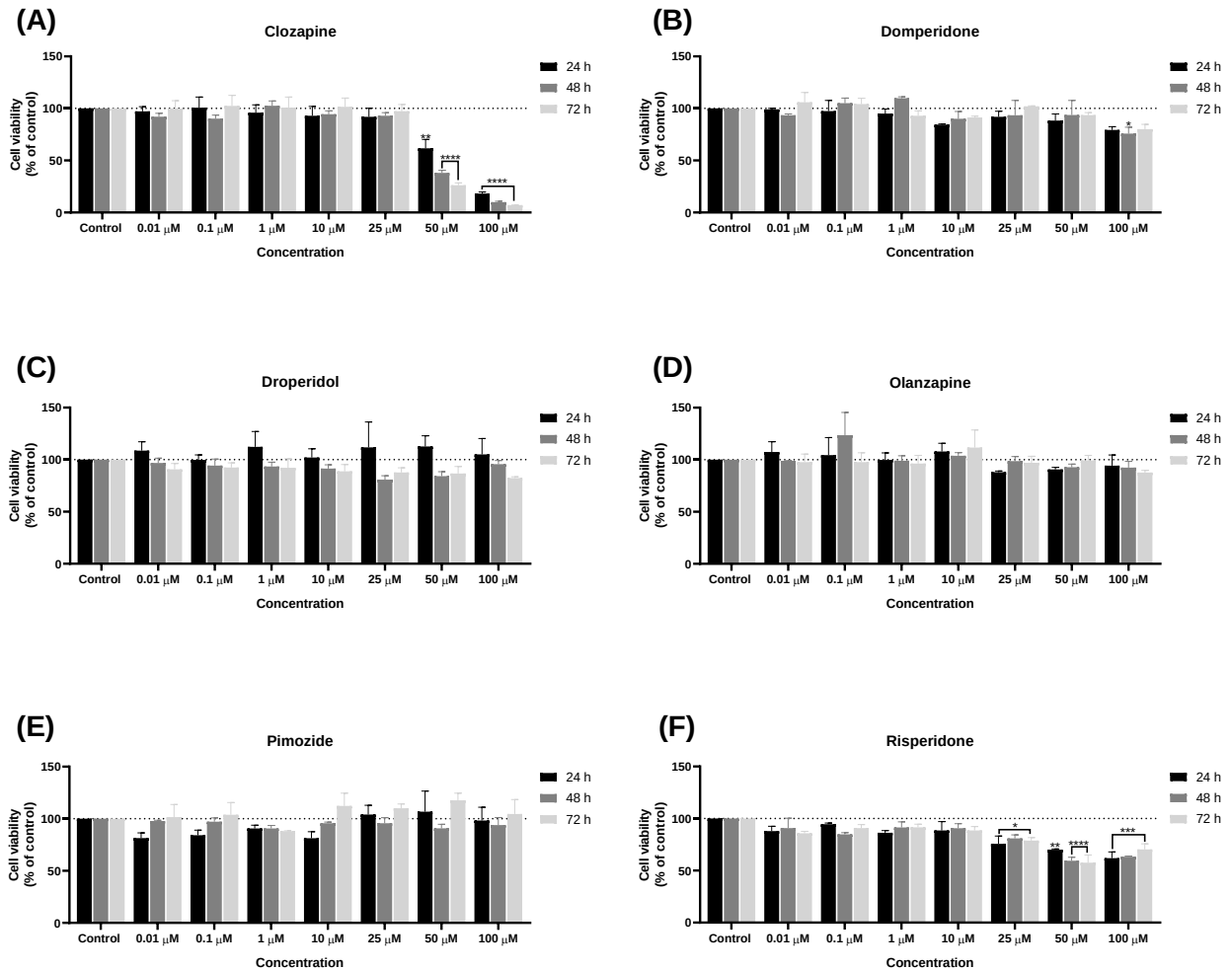
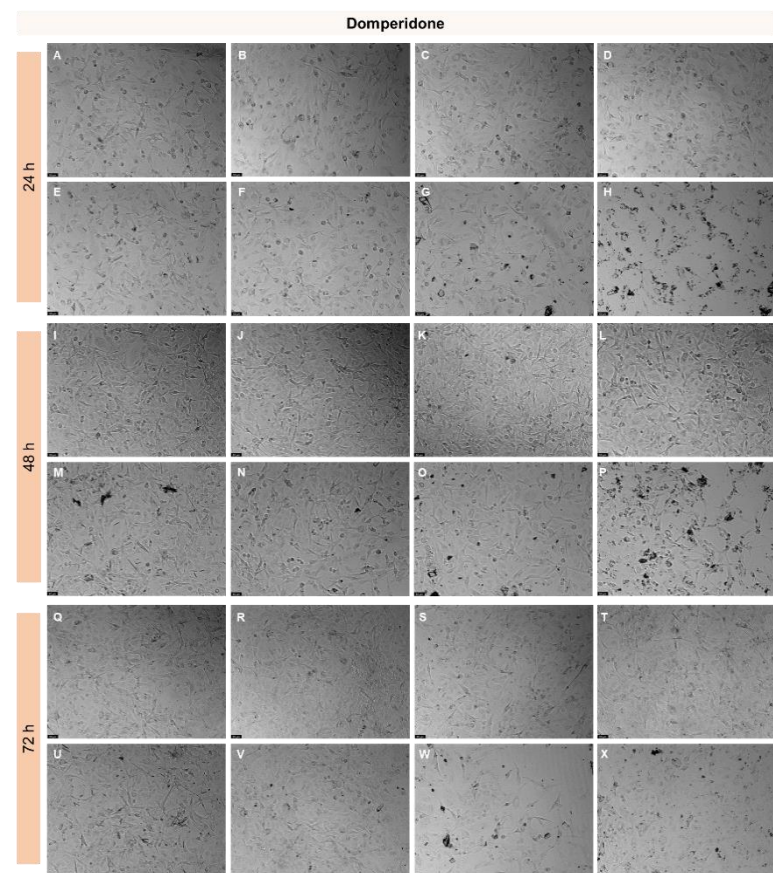
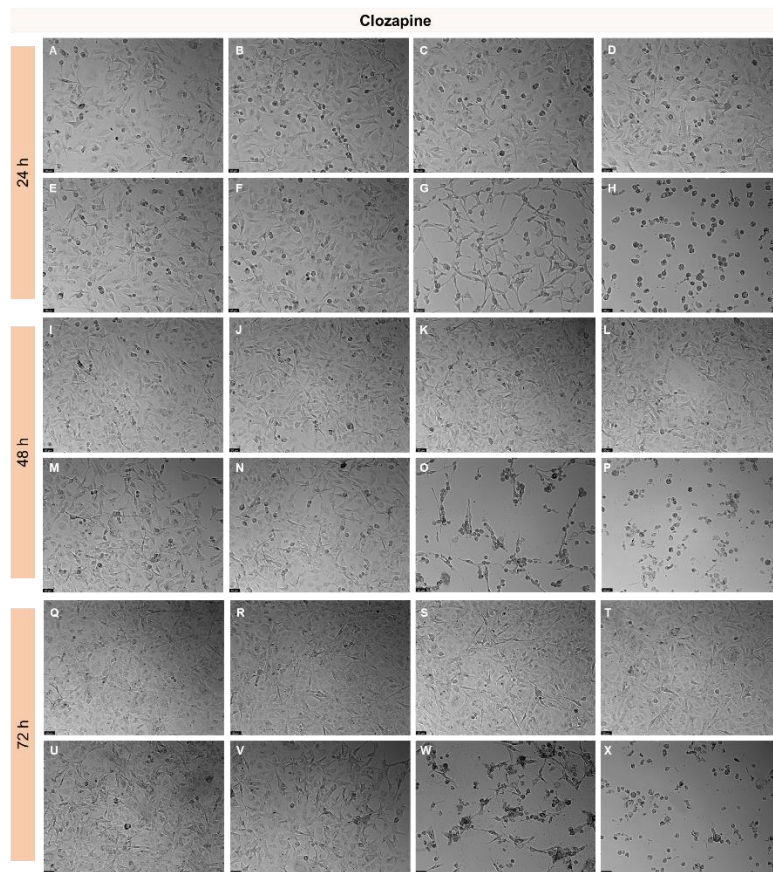
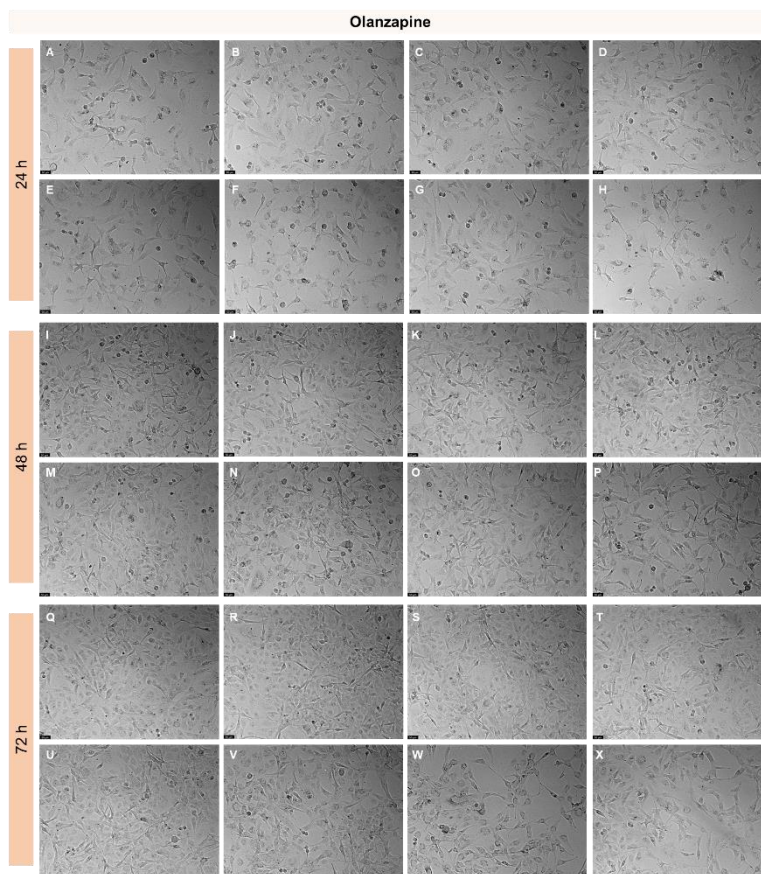
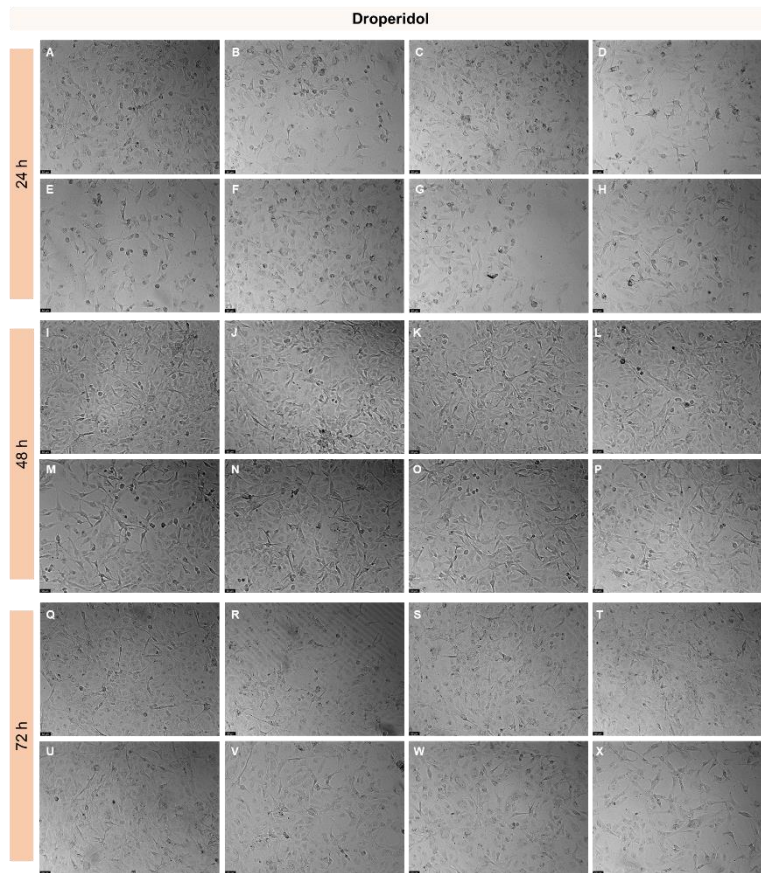


Figure 7. Effect of CLZ (A), DOM (B), DRO (C), OLZ (D), PIM (E) and RIS (F) on the viability of A172 cells. The cells were cultured in the presence of increasing concentrations of the six drugs and treated for 24, 48 and 72 hours. Then cell viability was determined using the MTT assay. Results are expressed as percentage of the vehicle-treated control $\pm$ SEM of three separate experiments. One-way ANOVA was used as statistical test. Statistically significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. control.





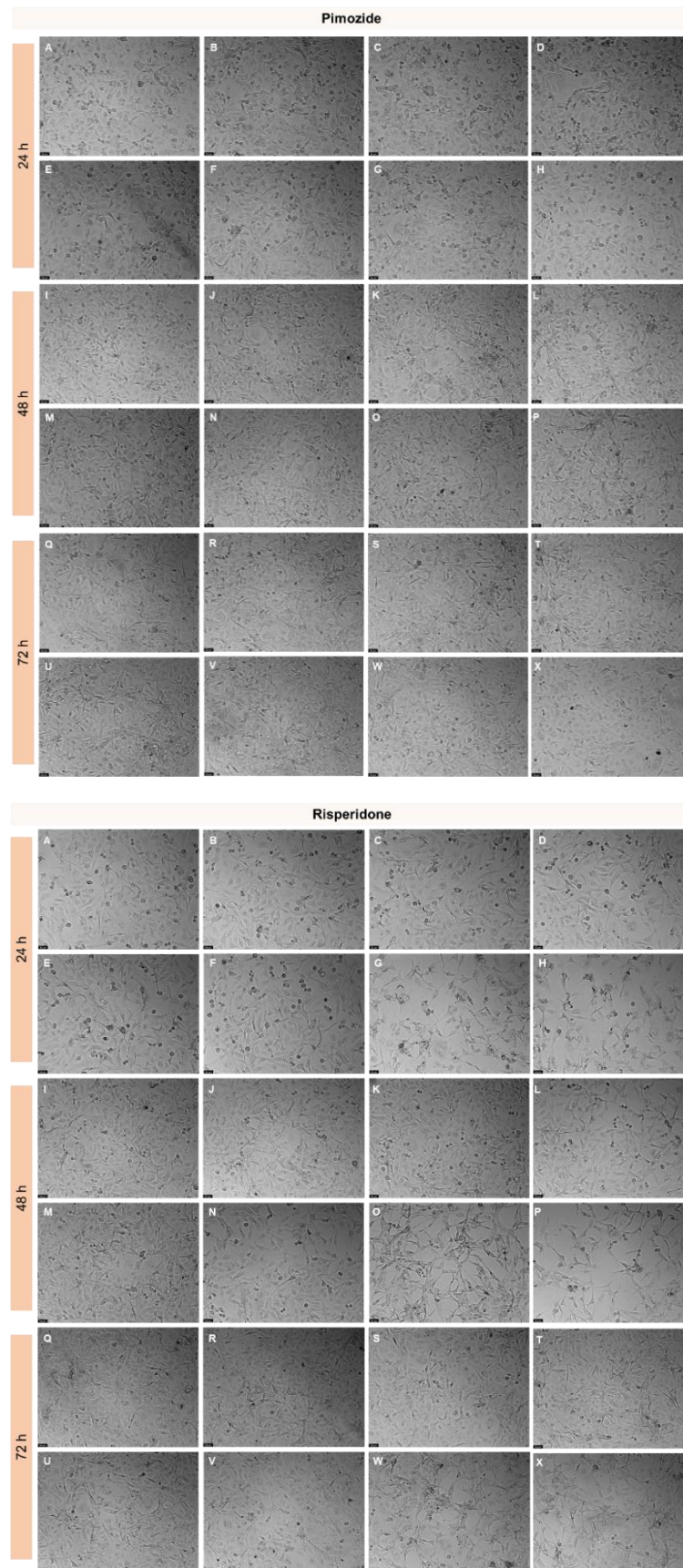


Figure 8. Microscopic visualization of the effects of CLZ, DOM, DRO, OLZ, PIM and RIS on the morphology of A172 cells over 24, 48 and 72 hours. Cells were treated with (A,I,Q) 0.1% DMSO

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(control), (B,J,R) 0.01 μ M, (C,K,S) 0.1 μ M, (D,L,T) 1 μ M, (E,M,U) 10 μ M, (F,N,V) 25 μ M, (G,O,W) 50 μ M and (H,P,X) 100 μ M of CLZ, DOM, DRO, OLZ, PIM and RIS. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

Analyzing the Figure 7, we found that only two of the six repurposed drugs, CLZ and RIS, showed toxic effects on the viability of the glioblastoma cell line A172. The other four drugs showed no cytotoxicity on these cells. On the contrary, two of them demonstrated neuroprotective activity.

DRO and PIM exhibited the most pronounced neuroprotective effects. The DRO evidenced this activity only at 24 hours, as evidenced by an increase in cell viability above 100%, particularly at the 50 μ M concentration, which reached approximately 113% (Figure 8 - Droperidol). Despite, this increase was not statistically significant when compared to control. Except for the concentrations of 0.1 μ M and 10 μ M, no significant increase in viability was observed.

As for PIM, this drug demonstrated neuroprotective activity only at higher concentrations and most notably at 72 hours. In the PIM graph in Figure 7, its clearly visible an increase in cell viability for the concentrations of 10 μ M and above. The highest increase was noted at 72 hours for the 50 μ M concentration. Similar to DRO, these increases were not statistically significant when compared to the control.

In relation to the drugs that demonstrated neurotoxic effects on the A172 cell line, the CLZ graph in Figure 7 shows that the cell viability remains constant and close to the 100% for the first five concentrations across all times. However, starting from a concentration of 25 μ M, a very marked decrease in cell viability is observed, which is dependent on both time and concentration, and is always statistically significant compared to the control. The lowest cell viability, approximately 7%, is observed for the 100 μ M concentration at 72h, indicating a cellular death of around 93% in the glioblastoma cells treated with the highest concentration of CLZ. These results are also corroborated by the microscopic images in Figure 8 (Clozapine - G, H, O, P, W e X) where it is visualized a considerably reduced number of cells at higher concentrations and noticeable differences in cell shape and size, indicating the anticancer activity of this drug in this cell line.

As for RIS, the graph in Figure 7 shows a slight decrease in cellular viability at all concentrations and times. However, this decrease becomes more pronounced and statistically significant at the highest concentrations (25, 50 and 100 μ M). The lowest cell viability, around 57%, is reached at 72h for the 50 μ M concentration, indicating about 43%

cell death. These findings are confirmed by the microscopic images in Figure 8 (Risperidone - G, H, O, P, W and X), which reveal changes in cell shape, with cells appearing more stretched, thinner and fewer in number. Despite not as active and effective in these cells as CLZ, RIS may also exhibit anticancer effects in this type of solid tumor.

To summarize all the results obtained from the two cell lines and assess the drug efficacy, Table 4 presents the half-maximal inhibitory concentration (IC₅₀) values for all the repurposed drugs. The IC₅₀ represents the concentration of a compound that is needed to inhibit 50% of a biological response or activity. As shown in Table 1, of the six repurposed drugs, CLZ was the only drug effective in both cell lines, with lower IC₅₀ values observed in SH-SY5Y cells. The lowest IC₅₀ value obtained was 27.4 μ M, indicating that a concentration of 27.4 μ M of CLZ is required to inhibit 50% of the viability of SH-SY5Y. Additionally, DOM and PIM were effective in the SH-SY5Y cell line, with IC₅₀ values of 17.6 μ M and 2.9 μ M at 72 hours, respectively. In contrast, for the A172 cell line, only CLZ achieved IC₅₀ values below 100 μ M, underlining its unique efficacy in this type of cell compared to the other drugs tested.

Overall, these findings suggest that CLZ is promising for treating both glioblastoma and neuroblastoma diseases affecting both cell lines, while DOM and PIM may be more selective for neuroblastoma cells.

Table 4. IC₅₀ (half-maximal inhibitory concentration) values of the six repurposed drugs in SY-SY5Y and A172 cells.

Repurposed Drugs	IC ₅₀ (SH-SY5Y, μ M)			IC ₅₀ (A172, μ M)		
	24 h	48 h	72h	24 h	48 h	72h
Clozapine	41	31.4	27.4	50.8	42.2	41.3
Domperidone	30.3	18.6	17.6	>100	>100	>100
Droperidol	>100	>100	>100	>100	>100	>100
Olanzapine	>100	>100	>100	>100	>100	>100
Pimozide	6.6	1.8	2.9	>100	>100	>100
Risperidone	>100	>100	>100	>100	51.6	>100

Effects of the Tyrosine on Cellular Viability of SH-SY5Y and A172 cell lines

To assess the impact of Tyr on the viability of SH-SY5Y and A172 cell lines, the cells were treated with this amino acid at concentrations of 25, 100, 250 and 500 μM , over incubation periods of 24, 48 and 72 hours. Cell viability percentage was measured using the MTT assay (Figures 9A and B) and morphological analyses were also performed on the cells treated with Tyr (Figure 10).

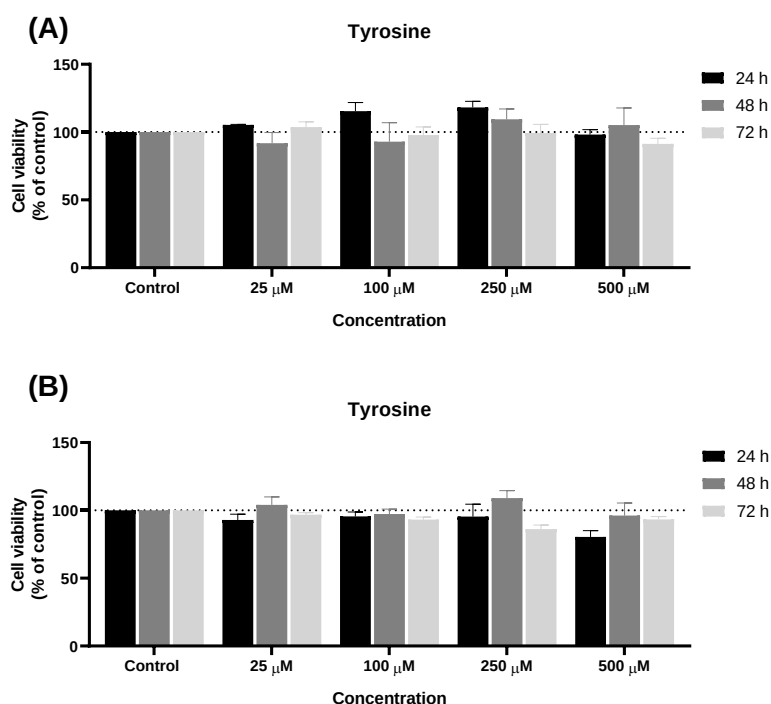


Figure 9. Effect of Tyr on the viability of SH-SY5Y (A) and A172 (B) cells. The cells were cultured in the presence of increasing concentrations of Tyr and treated with Tyr for 24, 48 and 72 hours. Then cell viability was determined using the MTT assay. Results are expressed as percentage of the vehicle-treated control $\pm$ SEM of three separate experiments.

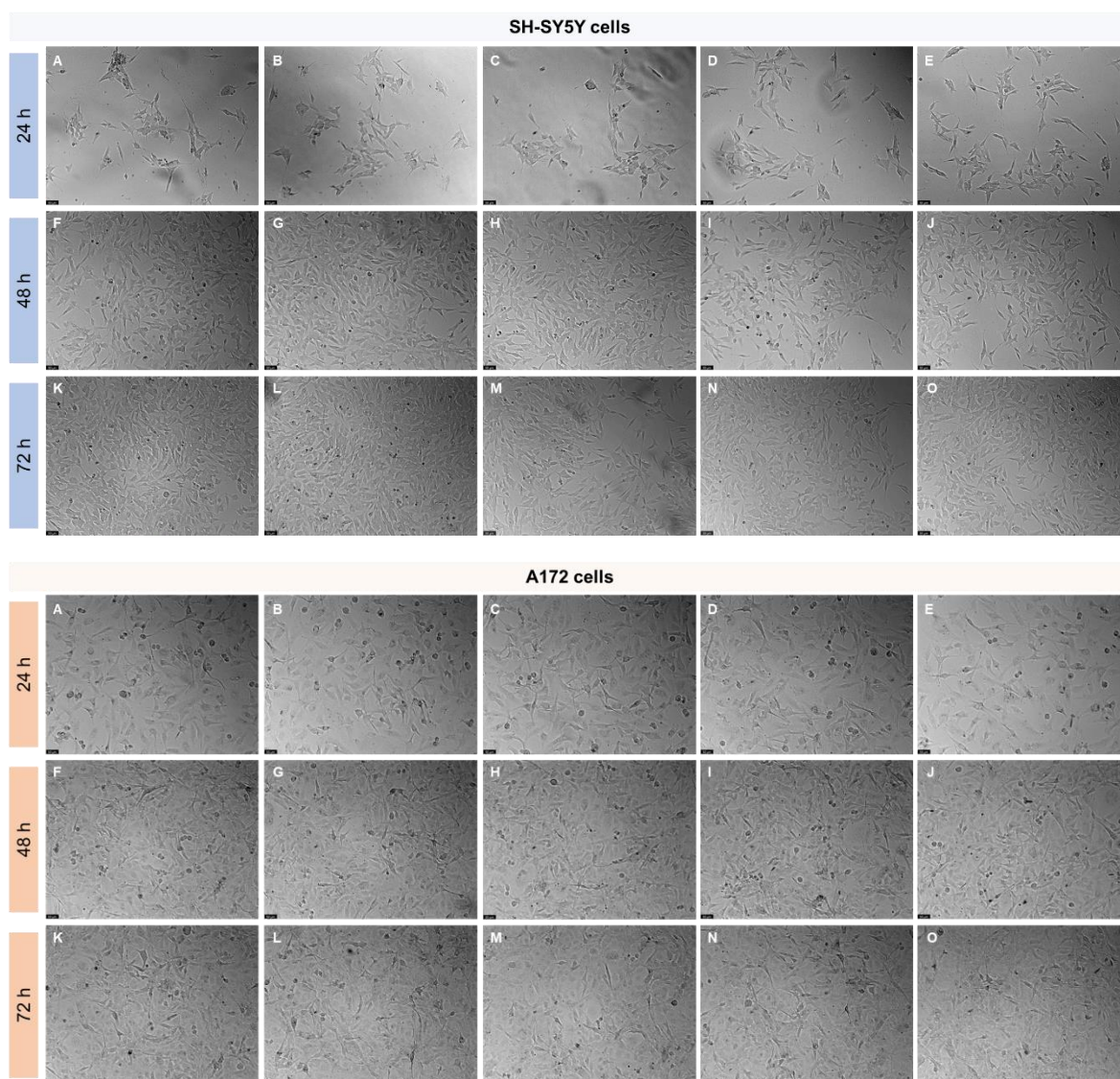


Figure 10. Microscopic visualization of the effects of Tyrosine on the morphology of SH-SY5Y and A172 cells over 24, 48 and 72 hours. Cells were treated with (A,F,K) 1% Water (control), (B,G,L) 25 μ M, (C,H,M) 100 μ M, (D,I,N) 250 μ M and (E,J,O) 500 μ M of Tyr. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ M.

The results showed that Tyr was not toxic to both cell lines at all concentrations and for all times (Figure 9). This lack of toxicity is further supported by the cell morphology, which showed no changes in the shape or size of the cells in either cell line (Figure 10). These findings were anticipated, as Tyr is a non-essential amino acid that can be produced by the body.

Effects of the Combinations of the Repurposed Drugs with Tyrosine on Cellular Viability of SH-SY5Y and A172 cell lines

To evaluate the combinations of the repurposed drugs with Tyr on the viability of SH-SY5Y and A172 cells, the cells were treated with two concentrations of the repurposed drugs and with increasing concentrations of Tyr, and then incubated for 24, 48, and 72 hours. Cell viability was determined using the MTT assay (Figures 11 and 15). Following this, morphological analyses were performed on the cells treated with these combinations (Figures 12, 13, 14, 16 and 17).

The two concentrations of the repurposed drugs were selected based on the IC₅₀ values previously determined. Specifically, we chose concentrations that we had previously tested, ensuring the IC₅₀ value fell between them. For CLZ, the concentrations of 25 μ M and 50 μ M were selected, as the highest IC₅₀ observed was around 40 μ M. Similarly, for DOM, the concentrations of 10 μ M and 25 μ M were chosen, for PIM, the concentration of 1 μ M and 10 μ M were selected and for RIS, the concentrations of 50 μ M and 100 μ M were picked, based on their respective IC₅₀ values.

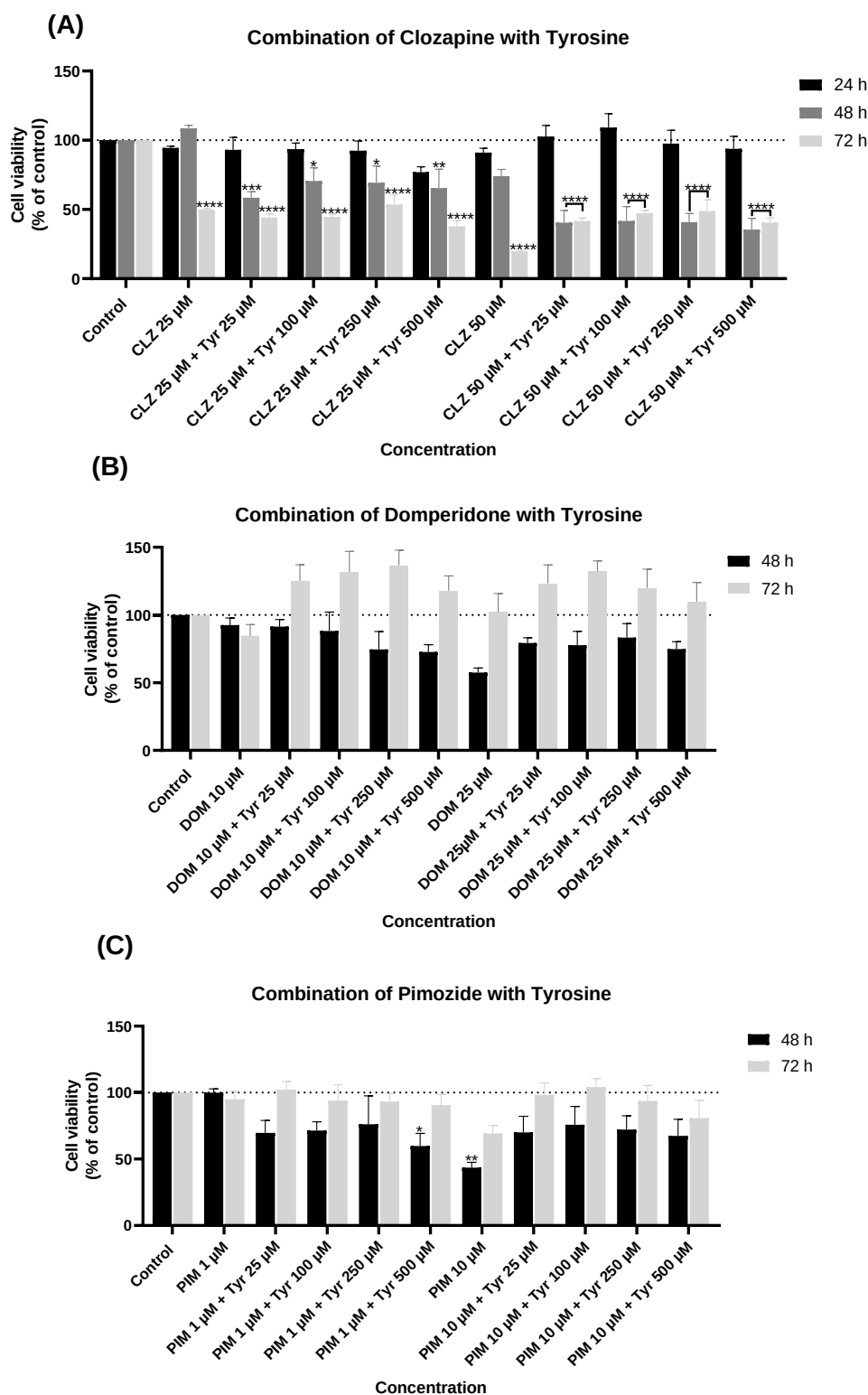


Figure 11. Effect of CLZ (A), DOM (B) and PIM (C) combined with Tyr on the viability of SH-SY5Y cells. The cells were cultured in the presence of two concentrations of CLZ, DOM and PIM and with increasing concentrations of Tyr and treated for 24, 48, and 72 hours. Then, cell viability was determined using the MTT assay. Results are expressed as a percentage of the vehicle-treated

control $\pm$ SEM of three separate experiments. Statistically significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. control.

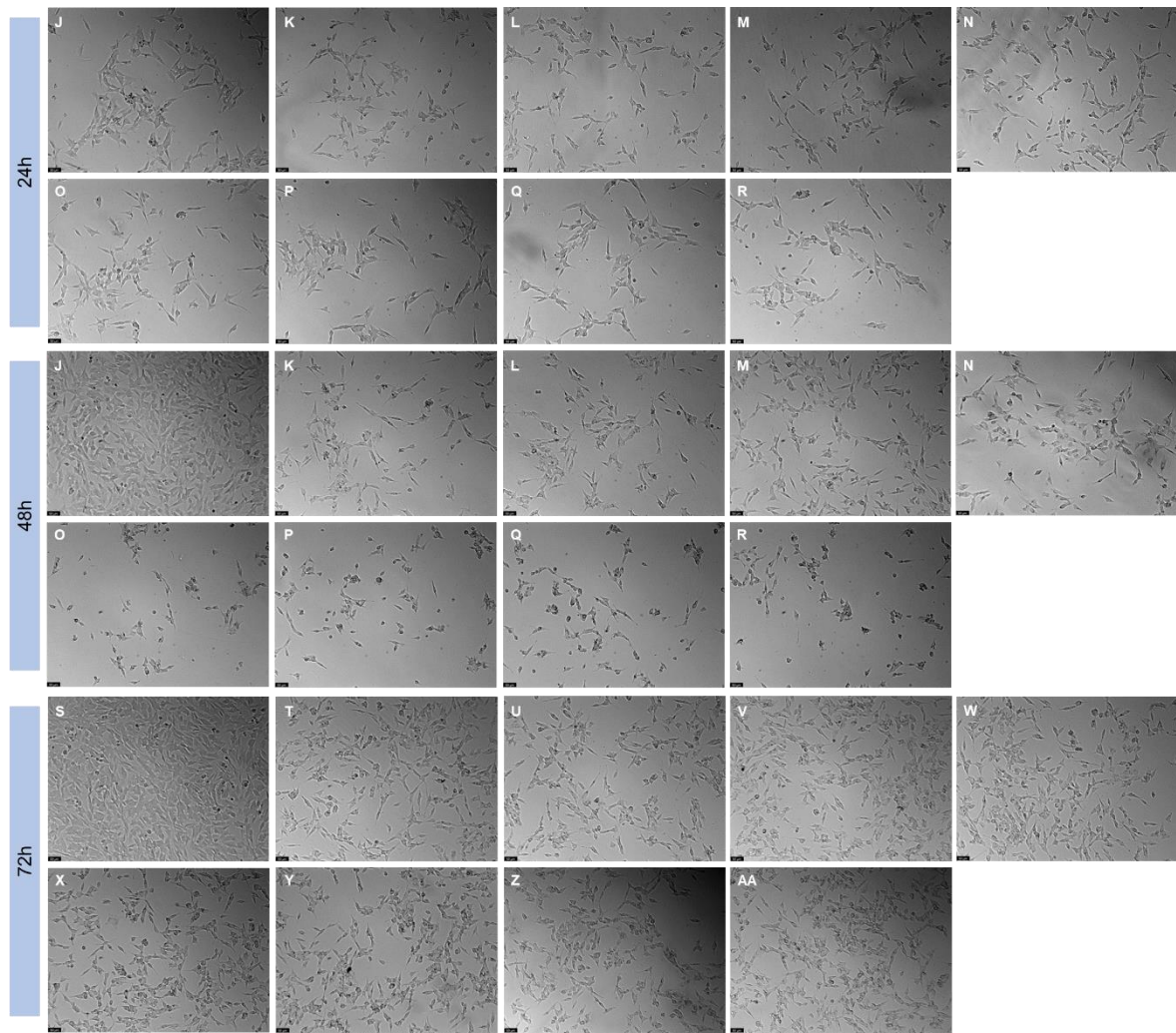


Figure 12. Microscopic visualization of the effects of Clozapine combined with Tyr on the morphology of SH-SY5Y cells over 24, 48 and 72 hours. Cells were treated with (**A,J,S**) 0.1% DMSO (control), (**B,K,T**) CLZ 25 μ M + Tyr 25 μ M, (**C,L,U**) CLZ 25 μ M + Tyr 100 μ M, (**D,M,V**) CLZ 25 μ M + Tyr 250 μ M, (**E,N,W**) CLZ 25 μ M + Tyr 500 μ M, (**F,O,X**) CLZ 50 μ M + Tyr 25 μ M, (**G,P,Y**) CLZ 50 μ M + Tyr 100 μ M, (**H,Q,Z**) CLZ 50 μ M + Tyr 250 μ M and (**I,R,AA**) CLZ 50 μ M + Tyr 500 μ M. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

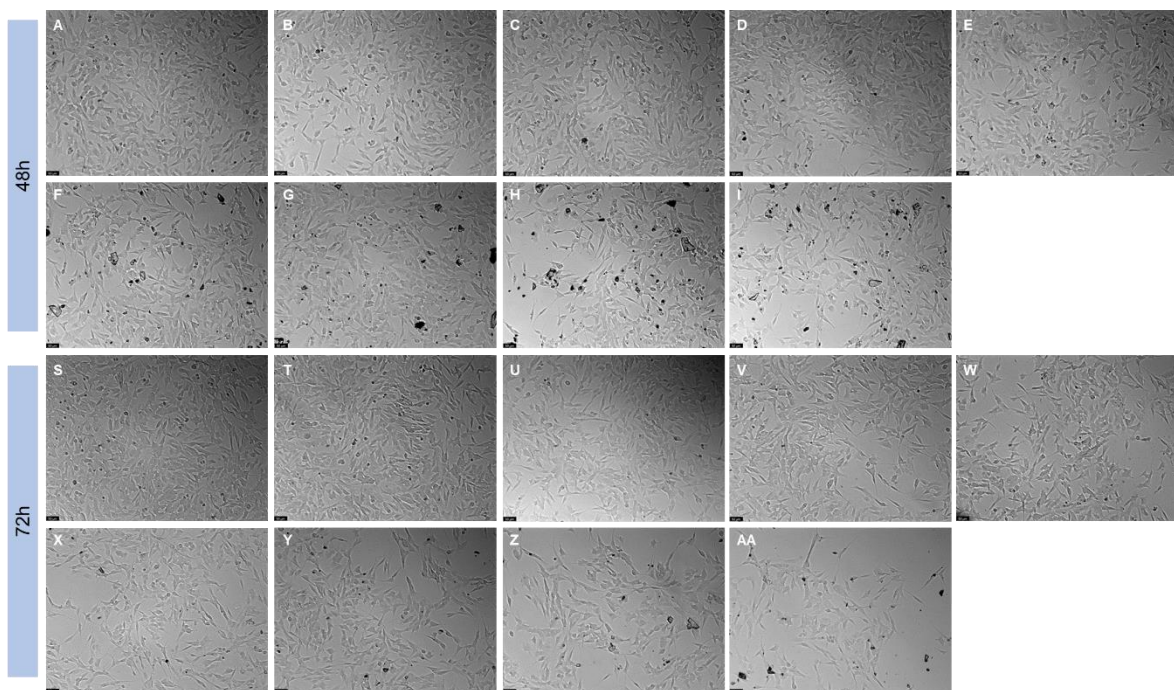


Figure 13. Microscopic visualization of the effects of DOM combined with Tyr on the morphology of SH-SY5Y cells over 48 and 72 hours. Cells were treated with (A,J,S) 0.1% DMSO (control), (B,K,T) DOM 10 μ M + Tyr 25 μ M, (C,L,U) DOM 10 μ M + Tyr 100 μ M, (D,M,V) DOM 10 μ M + Tyr 250 μ M, (E,N,W) DOM 10 μ M + Tyr 500 μ M, (F,O,X) DOM 25 μ M + Tyr 25 μ M, (G,P,Y) DOM 25 μ M + Tyr 100 μ M, (H,Q,Z) DOM 25 μ M + Tyr 250 μ M and (I,R,AA) DOM 25 μ M + Tyr 500 μ M. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

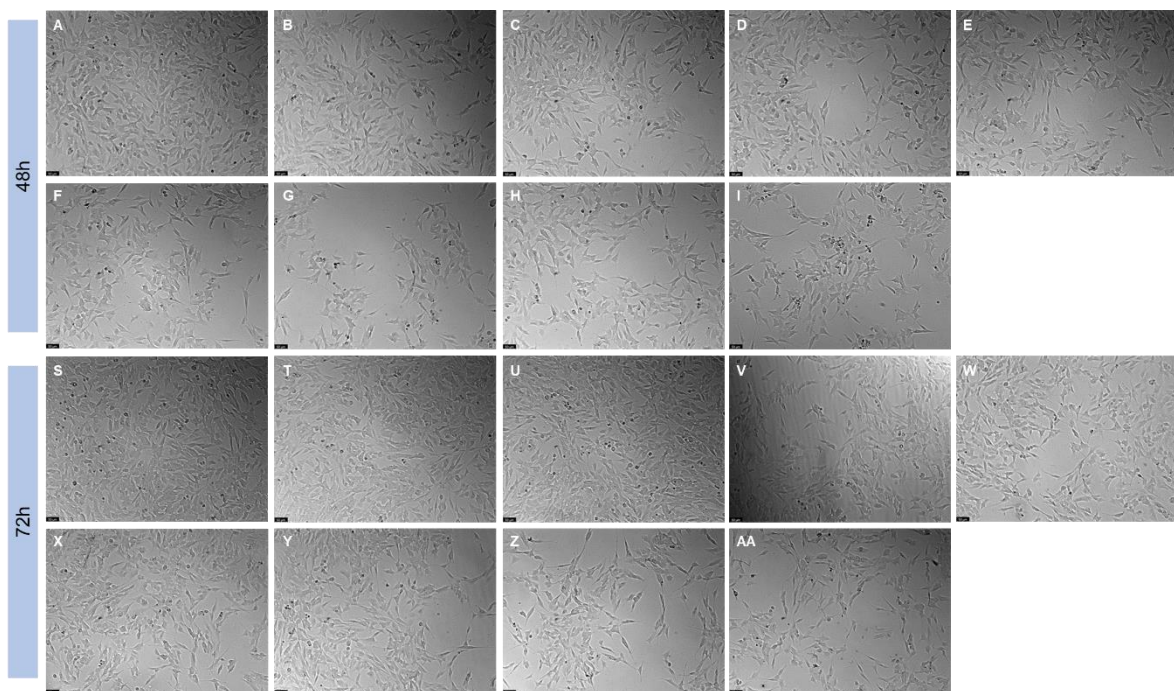


Figure 14. Microscopic visualization of the effects of PIM combined with Tyr on the morphology of SH-SY5Y cells over 48 and 72 hours. Cells were treated with (A,J,S) 0.1% DMSO (control), (B,K,T)

PIM 1 μ M + Tyr 25 μ M, (C,L,U) PIM 1 μ M + Tyr 100 μ M, (D,M,V) PIM 1 μ M + Tyr 250 μ M, (E,N,W) PIM 1 μ M + Tyr 500 μ M, (F,O,X) PIM 10 μ M + Tyr 25 μ M, (G,P,Y) PIM 10 μ M + Tyr 100 μ M, (H,Q,Z) PIM 10 μ M + Tyr 250 μ M and (I,R,AA) PIM 10 μ M + Tyr 500 μ M. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

For the combinations of CLZ with Tyr (Figures 11A and 12), no observable changes in cellular viability were seen at 24 hours. However, at 48 and 72 hours, there were significant decreases in cell viability. The most pronounced decreases were observed with the combinations of 50 μ M CLZ with Tyr, particularly at both 48 and 72 hours, and all statistically significant compared to the control. The combination that resulted in the lowest cell viability was the 50 μ M CLZ with 500 μ M Tyr, achieving around 35% viability at 48 hours, indicating nearly 65% cell death in neuroblastoma cells. These findings are further proved by microscopic images at 48 and 72 hours (Figure 12), showing significant morphological alterations and reduced cell numbers for all tested combinations.

In Figure 11B, we verified that the combination of DOM with Tyr was not effective or toxic in SH-SY5Y cells. At 72 hours, all the combinations showed an increase in the cell viability, which, although not statistically significant, was still notable. The most pronounced increase was observed with the combination of 10 μ M DOM and 250 μ M Tyr, reaching 137% of cell viability. At 48 hours, there was a greater decrease in cell viability, around 73%, for the combination of 10 μ M DOM with 500 μ M Tyr, but this was not statistically significant, as with all other combinations. All these findings are corroborated by microscopic images showing no alterations in cell morphology (Figure 13).

In Figure 11C, the combination of PIM with Tyr was more effective at 48 hours, as no decreased in cell viability of SH-SY5Y was observed at 72h. Although there were decreases in cell viability, only one combination, 1 μ M PIM with 500 μ M Tyr, showed a statistically significant decrease when compared to the control, achieving around 60% viability. Microscopic images in Figure 14 confirm these results, showing fewer cells at 48 hours compared to the control and even fewer cells compared to 72 hours.

Among all the combinations of the three repurposed drugs with Tyr in SH-SY5Y cells, we concluded that the most effective drug when combined with the amino acid was CLZ.

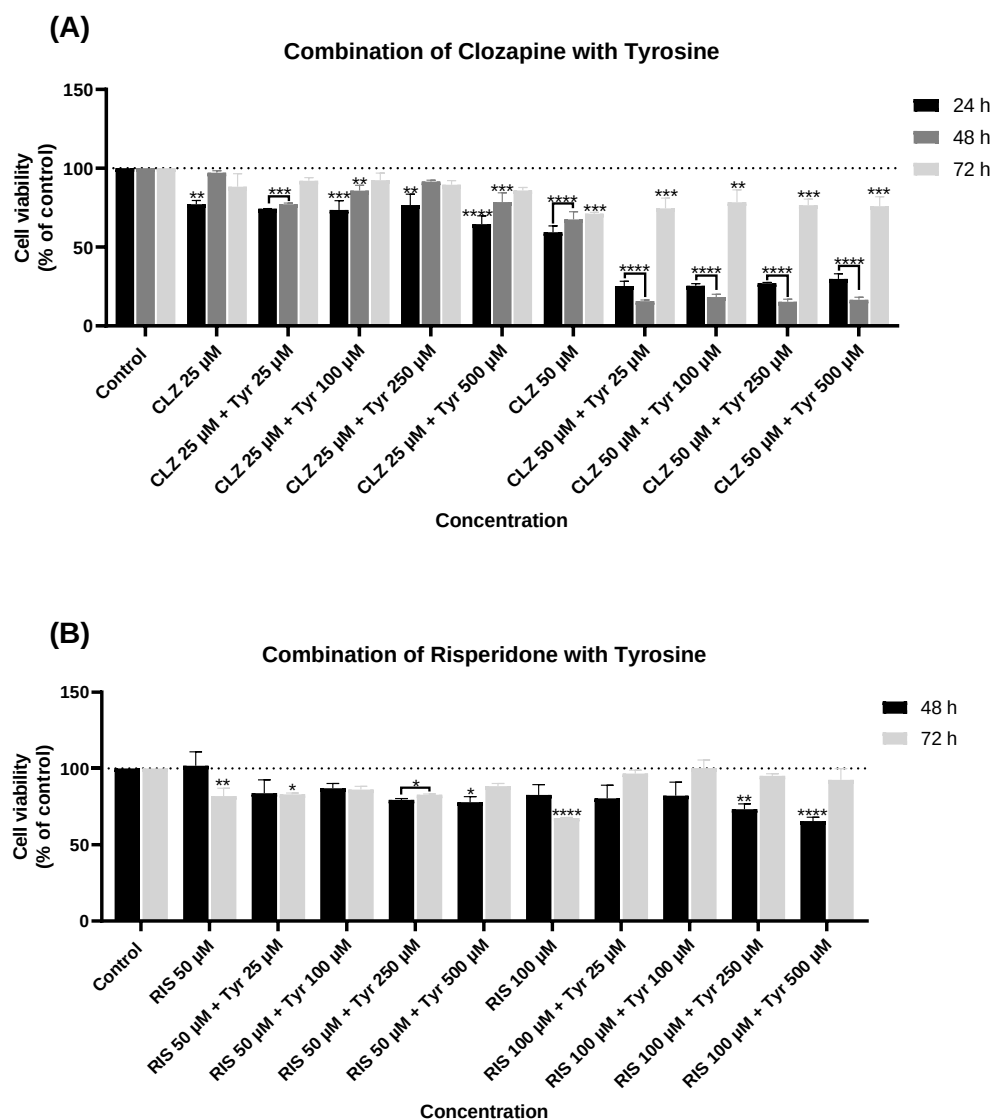


Figure 15. Effect of CLZ (A) and RIS (B) combined with Tyr on the viability of A172 cells. The cells were cultured in the presence of two concentrations of CLZ and RIS and with increasing concentrations of Tyr and treated for 24, 48, and 72 hours. Then, cell viability was determined using the MTT assay. Results are expressed as a percentage of the vehicle-treated control $\pm$ SEM of three separate experiments. Statistically significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. control.

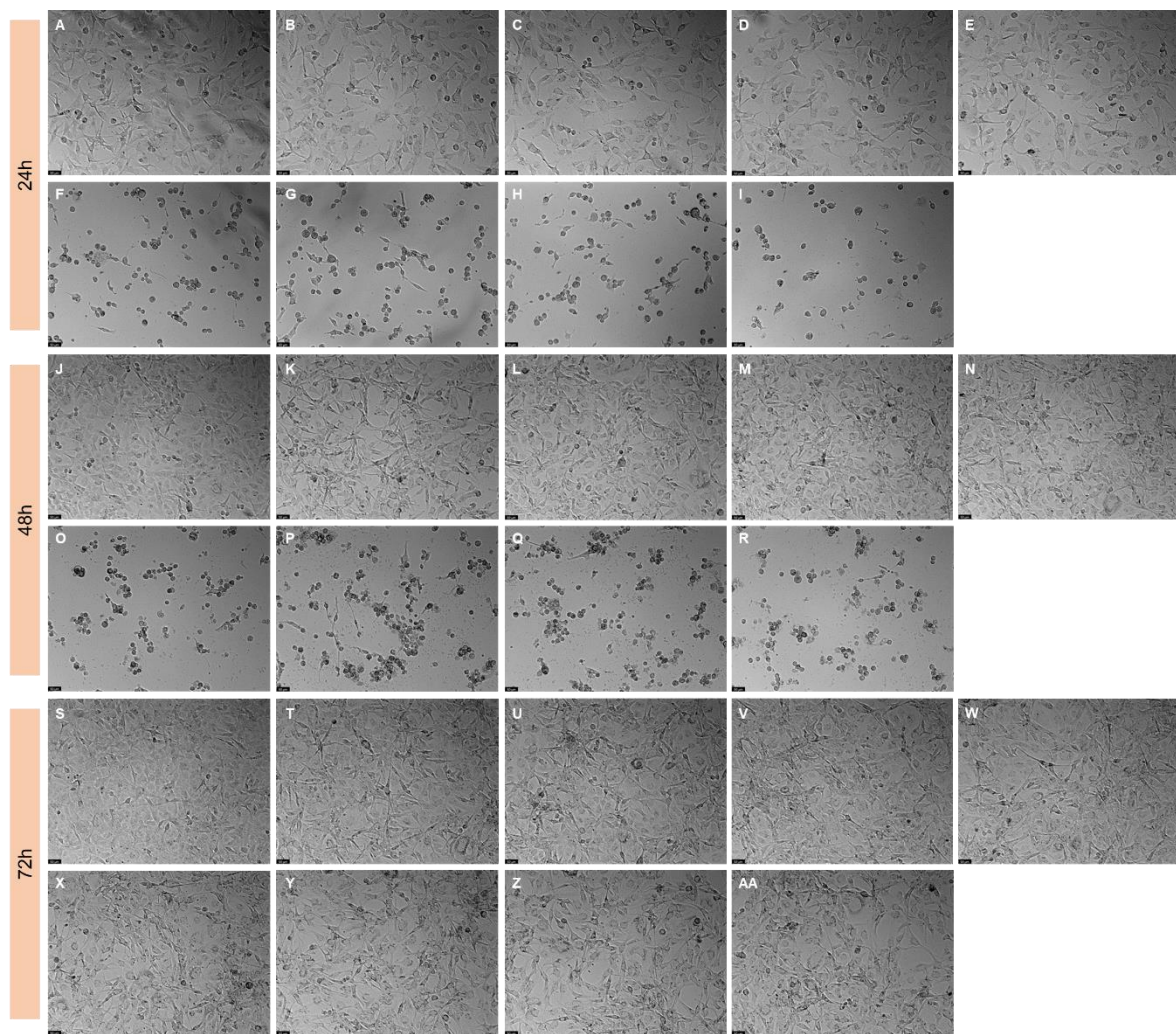


Figure 16. Microscopic visualization of the effects of CLZ combined with Tyr on the morphology of A172 cells over 24, 48 and 72 hours. Cells were treated with (A,J,S) 0.1% DMSO (control), (B,K,T) CLZ 25 μM + Tyr 25 μM, (C,L,U) CLZ 25 μM + Tyr 100 μM, (D,M,V) CLZ 25 μM + Tyr 250 μM, (E,N,W) CLZ 25 μM + Tyr 500 μM, (F,O,X) CLZ 50 μM + Tyr 25 μM, (G,P,Y) CLZ 50 μM + Tyr 100 μM, (H,Q,Z) CLZ 50 μM + Tyr 250 μM and (I,R,AA) CLZ 50 μM + Tyr 500 μM. Representative images were obtained with a high contrast (10×) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μm.

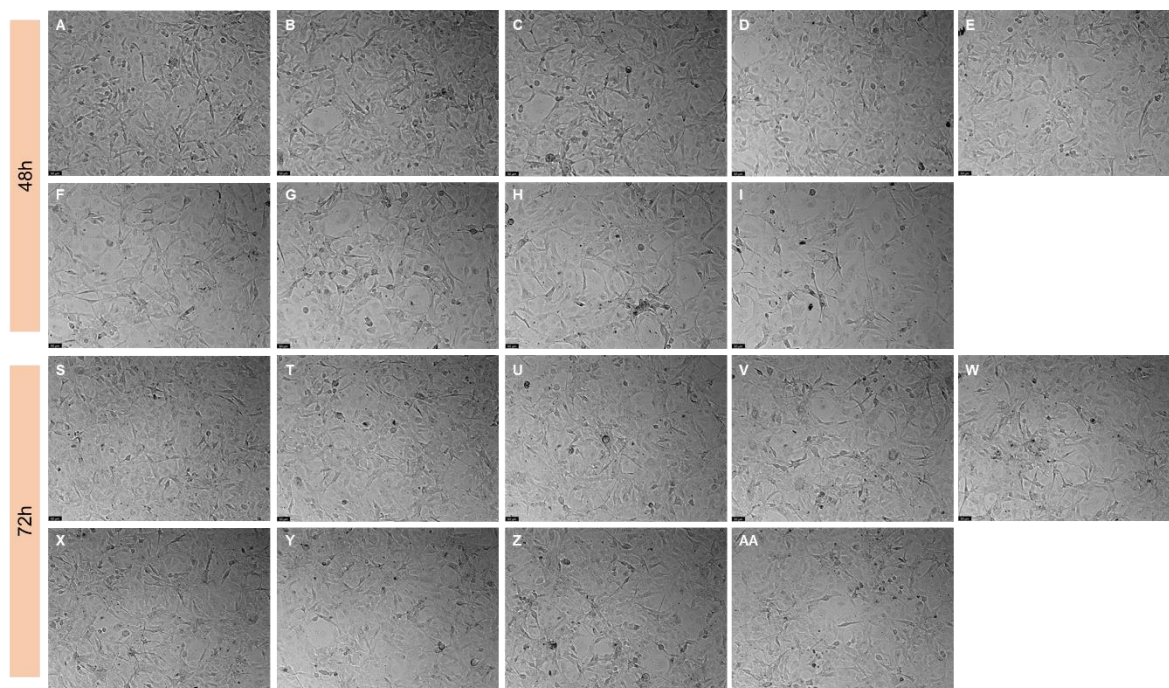


Figure 17. Microscopic visualization of the effects of RIS combined with Tyr on the morphology of A172 cells over 48 and 72 hours. Cells were treated with (**A,J,S**) 0.1% DMSO (control), (**B,K,T**) RIS 50 μ M + Tyr 25 μ M, (**C,L,U**) RIS 50 μ M + Tyr 100 μ M, (**D,M,V**) RIS 50 μ M + Tyr 250 μ M, (**E,N,W**) RIS 50 μ M + Tyr 500 μ M, (**F,O,X**) RIS 100 μ M + Tyr 25 μ M, (**G,P,Y**) RIS 100 μ M + Tyr 100 μ M, (**H,Q,Z**) RIS 100 μ M + Tyr 250 μ M and (**I,R,AA**) RIS 100 μ M + Tyr 500 μ M. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

For the combinations of CLZ with Tyr in A172 cells (Figure 15A and 16) we found that this combination was highly effective at 24 and 48 hours. When combined the highest concentration of CLZ (50 μ M) with Tyr, all these combinations were statistically significant compared to the control, with a p-value of less than 0.0001. The highest cell death occurred with the combination of 50 μ M CLZ and 250 μ M Tyr, achieving around 85% cell death, meaning a cell viability of 15%. These results are supported by the microscopic images in Figure 16, which show marked morphological alterations in the cells, such as a rounder and smaller shape, indicating that these cells are no longer viable (Figures 16F, G, H, I, O, P, Q and R). This demonstrates that these combinations were highly toxic to glioblastoma cells. The other combinations also showed statistically significant decreases in viability, though not as pronounced. These results persisted at 72 hours.

In Figure 15B, we visualized that although some combinations presented statistically significant data, the combination of RIS with Tyr was not very effective in reducing the cellular viability of A172 cells. The combination that achieved the lowest viability, around

65%, was the 100 μ M RIS with 500 μ M Tyr at 48h. This decrease is corroborated by image I at 48 hours in Figure 17, which shows a reduction in the number of cells for this combination compared to the control image (Figure 17A).

In conclusion, for the combinations of repurposed drugs with Tyr in A172 cells, CLZ proved to be the most effective drug when combined with Tyr, demonstrating significant neurotoxicity effects in glioblastoma cells.

Effects of the Combinations of the Repurposed Drugs with H₂O₂ on Cellular Viability of SH-SY5Y and A172 cell lines

To evaluate the effects of combining repurposed drugs with H₂O₂ on the viability of SH-SY5Y and A172 cells, the cells were exposed to two different concentrations of the repurposed drugs along with a concentration of 132 μ M of H₂O₂ (IC₅₀ value for H₂O₂ determined by the research group at 48 hours) [64]. The treatments were incubated for 24, 48, and 72 hours. Cell viability was assessed using the MTT assay (Figures 18 and 22). Subsequently, morphological analyses were performed on the cells treated with these combinations (Figures 19, 20, 21, 23 and 24).

The purpose of using H₂O₂ is to examine how cells respond to oxidative stress when combined with drug treatment. H₂O₂ is frequently employed in research to induce oxidative stress, as it has been shown to stimulate various cellular responses in cycle arrest, apoptosis and modulation of genes related to oxidative stress in breast cancer tumor cells [64,65]. The aim was to determine whether the induction of oxidative stress enhances the effectiveness of the repurposed drugs on the cell lines under study.

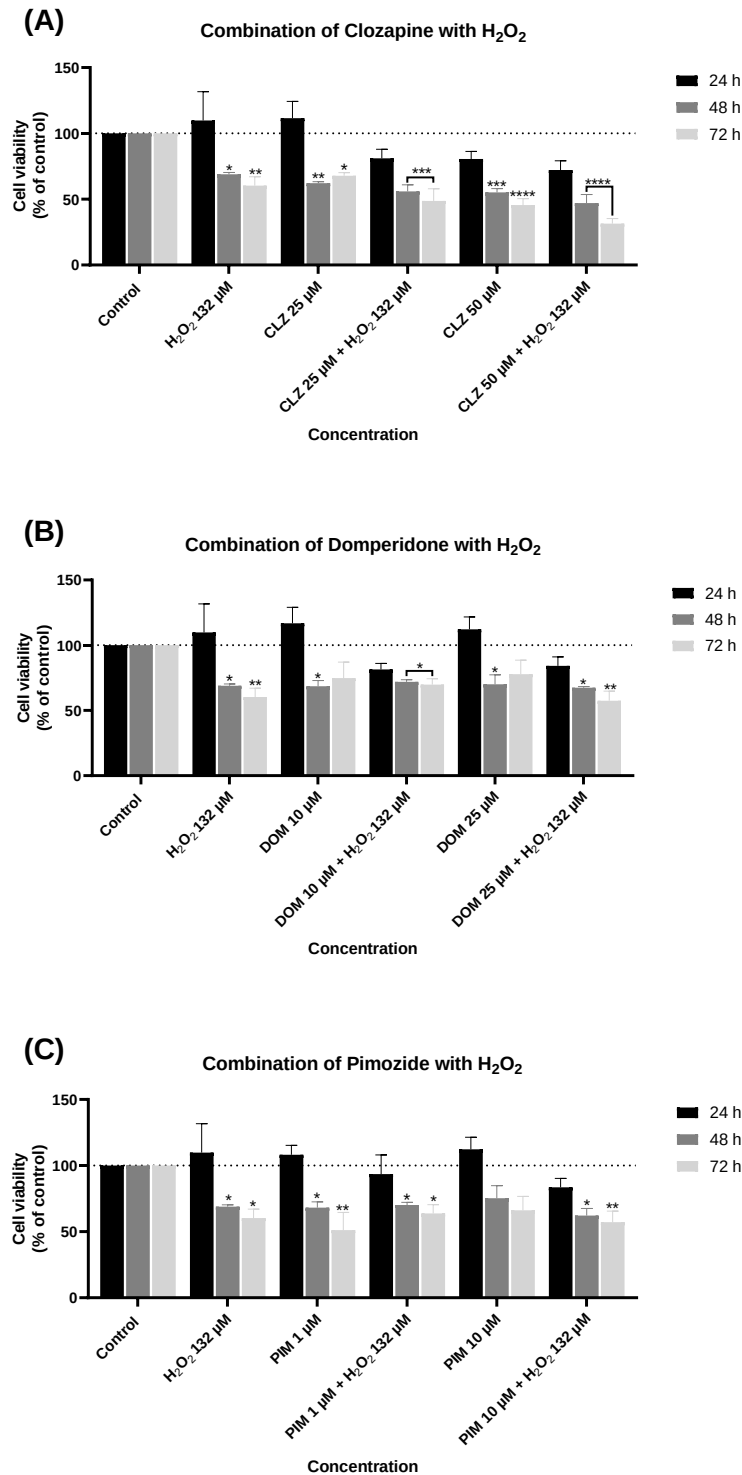


Figure 18. Effect of CLZ (A), DOM (B) and PIM (C) combined with H₂O₂ on the viability of SH-SY5Y cells. The cells were cultured in the presence of two concentrations of CLZ, DOM and PIM and with a unique concentration of H₂O₂ and treated for 24, 48, and 72 hours. Then, cell viability was determined using the MTT assay. Results are expressed as a percentage of the control and represent means $\pm$ SEM of three separate experiments. Statistically significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. control.

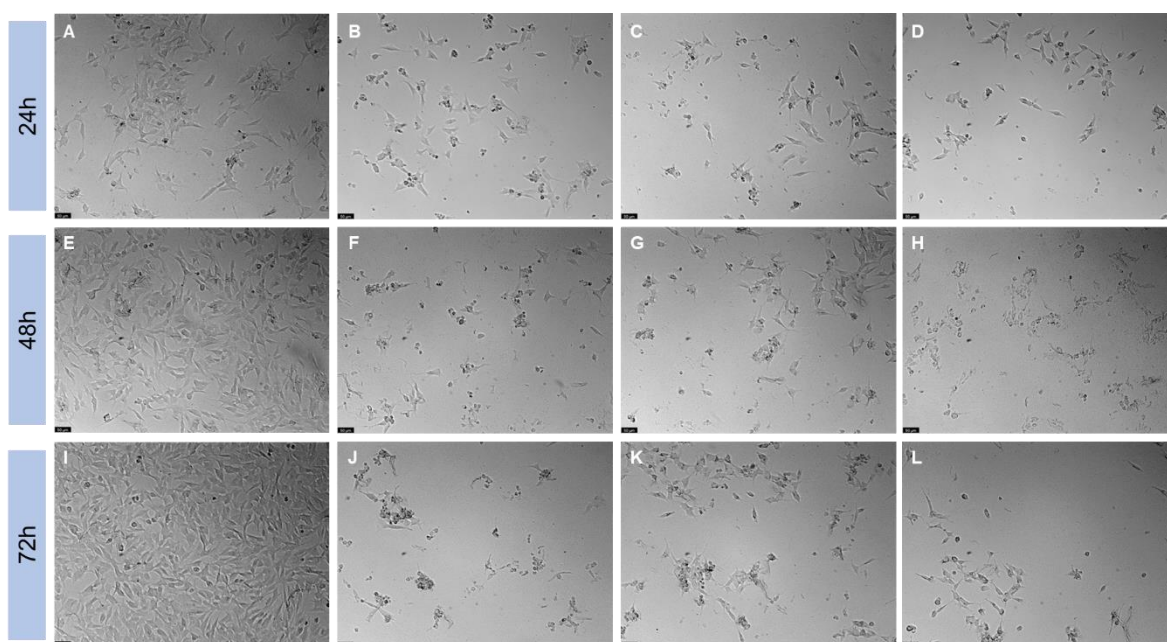


Figure 19. Microscopic visualization of the effects of CLZ combined with H₂O₂ on the morphology of SH-SY5Y cells over 24, 48 and 72 hours. Cells were treated with (**A,E,I**) 0.1% DMSO (control), (**B,F,J**) H₂O₂ 132 μM, (**C,L,U**) CLZ 25 μM + H₂O₂ 132 μM and (**D,M,V**) CLZ 50 μM + H₂O₂ 132 μM. Representative images were obtained with a high contrast (10×) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μm.

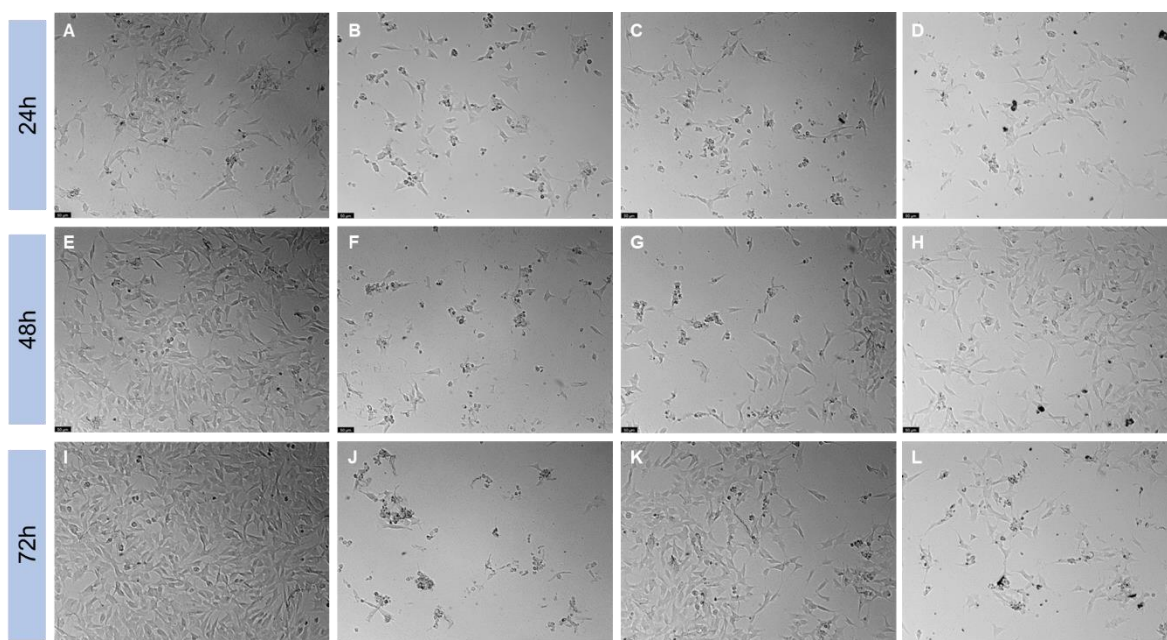


Figure 20. Microscopic visualization of the effects of DOM combined with H₂O₂ on the morphology of SH-SY5Y cells over 24, 48 and 72 hours. Cells were treated with (**A,E,I**) 0.1% DMSO (control), (**B,F,J**) H₂O₂ 132 μM, (**C,L,U**) DOM 10 μM + H₂O₂ 132 μM and (**D,M,V**) DOM 25 μM + H₂O₂ 132 μM.

Representative images were obtained with a high contrast (10×) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

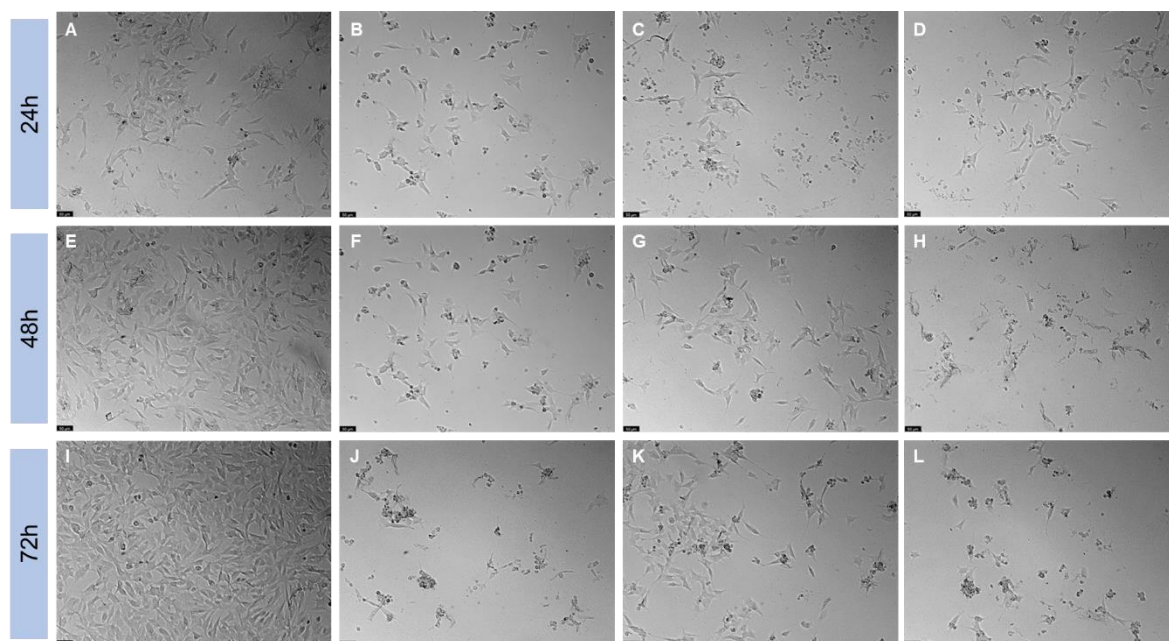


Figure 21. Microscopic visualization of the effects of PIM combined with H_2O_2 on the morphology of SH-SY5Y cells over 24, 48 and 72 hours. Cells were treated with (A,E,I) 0.1% DMSO (control), (B,F,J) H_2O_2 132 μ M, (C,L,U) PIM 1 μ M + H_2O_2 132 μ M and (D,M,V) PIM 10 μ M + H_2O_2 132 μ M. Representative images were obtained with a high contrast (10×) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

In the combination of CLZ with H_2O_2 (Figure 18A), we observed a significant decrease in the viability of SH-SY5Y cells. Comparing 25 μ M CLZ concentration alone with the combination of 25 μ M CLZ and H_2O_2 , we found that adding H_2O_2 resulted in significantly lower cell viability. For instance, at 72 hours, cell viability was approximately 47% with the combination combining, compared to about 68% for the 25 μ M CLZ concentration alone. This represents a further 21% reduction in cell viability with the addition of H_2O_2 . Similarly, for 50 μ M CLZ with H_2O_2 , although the reductions in cell viability were less pronounced, they remained statistically significant. At 72 hours, 50 μ M CLZ alone resulted in about 46% viability, while the combination reduced the viability to around 31%. The statistical significance underscored by p-values of <0.001 for 25 μ M CLZ + H_2O_2 and <0.0001 for 50 μ M CLZ + H_2O_2 . Furthermore, all these results are confirmed by microscopic images (Figure 19 C, D, G, H, K and L), showing that cells in combinations appeared rounder and smaller, indicating loss of viability of the neuroblastoma cells.

For the combination of DOM and H_2O_2 (Figure 18B), there was a noticeable decrease in SH-SY5Y cell viability, particularly at 48 and 72 hours, with statistically

significant results compared to the control. The most significant cell death was observed with 25 μM DOM with H_2O_2 at 72 hours, achieving around 43% cell death (approximately 57% viability). This was corroborated by microscopic images (Figure 20L), showing neuroblastoma cells becoming more rounded and fewer in number.

Regarding the combination of PIM with H_2O_2 (Figure 18C), this combination showed toxicity in SH-SY5Y cells, particularly at 48 and 72 hours. However, when we compared the combination of 1 μM PIM with H_2O_2 with the drug alone (1 μM), we found that the drug was more active in reducing cell viability alone than when combined with H_2O_2 . For example, at 72 hours, the drug achieved a cell viability of around 51%, while when combined with H_2O_2 it only achieved a viability of around 64% (13% higher than when the drug was alone). On the other hand, the combination of 10 μM PIM with H_2O_2 shows that when this concentration of the drug is combined with H_2O_2 , cell viability decreases more than when compared to the concentration of the drug alone. In other words, both at 48 and 72 hours this combination achieves viabilities of 62% and 57% respectively, statistically significant and lower than the viability of the 10 μM concentration of PIM, 75% at 48 hours and 66% at 72 hours. These results are also shown in Figure 21 through the cell morphology of the combinations in which we can see differences with the control cells.

With these findings we can conclude that clearly the best repurposed drug in combination with H_2O_2 was CLZ, which, together with H_2O_2 , managed to achieve high levels of toxicity in the cell line.

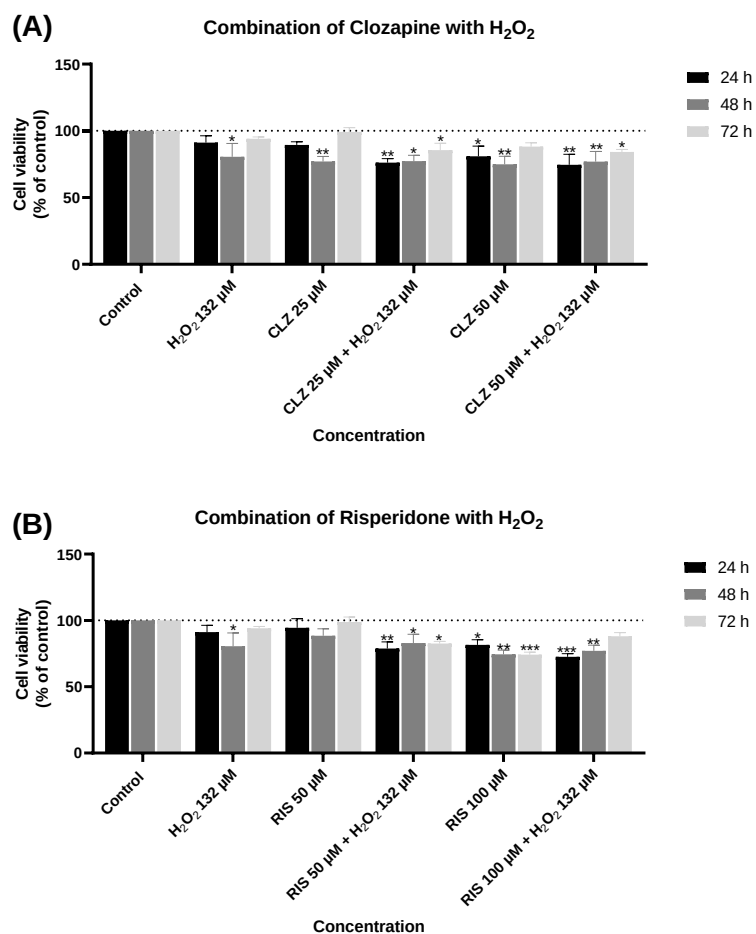


Figure 22. Effect of CLZ (A) and RIS (B) combined with H₂O₂ on the viability of A172 cells. The cells were cultured in the presence of two concentrations of CLZ and RIS and with a unique concentration of H₂O₂ and treated for 24, 48, and 72 hours. Then, cell viability was determined using the MTT assay. Results are expressed as a percentage of the vehicle-treated control $\pm$ SEM of three separate experiments. Statistically significant * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control.

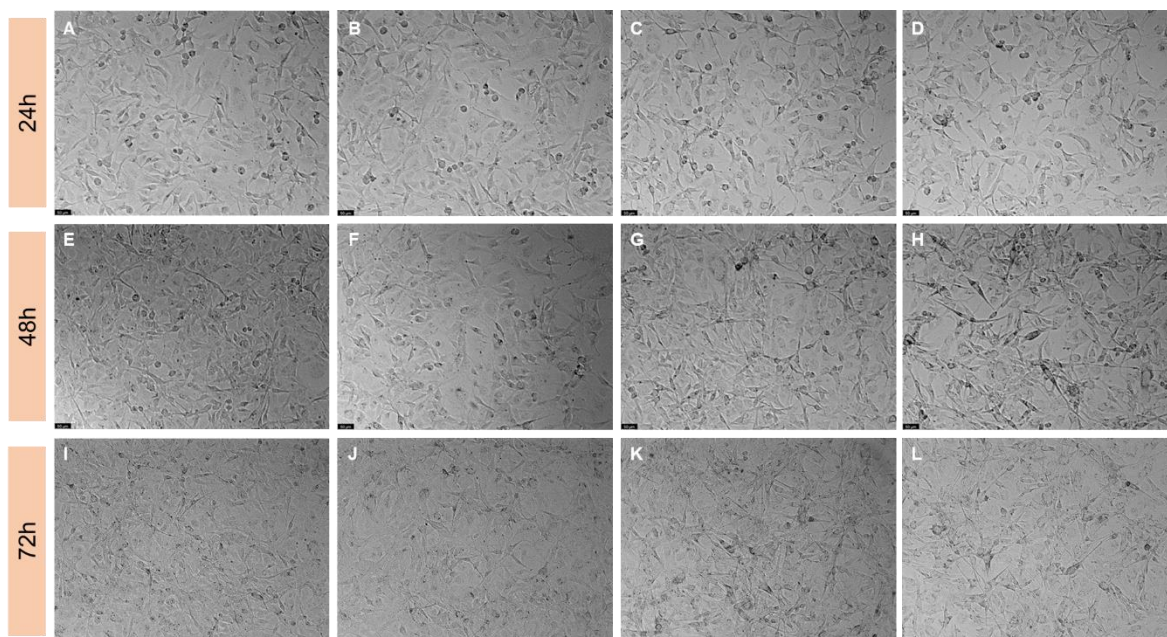


Figure 23. Microscopic visualization of the effects of CLZ combined with H₂O₂ on the morphology of A172 cells over 24, 48 and 72 hours. Cells were treated with (A,E,I) 0.1% DMSO (control), (B,F,J) H₂O₂ 132 μM, (C,L,U) CLZ 25 μM + H₂O₂ 132 μM and (D,M,V) CLZ 50 μM + H₂O₂ 132 μM. Representative images were obtained with a high contrast (10×) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μm.

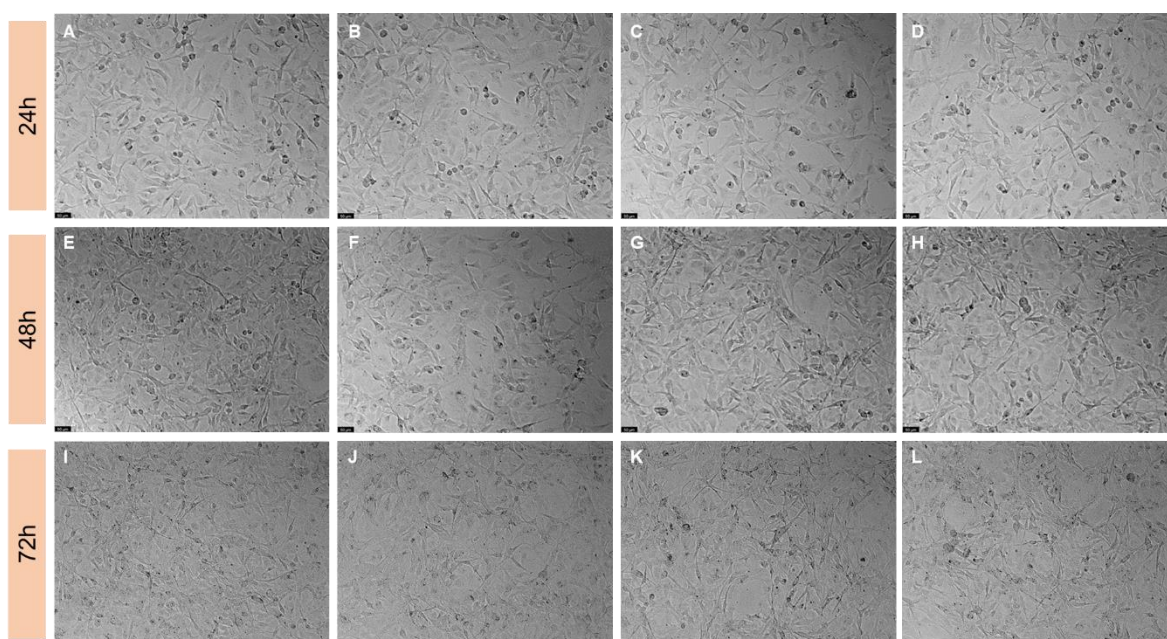


Figure 24. Microscopic visualization of the effects of RIS combined with H₂O₂ on the morphology of A172 cells over 24, 48 and 72 hours. Cells were treated with (A,E,I) 0.1% DMSO (control), (B,F,J) H₂O₂ 132 μM, (C,G,K) RIS 50 μM+ H₂O₂ 132 μM and (D,H,L) RIS 100 μM + H₂O₂ 132 μM. Representative images were obtained with a high contrast (10×) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μm.

The combination of CLZ with H₂O₂ in the A172 cells, shown in Figure 22A, did not exhibit significant toxicity to these cells. Although statistically significant differences were observed at all times for both combinations, the cell death was relatively low. The highest cell death was observed at 24 hours, with approximately 24% cell death (76% viability) for 25 µM CLZ with H₂O₂ and approximately 26% cell death (74% viability) for 50 µM CLZ with H₂O₂ was. Figure 23 supports these findings, showing no significant changes in cell size, shape or number across all conditions.

Similarly, the combination of RIS and H₂O₂, shown in Figure 22B, was not highly toxic to glioblastoma cells. The greatest toxicity was also observed at 24 hours, with viabilities of approximately 79% for 50 µM RIS with H₂O₂ and 72% for 100 µM RIS with H₂O₂. While most results were statistically significant compared to the control, the combination of 100 µM RIS with H₂O₂ at 72 hours was not. These results are further confirmed by Figure 24, showing no differences in cell morphology or number compared to the control image for all times and combinations tested.

In conclusion, the combinations of CLZ and RIS with H₂O₂ were not particularly effective in inducing toxicity in glioblastoma cells, indicating that the drugs were less effective when combined with H₂O₂ than when used alone.

Effects of the Combinations of the Repurposed Drugs with H₂O₂ and with Tyrosine on Cellular Viability of SH-SY5Y and A172 cell lines

To evaluate the effects of combining repurposed drugs with H₂O₂ and Tyr on the viability of SH-SY5Y and A172 cells, the cells were exposed to two different concentrations of the repurposed drugs, along with a 132 µM concentration of H₂O₂ and a 500 µM concentration of Tyr. The treatments were incubated for 24, 48, and 72 hours. Cell viability was assessed using the MTT assay (Figures 25 and 29). Following this, morphological analyses were performed on the cells treated with these combinations (Figures 26, 27, 28, 30 and 31).

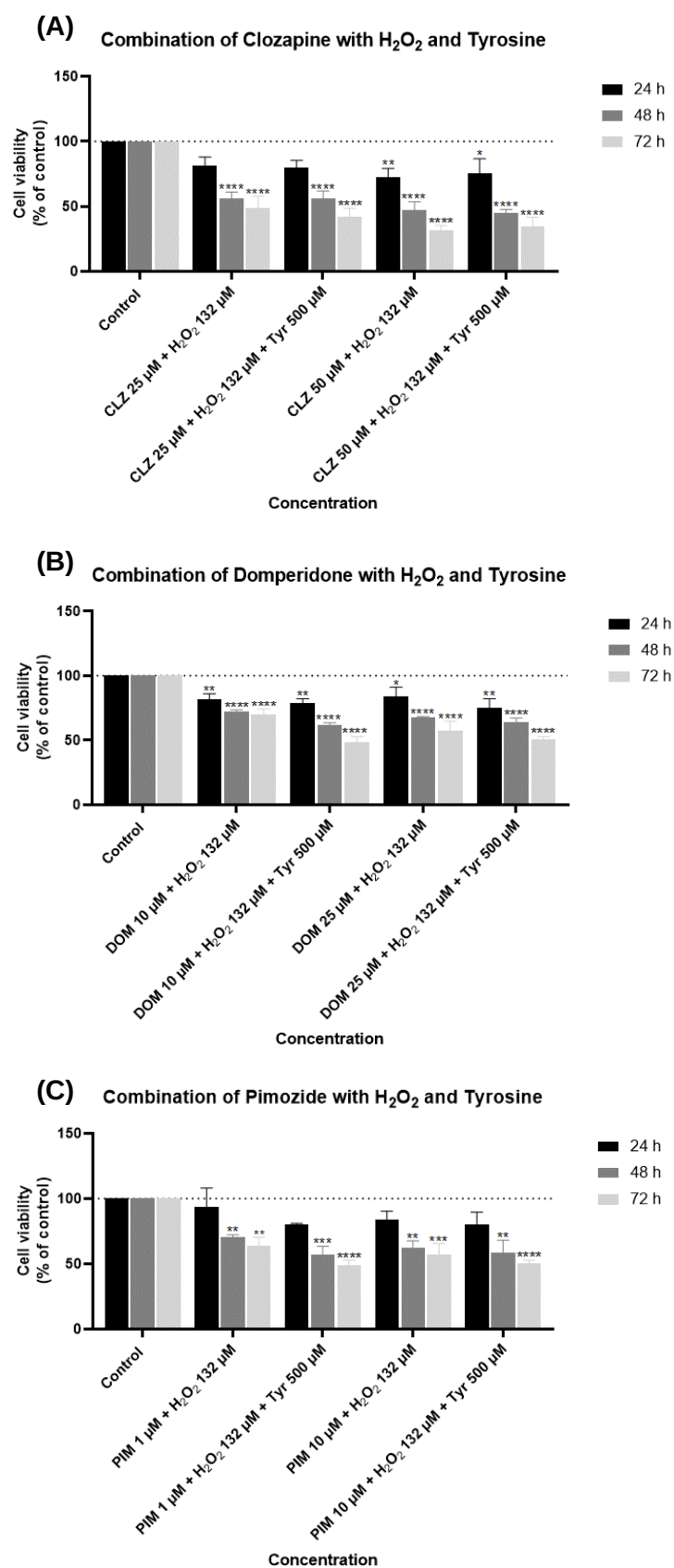


Figure 25. Effect of CLZ (A), DOM (B) and PIM (C) combined with H₂O₂ and with Tyr on the viability of SH-SY5Y cells. The cells were cultured in the presence of two concentrations of CLZ, DOM and

PIM and with a unique concentration of H₂O₂ and Tyr and treated for 24, 48, and 72 hours. Then, cell viability was determined using the MTT assay. Results are expressed as a percentage of the vehicle-treated control $\pm$ SEM of three separate experiments. Statistically significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. control.

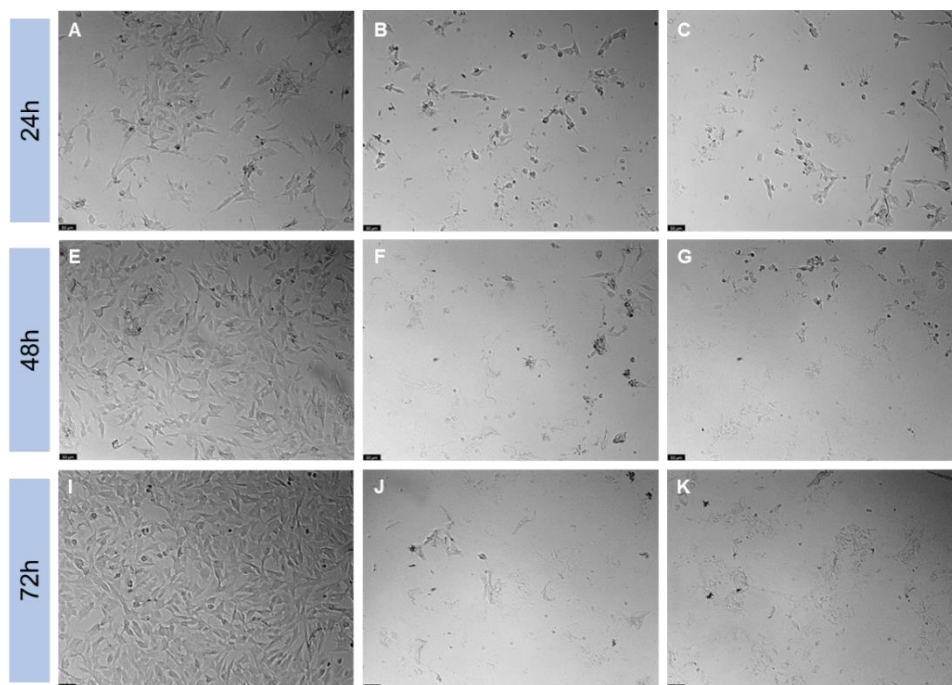


Figure 26. Microscopic visualization of the effects of Clozapine combined with H₂O₂ and with Tyr on the morphology of SH-SY5Y cells over 24, 48 and 72 hours. Cells were treated with (**A,D,G**) 0.1% DMSO (control), (**B,E,H**) CLZ 25 μ M + H₂O₂ 132 μ M + Tyr 500 μ M and (**C,F,I**) CLZ 50 μ M + H₂O₂ 132 μ M + Tyr 500 μ M. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

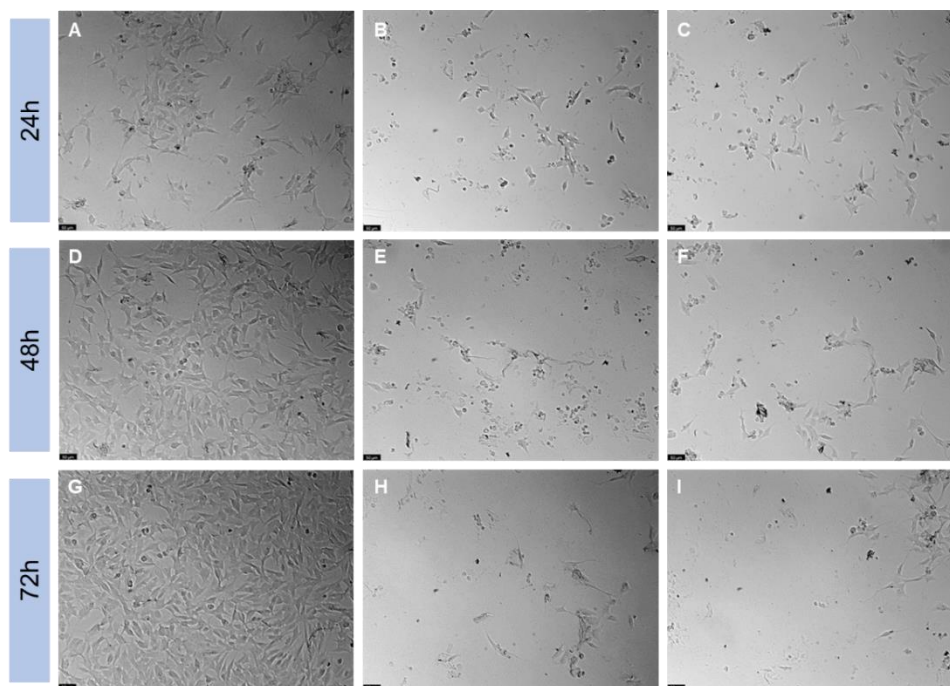


Figure 27. Microscopic visualization of the effects of DOM combined with H₂O₂ and with Tyr on the morphology of SH-SY5Y cells over 24, 48 and 72 hours. Cells were treated with (A,D,G) 0.1% DMSO (control), (B,E,H) DOM 10 μ M + H₂O₂ 132 μ M + Tyr 500 μ M and (C,F,I) DOM 25 μ M + H₂O₂ 132 μ M + Tyr 500 μ M. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

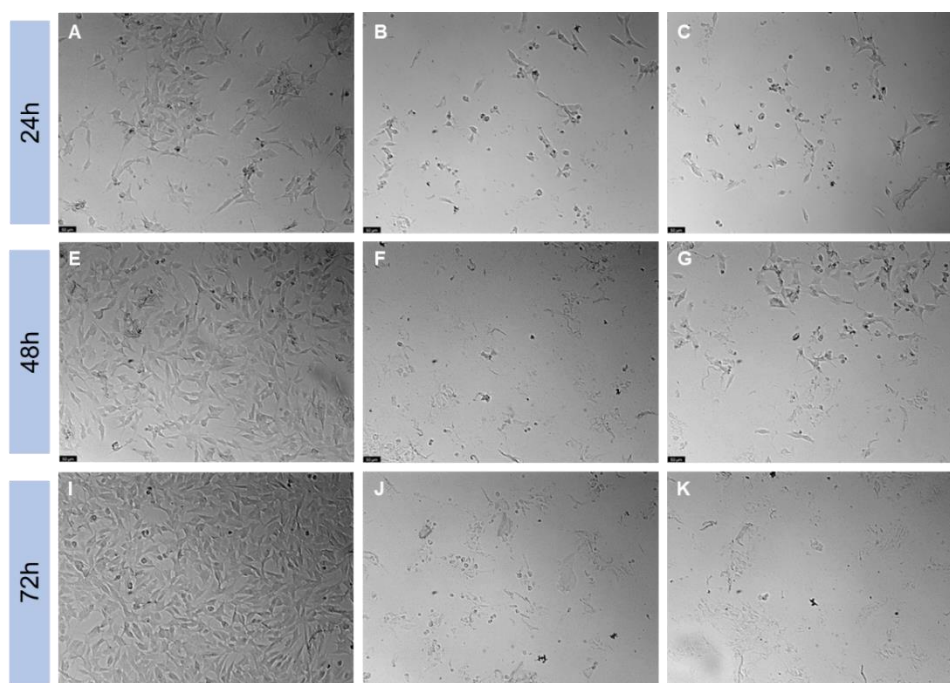


Figure 28. Microscopic visualization of the effects of PIM combined with H₂O₂ and with Tyr on the morphology of SH-SY5Y cells over 24, 48 and 72 hours. Cells were treated with (A,D,G) 0.1% DMSO (control), (B,E,H) PIM 1 μ M + H₂O₂ 132 μ M + Tyr 500 μ M and (C,F,I) PIM 10 μ M + H₂O₂ 132 μ M + Tyr 500 μ M.

Tyr 500 μ M. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

Figure 25A shows that the combination of CLZ with H₂O₂ and Tyr exhibit similarly toxicity as the combination of CLZ with H₂O₂ alone in SH-SY5Y cells. The addition of Tyr did not enhance toxicity. Despite the minimal differences, all the results obtained for this combination at 48 and 72 hours were statistically significant, with p-values < 0.0001 compared to the control, indicating a significant impact of this combination on cell viability. The highest cell death was observed with 50 μ M CLZ combined with H₂O₂ and Tyr at 72h, resulting in 65% cell death (35% viability), comparable to 69% cell death with 50 μ M CLZ with H₂O₂ alone. These results are corroborated by Figure 26, which shows a marked reduction in cell number and altered cell morphology (rounded and small) compared to the control cells (Figure 26A, D, G).

In figure 25B, the combination of DOM with H₂O₂ and Tyr was slightly more toxic to SH-SY5Y cells than the combination of DOM with H₂O₂, indicating that Tyr enhanced toxicity in this combination, contrary with CLZ. All conditions under study showed statistically significant results, with the lowest cell viability, around 49%, observed for 10 μ M DOM with H₂O₂ and Tyr at 72h. These findings are supported by the cell morphology and microscopic images in Figure 27.

For the combination of PIM with H₂O₂ and Tyr in SH-SY5Y cells, the addition of Tyr increased toxicity, resulting in greater cell death compared to the combination of PIM with H₂O₂ alone (Figure 25C). The most statistically significant results occurred at 72 hours, with approximately 51% and 49% cell death for the combination of 1 μ M and 10 μ M PIM with H₂O₂ and Tyr, respectively. Furthermore, these results are confirmed by the microscopic images in Figure 28, showing significant differences in cell morphology between the treated cells and the control cells, similar to the other combinations mentioned.

We concluded that all combinations were statistically significant and toxic to neuroblastoma cells. However, the combination of CLZ with H₂O₂ and Tyr achieved the highest levels of cell death, demonstrating that this triple combination has strong anticancer potential.

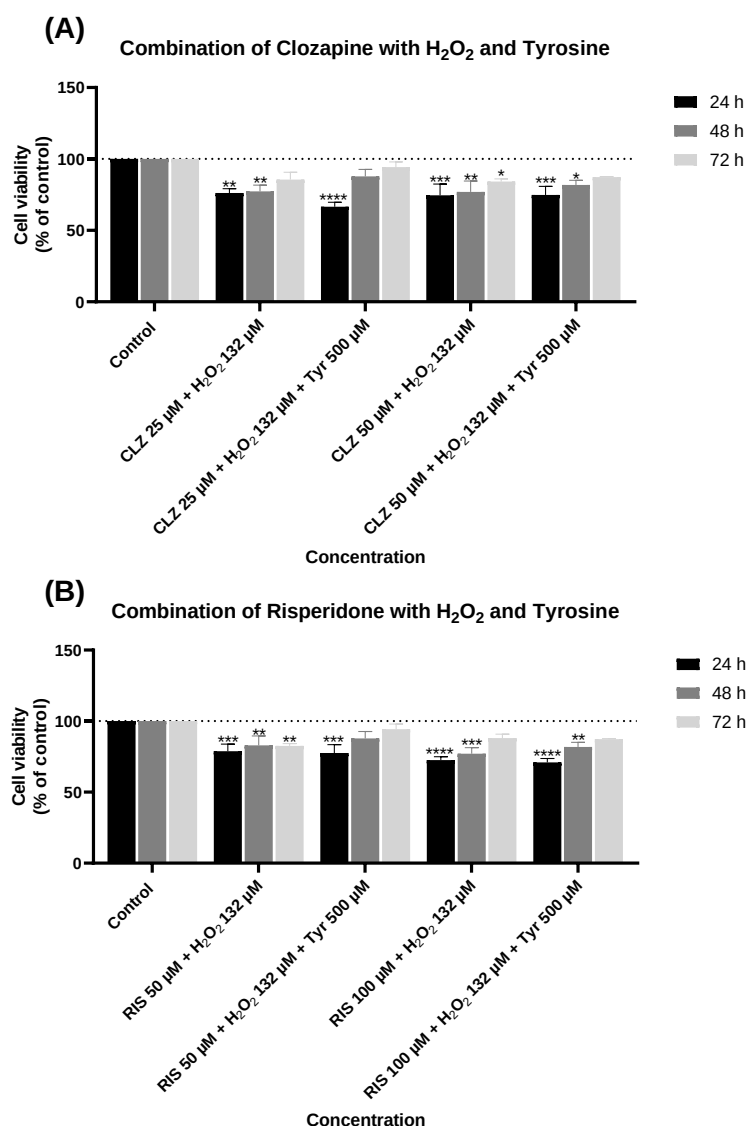


Figure 29. Effect of CLZ (A) and RIS (B) combined with H₂O₂ and with Tyr on the viability of A172 cells. The cells were cultured in the presence of two concentrations of CLZ and RIS and with a unique concentration of H₂O₂ and Tyr and treated for 24, 48, and 72 hours. Then, cell viability was determined using the MTT assay. Results are expressed as a percentage of the vehicle-treated control $\pm$ SEM of three separate experiments. Statistically significant * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ vs. control.

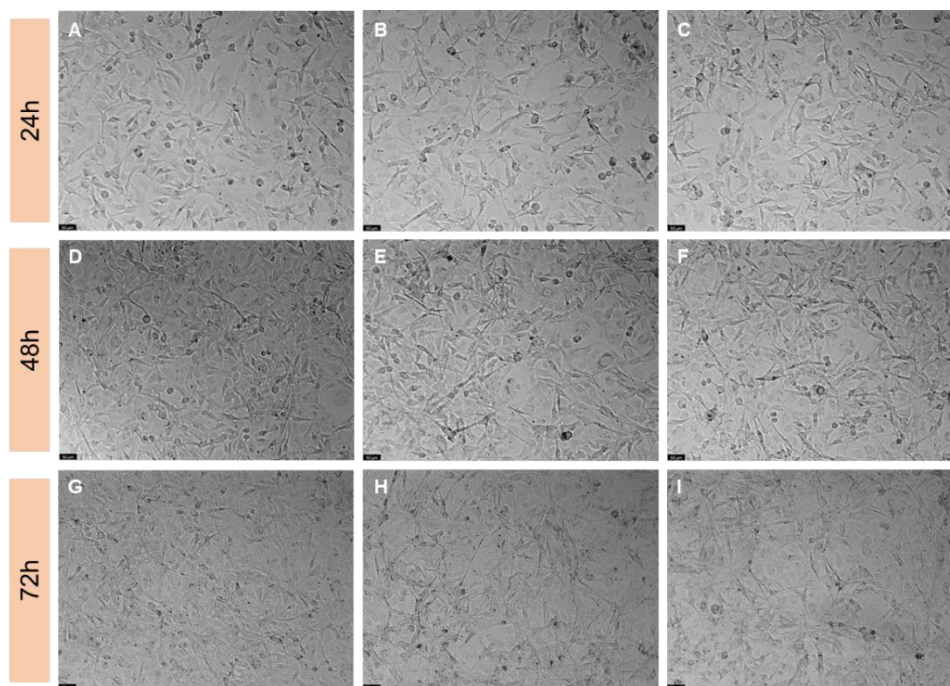


Figure 30. Microscopic visualization of the effects of CLZ combined with H₂O₂ and with Tyr on the morphology of A172 cells over 24, 48 and 72 hours. Cells were treated with (**A,D,G**) 0.1% DMSO (control), (**B,E,H**) CLZ 25 μM + H₂O₂ 132 μM + Tyr 500 μM and (**C,F,I**) CLZ 50 μM + H₂O₂ 132 μM + Tyr 500 μM. Representative images were obtained with a high contrast (10×) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μm.

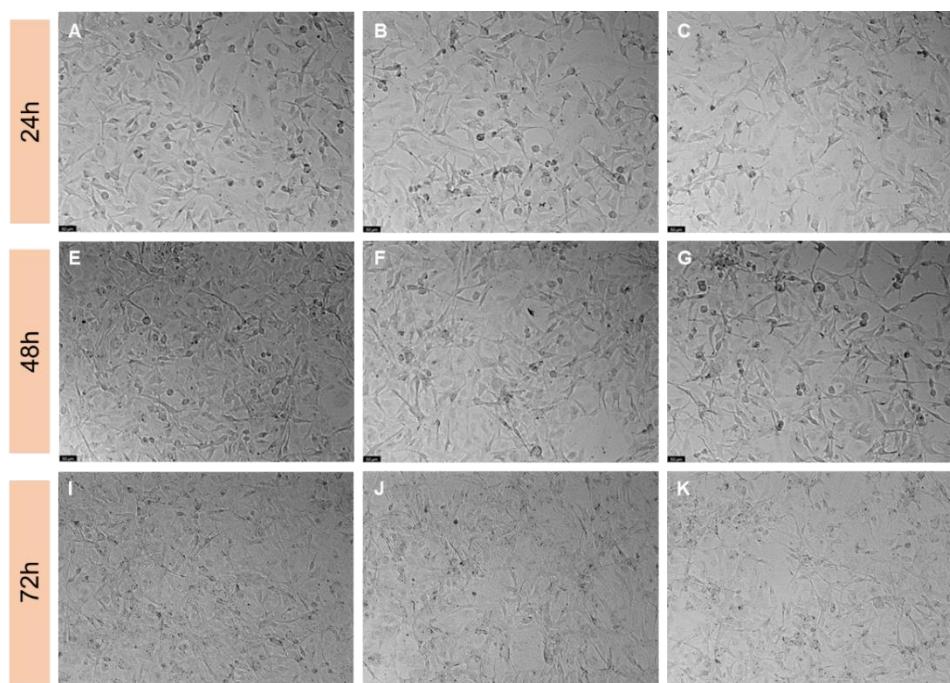


Figure 31. Microscopic visualization of the effects of RIS combined with H₂O₂ and with Tyr on the morphology of A172 cells over 24, 48 and 72 hours. Cells were treated with (**A,E,I**) 0.1% DMSO (control), (**B,E,H**) RIS 50 μM + H₂O₂ 132 μM + Tyr 500 μM and (**C,F,I**) RIS 100 μM + H₂O₂ 132 μM + Tyr 500 μM. Representative images were obtained with a high contrast (10×) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μm.

Tyr 500 μ M. Representative images were obtained with a high contrast (10 $\times$) bright field objective (LionHeart FX Automated Microscope) from three independent experiments. Scale bar: 50 μ m.

The combination of CLZ with H₂O₂ and Tyr (Figure 29A) showed low toxicity in the A172 cell line, with statistically significant results observed only at 24 hours for both combinations and at 48 hours for the 50 μ M CLZ with H₂O₂ and Tyr combination. The decrease in cell viability was notable only at 24 hours, with reductions to around 66% and 75% for the combinations of 25 μ M and 50 μ M CLZ with H₂O₂ and Tyr, respectively. These findings are validated by the microscopic images in Figure 30, which show fewer cells at 24 hours in the combination treatments compared to the control images and the other times.

In Figure 29B, we observed that the combination of RIS with H₂O₂ and with Tyr also exhibited low toxicity in the A172 cells. The statistically significant decrease in cell viability were observed at 24 hours for both combinations and at 48 hours for the 100 μ M RIS combination. However, the reductions in cell viability at 24 hours were minimal, only about 1% and 2% lower than RIS with H₂O₂ alone, resulting in cell deaths of approximately 22% and 29% for the combinations of 50 μ M RIS and 100 μ M RIS, respectively. These results are corroborated by the microscopic images in Figure 31, specifically the B and C images corresponding to 24 hours.

In conclusion, the combinations of the two repurposed drugs with H₂O₂ and Tyr in glioblastoma cells did not demonstrate significant toxicity. Therefore, these three compounds together do not show substantial anticancer potential in this cell line.

Synergistic Combinations of Repurposed Drugs with Tyrosine and H₂O₂

To investigate the effects of the synergistic combinations of the repurposed drugs with H₂O₂ and with Tyr in SH-SY5Y and A172 cells, the combination index (CI) was calculated according to the Chou–Talalay method using CompuSyn software. The Chou–Talalay method is based on the median effect equation derived from the law of mass action, integrates the Michaelis–Menten, Hill, Henderson–Hasselbalch, and Scatchard equations in biochemistry and biophysics. This method provides a quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations [66].

The combination of CLZ with Tyr in SH-SY5Y cells (Table 5) demonstrated synergism only at 72 hours, with all combination pairs showing CI values below 1. For the combination of 25 μ M of CLZ with 25 μ M of Tyr at 24 and 48 hours, and the combination of 50 μ M CLZ with 25 μ M Tyr at 48h, an additive effect was observed in this cell line. All other combinations had CI values greater than 1, indicating antagonism between CLZ and Tyr.

These results are illustrated in Figure 32, where the blue circle indicates the combination of 25 μM of CLZ with 25 μM Tyr at 24 hours with a CI value greater than 1. The red squares represent the combinations of 25 μM CLZ with 25 μM Tyr and 50 μM CLZ with 25 μM Tyr at 48 hours, both with CI values greater than 1. The green triangles show all the tested combinations of CLZ with Tyr at 72 hours, with CI values below to 1.

Table 5. Combination index (CI) values of CLZ and Tyr combinations in SH-SY5Y cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Clozapine (μM)	Dose Tyrosine (μM)	SH-SY5Y		
		CI (24h)	CI (48h)	CI (72h)
25	25	1.08550	1.20889	0.34979
25	100	2.84445	4.39586	0.37308
25	250	15.1900	11.5192	0.43125
25	500	46.8675	24.7252	0.39633
50	25	595.613	1.54442	0.68032
50	100	595.615	6.14139	0.75946
50	250	595.619	14.8158	0.74540
50	500	17.4854	31.3872	0.76281

The combination of CLZ with H_2O_2 in SH-SY5Y cells (Table 6) demonstrated synergism at all time points tested, with all combination pairs showing CI values below 1, indicating that this combination is very promising. Figure 32 supports these results, with all combination pairs appearing in the graph. The pink triangle represents the combinations at 24 hours, the blue diamond represents the combinations at 48 hours, and the red cross represents the combinations at 72 hours, all with CI values below 1.

Table 6. Combination index (CI) values of CLZ and H₂O₂ combinations in SH-SY5Y cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Clozapine (μM)	Dose H ₂ O ₂ (μM)	SH-SY5Y		
		CI (24h)	CI (48h)	CI (72h)
25	132	0.39614	0.53720	0.36572
50	132	0.19361	0.53572	0.62720

The combination of CLZ with H₂O₂ and Tyr in SH-SY5Y cells (Table 7) showed synergism only at 72 hours, as indicated by CI values below 1. In contrast, combinations at 24 and 48 hours had CI values greater than 1, indicating antagonistic effects. These findings are illustrated in Figure 32, where the blue squares represent the combinations clearly showing CI values below 1 at 72 hours.

Table 7. Combination index (CI) values of CLZ, H₂O₂ and Tyr combinations in SH-SY5Y cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Clozapine (μM)	Dose H ₂ O ₂ (μM)	Dose Tyrosine (μM)	SH-SY5Y		
			CI (24h)	CI (48h)	CI (72h)
25	132	500	37.3339	25.6252	0.70109
50	132	500	35.4844	27.7150	0.72783

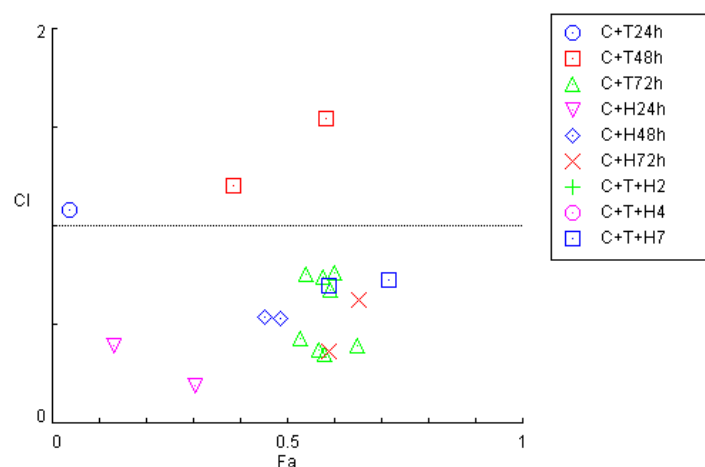


Figure 32. Fa-CI plot of combinations with CLZ in the SH-SY5Y cell line. Combinations are represented as follows: CLZ + Tyr 24h (blue circle), CLZ + Tyr 48h (red square), CLZ + Tyr 72h

(green triangle), CLZ + H₂O₂ 24h (pink triangle), CLZ + H₂O₂ 48h (blue diamond), CLZ + H₂O₂ 72h (red cross), CLZ + H₂O₂ + Tyr 24h (green plus sign), CLZ + H₂O₂ + Tyr 48h (pink circle) and CLZ + H₂O₂ + Tyr 72h (blue square).

In conclusion, the most effective synergism using the CLZ is achieved when it is combined with H₂O₂.

In SH-SY5 cells, the combination of PIM with Tyr (Table 8) demonstrated synergism only at 72 hours, except for the combinations of 10 μ M PIM with 25, 100 and 250 μ M Tyr, which had CI values greater than 1. These results are supported by Figure 33. The two blue circles in the figure represent the combinations at 24 hours that had CI values close to 1 but exhibited antagonistic effects in the cells. The five combinations that resulted in synergism between the two compounds are represented by the red squares, showing CI values below 1 in the graph, thus confirming the synergistic interaction between PIM and Tyr at 72 hours.

Table 8. Combination index (CI) values of PIM and Tyr combinations in SH-SY5Y cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Pimozide (μ M)	Dose Tyrosine (μ M)	SH-SY5Y	
		CI (48h)	CI (72h)
1	25	1.10047	0.20645
1	100	4.12193	0.08279
1	250	11.1538	0.30071
1	500	25.4684	0.34390
10	25	1.19915	31900.4
10	100	3.92125	31901.8
10	250	11.0843	31904.7
10	500	22.7102	0.44062

The combination of PIM with H₂O₂ in SH-SY5Y cells (Table 9) evidenced synergisms for all combinations and times tested, except for the 1 μ M PIM with 132 μ M H₂O₂ combination at 24 hours, which had a CI value greater than 1. Figure 33 corroborates these findings, with the green triangles indicating the 24 hours combinations, one (1 μ M PIM with 132 μ M H₂O₂) having a CI value greater than 1 and another (10 μ M PIM with 132 μ M H₂O₂) having a CI value below 1. The pink triangles represent the 48 hours combinations, and the

blue diamonds represent the 72 hours combinations, all with CI values less than 1, showing synergism between PIM and H₂O₂.

Table 9. Combination index (CI) values of PIM and H₂O₂ combinations in SH-SY5Y cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Pimozide (μM)	Dose H ₂ O ₂ (μM)	SH-SY5Y		
		CI (24h)	CI (48h)	CI (72h)
1	132	1.76955	0.98025	0.93454
10	132	0.84971	0.97425	0.66654

In SH-SY5Y cells, the combination of PIM, H₂O₂ and Tyr (Table 10) exhibited synergistic effects at 72 hours and at 24 hours for the combination of 1 μM PIM with 132 μM H₂O₂ and 500 μM Tyr, with CI values less than 1. The combination of 10 μM PIM with 132 μM H₂O₂ and with 500 μM Tyr showed an additive effect, achieving a CI value of 1. At 48 hours, both combinations demonstrated CI values greater than 1, indicating antagonism effect at this time. These findings are illustrated in Figure 33, where the combinations at 24 hours are represented by the two red crosses, one close to 1 and the other close to 0, and the combinations at 72 hours are represented by the two pink circles.

Table 10. Combination index (CI) values of PIM, H₂O₂ and Tyr combinations in SH-SY5Y cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Pimozide (μM)	Dose H ₂ O ₂ (μM)	Dose Tyrosine (μM)	SH-SY5Y		
			CI (24h)	CI (48h)	CI (72h)
1	132	500	0.23416	24.2442	0.30503
10	132	500	1.04354	21.3904	0.24447

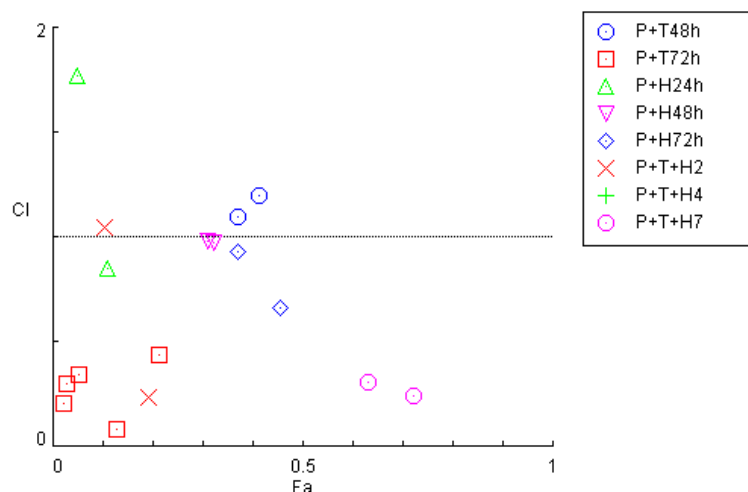


Figure 33. Fa-CI plot of combinations with PIM in the SH-SY5Y cell line. Combinations are represented as follows: PIM + Tyr 48h (blue circle), PIM + Tyr 72h (red square), PIM + H₂O₂ 24h (green triangle), PIM + H₂O₂ 48h (pink triangle), PIM + H₂O₂ 72h (blue diamond), PIM + H₂O₂ + Tyr 24h (red cross), PIM + H₂O₂ + Tyr 48h (green plus sign) and PIM + H₂O₂ + Tyr 72h (pink circle).

In conclusion, the most synergistic effects for PIM were observed in its combinations with H₂O₂. The combinations of PIM with H₂O₂ and Tyr at 72 hours exhibited the lowest CI values, around 0.2, indicating the most intense synergism. By relating the CI value to the Fa value (with Fa close to 0 indicating no cell death and Fa close to 1 indicating complete cell death [66]), we can see that these combinations achieved the highest levels of cell death along with the best synergistic effects (Figure 33).

The combination of DOM with Tyr (Table 11) demonstrated synergism only in the combination of 10 μ M DOM with 25 μ M Tyr at 48 hours, with a CI value of around 0.9. All other combinations evidenced CI values greater than 1, indicating antagonistic effects in the SH-SY5Y cells. These results are illustrated in Figure 34 by the blue circle close to 1.

Table 11. Combination index (CI) values of DOM and Tyr combinations in SH-SY5Y cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Domperidone (μM)	Dose Tyrosine (μM)	SH-SY5Y	
		CI (48h)	CI (72h)
10	25	0.94555	42935.0
10	100	2.91032	42936.5
10	250	8.21662	42939.4
10	500	19.9286	42944.3
25	25	1.17424	107337.
25	100	3.38364	107338.
25	250	7.53975	107341.
25	500	17.1888	107346.

In SH-SY5Y cells, the combination of PIM with H_2O_2 (Table 12) exhibited synergistic effects exclusively at 24 hours, achieving CI values below 1. At 48 hours, only the combination of 25 μM DOM with 132 μM H_2O_2 showed additive effects, with a CI value of 1. These findings are also represented in Figure 34, where the green triangle represents the two synergistic combinations at 24 hours, and the pink triangle close to 1 represents the only combination with additivity effect.

Table 12. Combination index (CI) values of DOM and H_2O_2 combinations in SH-SY5Y cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Domperidone (μM)	Dose H_2O_2 (μM)	SH-SY5Y		
		CI (24h)	CI (48h)	CI (72h)
10	132	0.13006	1.13504	2.04764
25	132	0.67824	1.08001	2.88246

The combination of DOM with H_2O_2 and Tyr in SH-SY5Y cells (Table 13) showed synergism only at 24 hours, as indicated by CI values below 1. In contrast, combinations at 48 and 72 hours had CI values greater than 1, indicating antagonistic effects, except for the

combination of 10 μM DOM with H_2O_2 and Tyr at 72 hours, which exhibited CI value of 1, indicating additive effects. These findings are illustrated in Figure 34, where the red crosses represent the combinations at 24 hours, clearly showing CI values below 1, and the pink circle represent the combinations with additive effects at 72 hours.

Table 13. Combination index (CI) values of DOM, H_2O_2 and Tyr combinations in SH-SY5Y cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Domperidone (μM)	Dose H_2O_2 (μM)	Dose Tyrosine (μM)	SH-SY5Y		
			CI (24h)	CI (48h)	CI (72h)
10	132	500	0.26510	22.9817	0.98122
25	132	500	0.32063	22.2473	1.78671

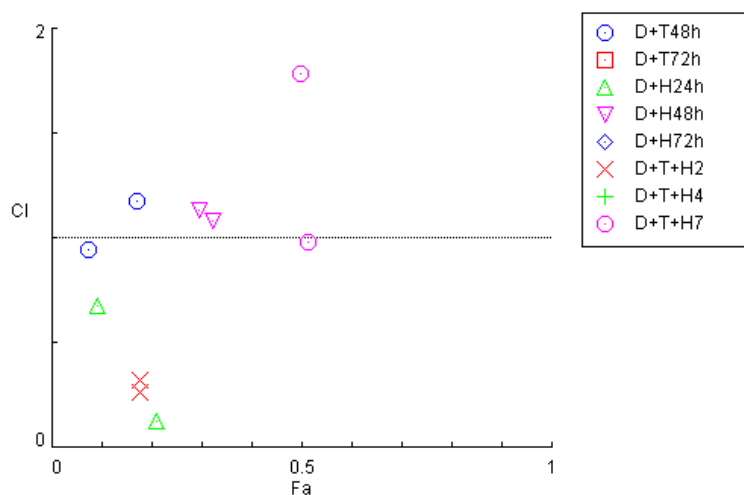


Figure 34. Fa–CI plot of combinations with DOM in the SH-SY5Y cell line. Combinations are represented as follows: DOM + Tyr 48h (blue circle), DOM + Tyr 72h (red square), DOM + H_2O_2 24h (green triangle), DOM + H_2O_2 48h (pink triangle), DOM + H_2O_2 72h (blue diamond), DOM + H_2O_2 + Tyr 24h (red cross), DOM + H_2O_2 + Tyr 48h (green plus sign) and DOM + H_2O_2 + Tyr 72h (pink circle).

In conclusion, the combinations with DOM in neuroblastoma cells demonstrated synergism only at 24 hours, with the most effective results occurring in the combination of 10 μM DOM with 132 μM H_2O_2 .

The next step was to analyze the synergistic effects of different combinations of CLZ and RIS in A172 cells.

The combination of CLZ with Tyr in A172 cells (Table 14) demonstrated synergism only at 24 hours for all the combinations, with the other combinations and times demonstrated antagonistic effects with CI values exceeding 1. Notably, all the CI values obtained at 24 hours were very low, approaching 0, highlighting significant synergistic effects between CLZ and Tyr. These results are represented in Figure 35 by the blue circles under 1, indicating the synergy between these two compounds.

Table 14. Combination index (CI) values of CLZ and Tyr combinations in A172 cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Clozapine (μM)	Dose Tyrosine (μM)	A172		
		CI (24h)	CI (48h)	CI (72h)
25	25	0.36339	8.228E8	84.6604
25	100	0.33301	7.715E8	70.7814
25	250	0.41372	7.822E8	20.4841
25	500	0.29223	9.885E9	8.72126
50	25	0.25760	1.30E11	1.53466
50	100	0.27298	3.68E11	7.65803
50	250	0.27368	1.73E12	3.04065
50	500	0.29123	2.87E12	6.10855

In A172 cells, the combination of CLZ with H₂O₂ (Table 15) showed synergistic effects at 24 hours for both combinations, and at 48 hours specifically for the combination of 25 μM CLZ with 132 μM H₂O₂, all with CI values below 1. Figure 35 illustrates these results with pink triangles and a blue diamond under 1, indicating the cooperative interaction between these two substances.

Table 15. Combination index (CI) values of CLZ and H₂O₂ Tyr combinations in A172 cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Clozapine (μM)	Dose H ₂ O ₂ (μM)	A172		
		CI (24h)	CI (48h)	CI (72h)
25	132	0.45249	0.70985	8.69543
50	132	0.80732	1.64899	15.5280

In Table 16, the combination of CLZ, H₂O₂ and Tyr exhibited synergism only at 24 hours, with CI values less than 1, while all other combinations showed CI values above 1. These findings are demonstrated in Figure 35 with the green plus signs presented on the graph.

Table 16. Combination index (CI) values of CLZ, H₂O₂ and Tyr combinations in A172 cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Clozapine (μM)	Dose H ₂ O ₂ (μM)	Dose Tyrosine (μM)	A172		
			CI (24h)	CI (48h)	CI (72h)
25	132	500	0.36817	1.09E10	190.077
50	132	500	0.95749	1.41E10	33.3389

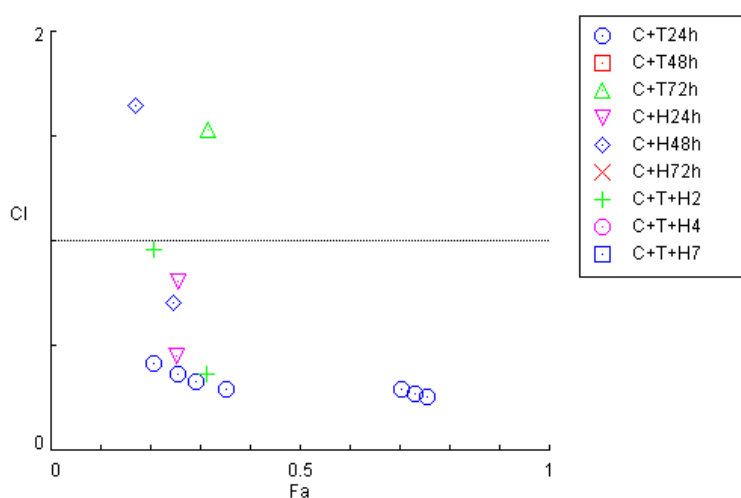


Figure 35. Fa–CI plot of combinations with CLZ in the A172 cell line. Combinations are represented as follows: CLZ + Tyr 24h (blue circle), CLZ + Tyr 48h (red square), CLZ + Tyr 72h (green triangle),

CLZ + H₂O₂ 24h (pink triangle), CLZ + H₂O₂ 48h (blue diamond), CLZ + H₂O₂ 72h (red cross), CLZ + H₂O₂ + Tyr 24h (green plus sign), CLZ + H₂O₂ + Tyr 48h (pink circle) and CLZ + H₂O₂ + Tyr 72h (blue square).

To conclude, the best synergistic effect was observed when combining CLZ with Tyr at 24 hours in glioblastoma cells, exhibiting the lowest CI values and the highest Fa values. Specifically, the combinations with higher concentrations of CLZ achieved Fa values between 0.7 and 0.8, indicating cell death rates of 70% and 80%.

The combination of RIS with Tyr in A172 cells (Table 17) demonstrated no synergistic effect at any tested concentration or times, as evidenced by CI values exceeding 1 for all concentration pairs.

Table 17. Combination index (CI) values of RIS and Tyr combinations in A172 cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Risperidone (μM)	Dose Tyrosine (μM)	A172	
		CI (48h)	CI (72h)
50	25	7.145E8	52.0120
50	100	5.613E8	422.123
50	250	6.455E9	33.3938
50	500	1.37E10	671.597
100	25	2.485E8	5.299E7
100	100	6.387E8	3.94E52
100	250	1.33E10	924479.
100	500	3.70E10	2.931E7

As shown in Table 18, the combination of RIS with H₂O₂ exhibited synergism at only one concentration after 24 hours, with CI values below 1. All other combinations had CI values above 1. These findings are illustrated in Figure 36 by the green triangle on the graph.

Table 18. Combination index (CI) values of RIS and H₂O₂ combinations in A172 cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Risperidone (μM)	Dose H ₂ O ₂ (μM)	A172		
		CI (24h)	CI (48h)	CI (72h)
50	132	2.30127	3.71558	14.4457
100	132	0.87690	267.509	10517.6

In A172 cells, the combination of RIS with H₂O₂ and Tyr showed synergism only at 24 hours, with CI values below 1 (Table 19). All other combinations had CI values above 1. These results are illustrated in Figure 36 by the red crosses below the CI value of 1 on the graph.

Table 19. Combination index (CI) values of RIS, H₂O₂ and Tyr combinations in A172 cells at 24, 48 and 72 hours: CI < 1 indicates synergism, CI = 1 indicates additivity, and CI > 1 indicates antagonism.

Dose Risperidone (μM)	Dose H ₂ O ₂ (μM)	Dose Tyrosine (μM)	A172		
			CI (24h)	CI (48h)	CI (72h)
50	132	500	0.64749	2.00E10	9.65919
100	132	500	0.45423	1.25E10	16.7132

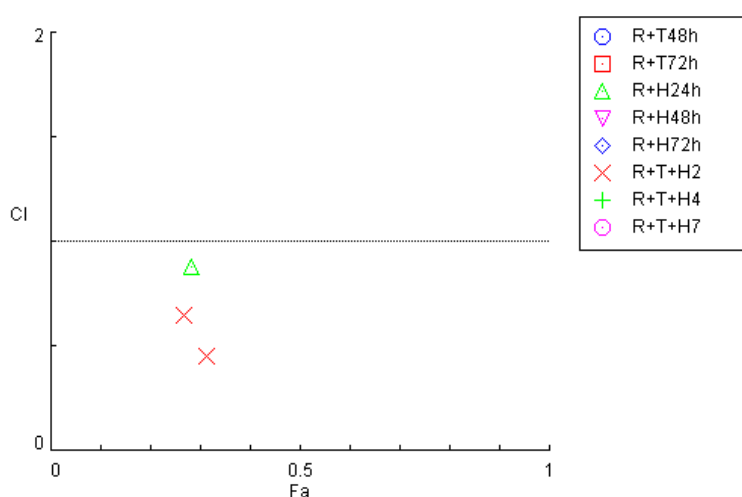


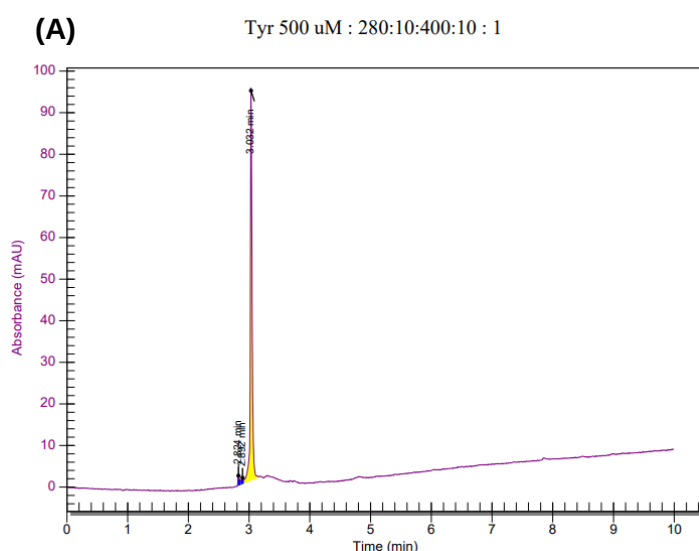
Figure 36. Fa–CI plot of combinations with RIS in the A172 cell line. Combinations are represented as follows: RIS + Tyr 48h (blue circle), RIS + Tyr 72h (red square), RIS + H₂O₂ 24h (green triangle),

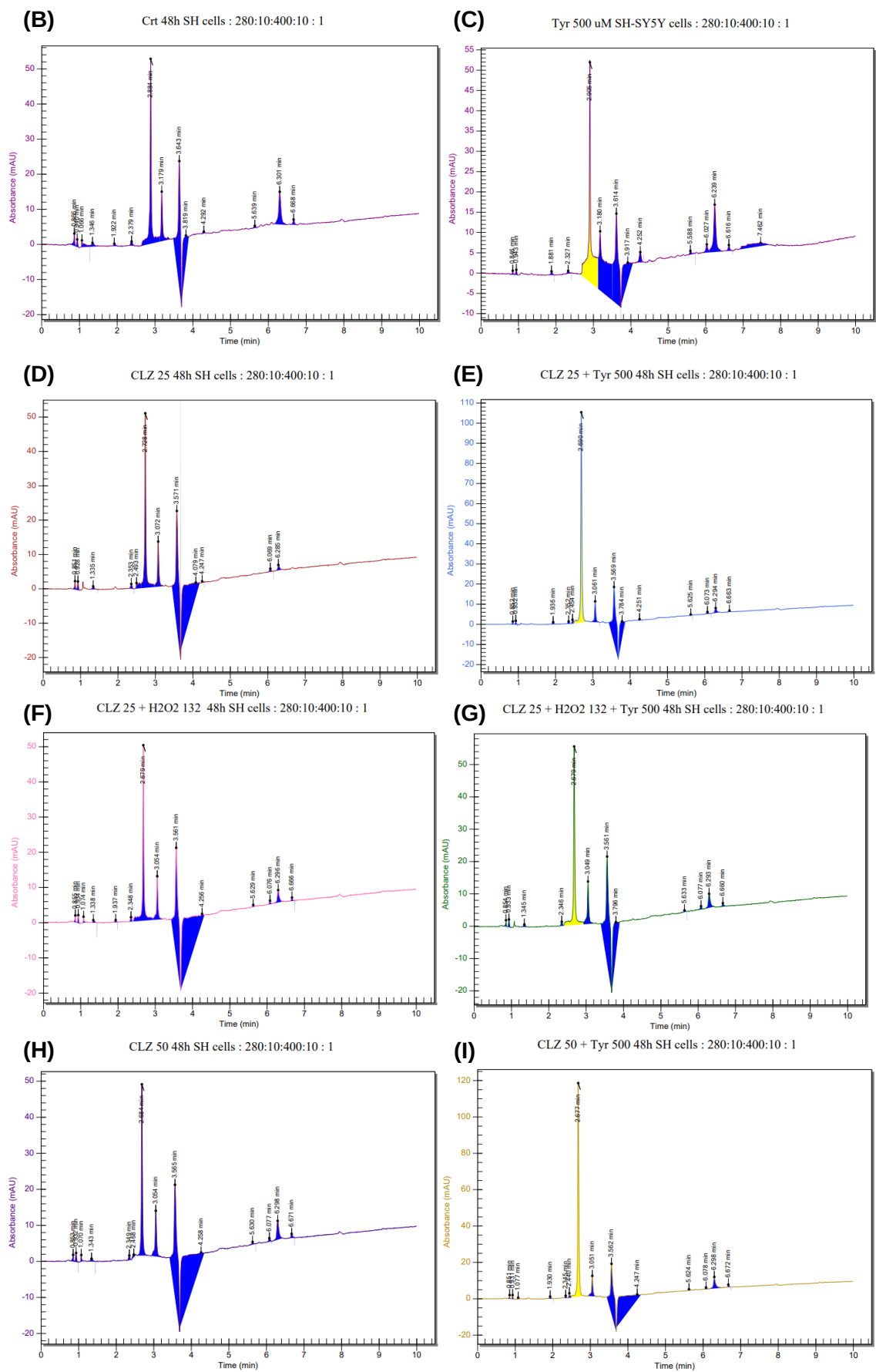
RIS + H₂O₂ 48h (pink triangle), RIS + H₂O₂ 72h (blue diamond), RIS + H₂O₂ + Tyr 24h (red cross), RIS + H₂O₂ + Tyr 48h (green plus sign) and RIS + H₂O₂ + Tyr 72h (pink circle).

In summary, the combinations with RIS showed limited synergism, with only three combinations achieving CI values below 1. Among all tested combinations in both cell lines, those involving RIS were the least promising, presenting the weakest synergistic effects.

Impacts of Clozapine and its Combinations with Tyrosine and H₂O₂ on Extracellular Tyrosine and Dopamine Levels in SH-SY5Y and A172 cell lines

Given that CLZ emerged as the most promising drug in the combinations tested on both cell lines, we decided to further investigate its effects and those of its combinations using HPLC to observe the concentrations of Tyr and DA. To assess the influence of the 48 hours treatment with CLZ and its combinations with Tyr and H₂O₂ on extracellular Tyr and DA concentrations in the SH-SY5Y and A172 cell lines, we treated the cells with CLZ at concentrations of 25 and 50 μ M, and Tyr and H₂O₂ at concentrations of 500 and 132 μ M, respectively. The high concentration of Tyr was chosen to ensure a measurable response, allowing a more precise interpretation of the HPLC results (Figures 37 and 38). After exposing the cells to the different treatment conditions, the extracellular medias were collected, with the aim of understanding the effect of these treatments on the Tyr and DA extracellular concentration (Tables 20 and 21).





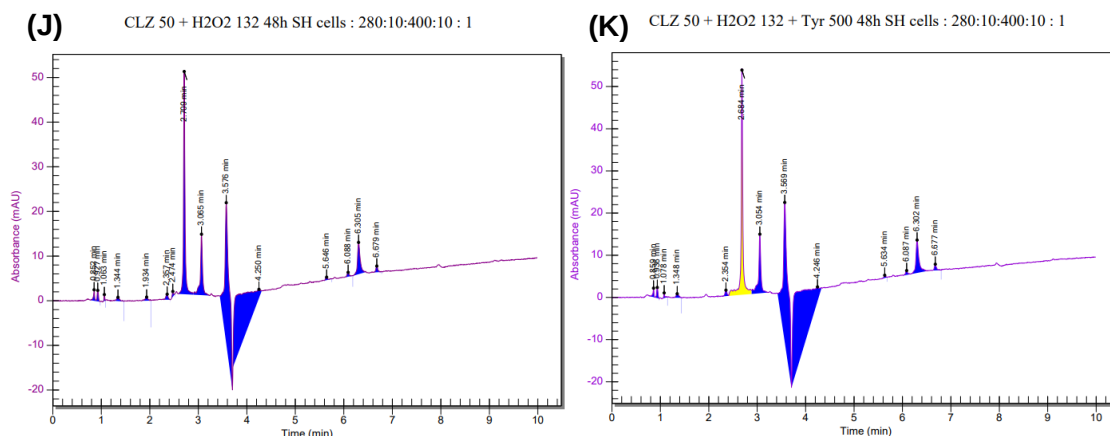
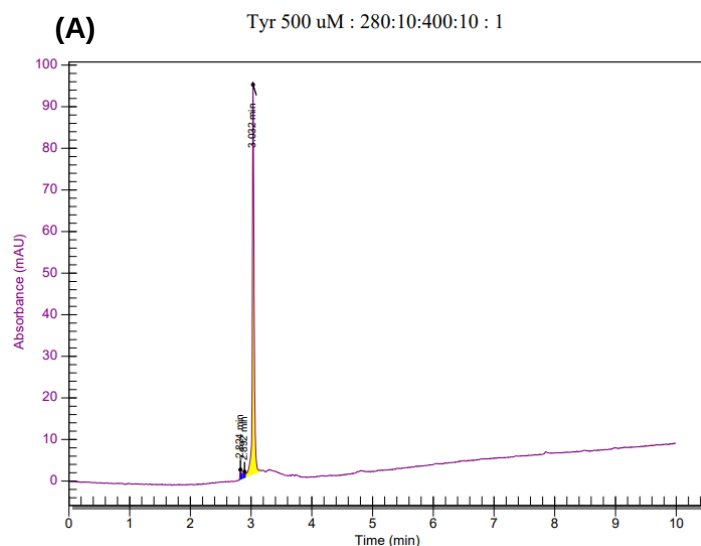


Figure 37. Analysis of the CLZ combinations with Tyr and H₂O₂ in the SH-SY5Y cell line. Representative chromatograms of (A) internal standard containing the indicated Tyr, (B) cell culture medium sample of untreated cells (control), (C) cell culture medium sample of Tyr 500 μ M, (D) cell culture medium sample of CLZ 25 μ M, (E) cell culture medium sample of CLZ 25 μ M with Tyr 500 μ M, (F) cell culture medium sample of CLZ 25 μ M with H₂O₂ 132 μ M, (G) cell culture medium sample of CLZ 25 μ M with H₂O₂ 132 μ M and Tyr 500 μ M, (H) cell culture medium sample of CLZ 50 μ M, (I) cell culture medium sample of CLZ 50 μ M with Tyr 500 μ M, (J) cell culture medium sample of CLZ 50 μ M with H₂O₂ 132 μ M, (K) cell culture medium sample of CLZ 25 μ M with H₂O₂ 132 μ M and Tyr 500 μ M. Representative chromatograms were obtained in three independent experiments ($n = 3$).

Our data demonstrated that after 48 hours of Tyr exposure in SH-SY5Y cells, the concentration of Tyr in the extracellular medium decreased approximately 147 μ M. When Tyr was combined with CLZ at 25 μ M, Tyr levels in the extracellular medium decreased by about 115 μ M, and with CLZ at 50 μ M, Tyr levels by around 207 μ M (Table 20). These findings are supported by chromatograms C, E and I in Figure 37, which illustrates that the Tyr peak (shown in yellow) is smaller compared to the Tyr standard peak (Figure 37 A). Additionally, when Tyr was not added to the medium, no Tyr was detected in any of the samples. Furthermore, the addition of H₂O₂ to the combination of CLZ and Tyr resulted in undetectable Tyr levels, indicating that Tyr was rapidly and completely consumed by the cells. This consumption likely occurred because the cells, under oxidative stress, utilized all the available nutrients and components from the extracellular medium in an attempt to counteract the stress and restore balance. As SH-SY5Y cells are known to be a valuable tool in research involving DA synthesis [67], no DA concentration was detected in the extracellular medium, contrary to expectations. This absence suggests that DA was either completely consumed by the cells or was present at such low levels that it could not be detected by HPLC.

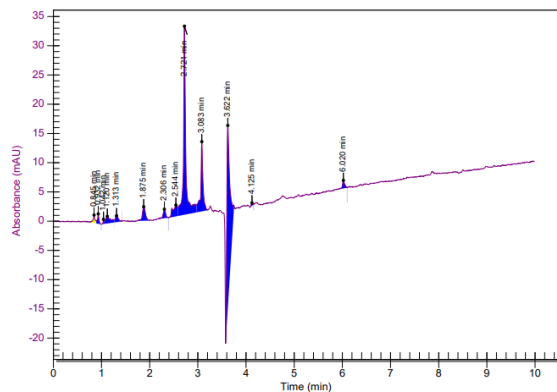
Table 20. Retention times and Areas used to calculate variation in Tyr concentrations in the cell medium of CLZ and its combinations in SH-SY5Y cells, determined by HPLC.

SH-SY5Y cells (48h)	Retention Time (min)	Total Area	Tyr Area	Final Area	Tyr Concentration (μM)
Standard	2.694	-	195139.56	-	-
Control	2.884	-	-	-	-
Tyr 500 μM	2.905	234969.88	91406.03	103733.53	353,19
CLZ 25 μM	2.728	-	-	-	-
CLZ 25 μM + Tyr 500 μM	2.690	225583.15	82019.33	113120.23	385,22
CLZ 25 μM + H_2O_2 132 μM	2.679	-	-	-	-
CLZ 25 μM + H_2O_2 132 μM + Tyr 500 μM	2.679	143483.79	-80.3	Tyr was all consumed	-
CLZ 50 μM	2.684	-	-	-	-
CLZ 50 μM + Tyr 500 μM	2.677	252686.25	109122.43	86017.13	292,73
CLZ 50 μM + H_2O_2 132 μM	2.709	-	-	-	-
CLZ 50 μM + H_2O_2 132 μM + Tyr 500 μM	2.684	137808.98	-5754.84	Tyr was all consumed	-

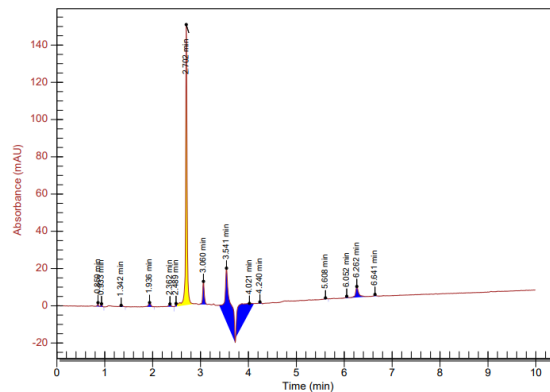


(B)

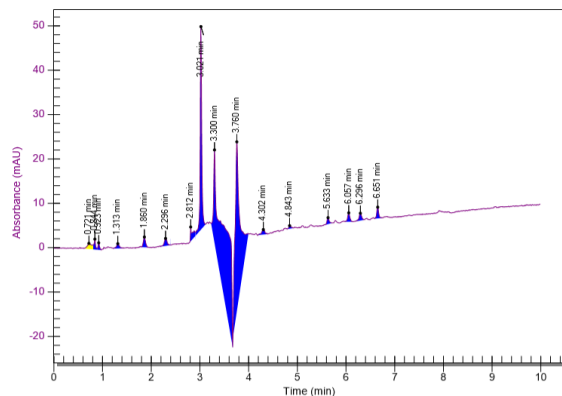
Crt 48h A172 cells : 280:10:400:10 : 1

**(C)**

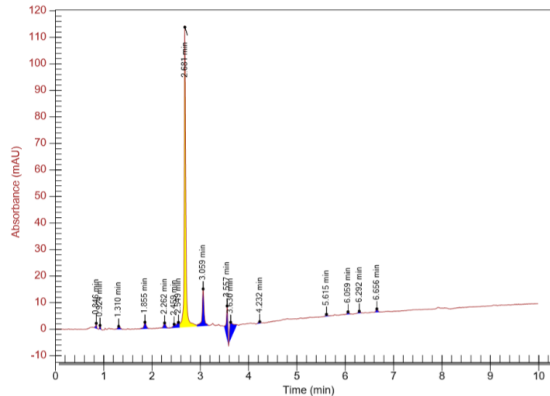
Tyr 500 uM A172 cells : 280:10:400:10 : 1

**(D)**

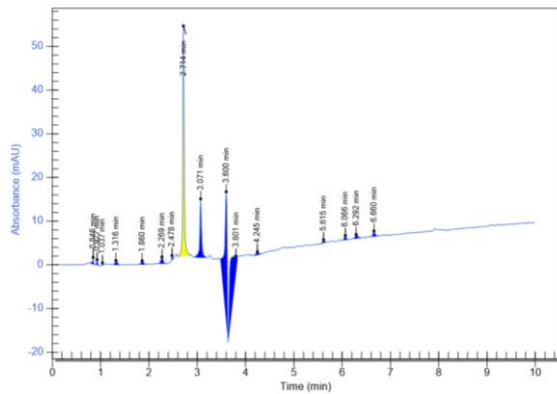
CLZ 25 48h A172 cells : 280:10:400:10 : 1

**(E)**

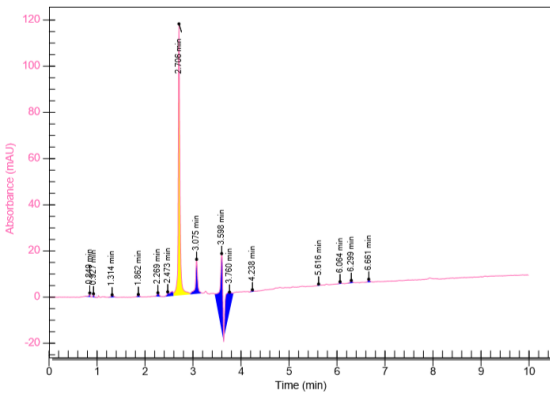
CLZ 25 + Tyr 500 48h A172 cells : 280:10:400:10 : 1

**(F)**

CLZ 25 + H2O2 132 48h A172 cells : 280:10:400:10 : 1

**(G)**

CLZ 25 + H2O2 132 + Tyr 500 48h A172 cells : 280:10:400:10 : 1



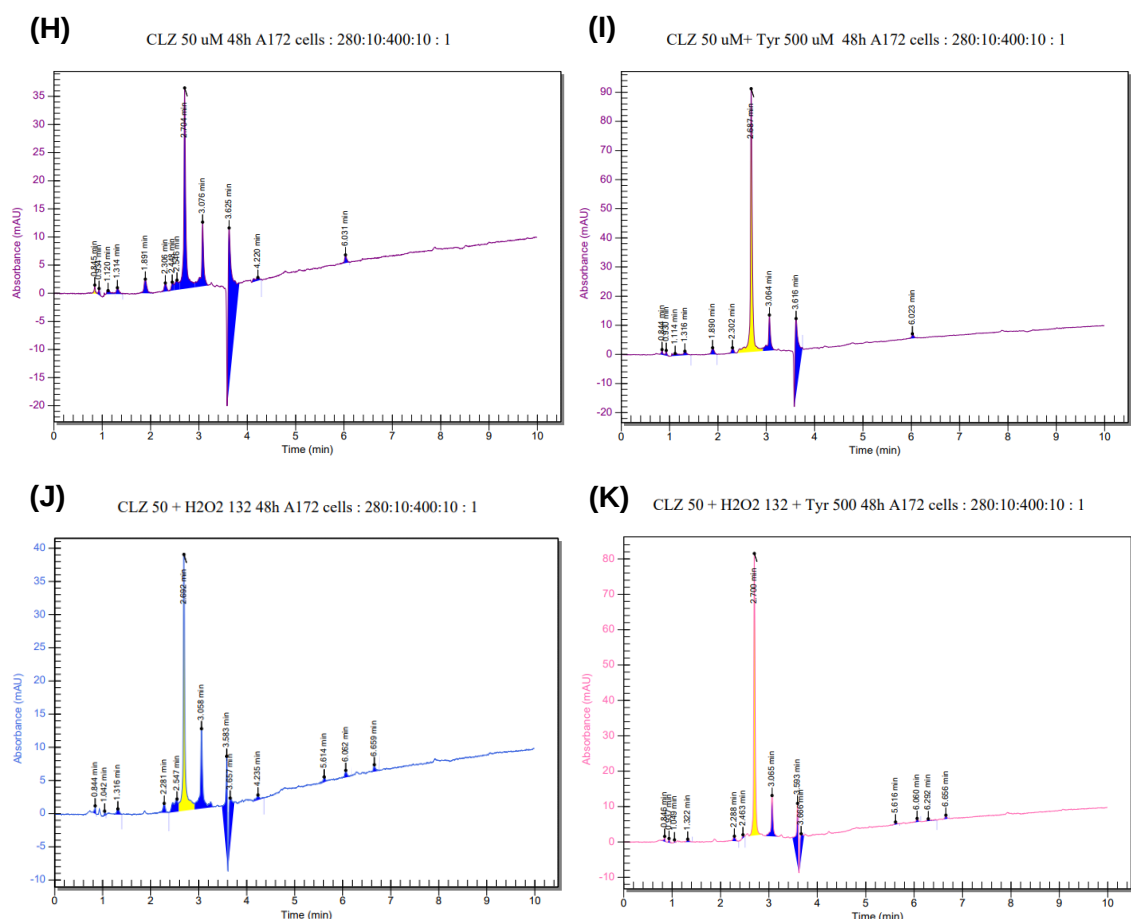


Figure 38. Analysis of the CLZ combinations with Tyr and H₂O₂ in the A172 cell line. Representative chromatograms of (A) internal standard containing the indicated Tyr, (B) cell culture medium sample of untreated cells (control), (C) cell culture medium sample of Tyr 500 μ M, (D) cell culture medium sample of CLZ 25 μ M, (E) cell culture medium sample of CLZ 25 μ M with Tyr 500 μ M, (F) cell culture medium sample of CLZ 25 μ M with H₂O₂ 132 μ M, (G) cell culture medium sample of CLZ 25 μ M with H₂O₂ 132 μ M and Tyr 500 μ M, (H) cell culture medium sample of CLZ 50 μ M, (I) cell culture medium sample of CLZ 50 μ M with Tyr 500 μ M, (J) cell culture medium sample of CLZ 50 μ M with H₂O₂ 132 μ M, (K) cell culture medium sample of CLZ 25 μ M with H₂O₂ 132 μ M and Tyr 500 μ M. Representative chromatograms were obtained in three independent experiments (n = 3).

Our results showed that after 48 hours of Tyr exposure in A172 cells, the concentration of Tyr in the extracellular medium increased by approximately 86 μ M. When combined with CLZ at 25 μ M, Tyr levels decreased by about 385 μ M, and with CLZ at 50 μ M, Tyr levels decreased by around 361 μ M (Table 21). When Tyr was combined with CLZ at 25 μ M and H₂O₂, Tyr levels increased by approximately 103 μ M, and with CLZ 50 μ M and H₂O₂, Tyr levels decreased by around 299 μ M (Table 21). These findings are corroborated by chromatograms in Figure 38. Combinations resulting in a decrease in Tyr concentration show slightly smaller peaks compared to the Tyr standard peak (chromatograms E, I and K). Conversely, combinations with an increase in Tyr levels exhibit

slightly higher peaks than the Tyr standard peak (chromatograms C and G). Additionally, when Tyr was not added to the medium, no Tyr was detected in any samples. Furthermore, unlike the results in the SH-SY5Y cells, the addition of H₂O₂ to the combination of CLZ and Tyr did not result in undetectable levels of Tyr, and one combination even showed higher Tyr levels than the standard. In this cell line, no DA levels were detected in the extracellular medium either.

Table 21. Retention times and Areas used to calculate variation in Tyr concentrations in the cell medium of CLZ and its combinations in A172 cells, determined by HPLC.

A172 cells (48h)	Retention Time (min)	Total Area	Tyr Area	Final Area	Tyr Concentration (µM)
Standard	2.694	-	195139.56	-	-
Control	2.721	-	-	-	-
Tyr 500 µM	2.702	332258.91	220009.74	-24870.18	- 85,69954269
CLZ 25 µM	3.021	-	-	-	-
CLZ 25 µM + Tyr 500 µM	2.681	274430.57	162181.4	32958,16	111,65
CLZ 25 µM + H ₂ O ₂ 132 µM	2.714	-	-	-	-
CLZ 25 µM + H ₂ O ₂ 132 µM + Tyr 500 µM	2.706	294476.33	182227.16	176912,4	602,93
CLZ 50 µM	2.704	-	-	-	-
CLZ 50 µM + Tyr 500 µM	2.687	266350.71	154101.54	41038.02	139,23
CLZ 50 µM + H ₂ O ₂ 132 µM	2.692	-	-	-	-
CLZ 50 µM + H ₂ O ₂ 132 µM + Tyr 500 µM	2.700	171386.49	59137.32	59137.32	200,99

Discussion

It's important not only to investigate more forms of treatment for primary brain tumors, but also because between 9% and 17% of the most common and deadly adult cancers, such as lung, breast, kidney, colon and melanoma, metastasize to the brain [68]. Since therapies for primary brain tumors are often ineffective, they also tend to be ineffective against metastases from other tumor types [69]. Whether dealing with a primary brain tumor or a metastasis, the severity and location of the tumor make treatment equally difficult to treat, necessitating further research to discover new treatment options [70]. Additionally, the presence of the blood-brain barrier hinders the effectiveness of therapies like chemotherapy [68,71].

Over the years, it has become evident that cancer is a disease characterized by mutations in various pathways, contributing to its development and progression. Despite advances in cancer treatment, achieving a complete cure remains difficult. Therefore, it is crucial to identify as many mutated pathways as possible in this disease, whether they are genetic, epigenetic, metabolic, cell signaling, or neuronal. The tryptophan pathway is frequently mutated in various types of cancer, and the serotonergic signaling system, which develops from tryptophan, is often deregulated, influencing tumor growth depending on serotonin concentration and receptors involvement [72]. Additionally, neurotransmitters such as serotonin can affect tumor progression by altering the tumor microenvironment [73].

Since neurotransmitter pathways originate in the brain, they could be used in the treatment of brain tumors. Many conventional treatments are ineffective due to the blood-brain barrier, which prevents therapeutic agents from reaching their targets. By utilizing pathways already present in the brain and employing drugs that inhibit these pathways, we could potentially overcome this barrier more effectively and enhance treatment. This approach could improve the administration and effectiveness of treatments, providing a more targeted and efficient method for combating brain tumors. In addition, the use of endogenous pathways could reduce side effects and improve patient outcomes by minimizing the need for high doses of systemic drugs. Given these insights, we decided to investigate the tyrosine pathway involved in synthesizing dopamine to determine if it is also associated with cancer progression.

DA plays a dual role in the body, acting as a neurotransmitter in the CNS and as a circulating hormone in peripheral tissues, such as the adrenal glands, spleen, pancreas, and sympathetic nerves. It is synthesized in the brain from amino acid Tyr through a series of enzymatic steps [73,74]. DRs are expressed in various tissues and organs such as the

brain, retina, heart, coronary arteries, gastrointestinal tract and sympathetic ganglia [74]. Dysregulation of the dopaminergic system is a fundamental mechanism in many diseases, such as schizophrenia and Parkinson's, with the DRs being key targets in their treatments [74].

Epidemiological and molecular biological studies have indicated that patients with schizophrenia and PD exhibit different susceptibilities to various cancers, with varying effects even within the same type of cancer. This suggest that an imbalance in the dopaminergic system may be related to the development of cancer [73]. Additionally, patients treated with antipsychotics have been observed to have a lower incidence of certain cancers, including prostate, breast and rectal cancer [75]. And interestingly, different dopamine receptors may exhibit varying responses and predispositions in different tumors and cancer types [73]. These observations collectively suggest a potential connection between the dopaminergic system and carcinogenesis [74]. Thus, DA has been observed to play a crucial role in inducing cell proliferation, apoptosis, tumor angiogenesis, migration and gene expression through the specific activation of DRs and this process may promote tumor and disease progression, as demonstrated in several in vivo and in vitro studies [74,75].

To elucidate the connection between the dopamine pathway and neuro-oncology, we started to test six dopamine receptor drugs on the SH-SY5Y and A172 cell lines. Our findings revealed that the most cytotoxic drugs in SH-SY5Y cells were CLZ, DOM and PIM, while in A172 cells, CLZ and RIS had the greatest effect on decreasing cell viability. These drugs share a common mechanism of action as DA antagonists, blocking DRs and reducing the effects of DA on cells. Notably, except for DOM, all the drugs demonstrating significant anticancer potential are antipsychotics, aligning with previous studies suggesting that antipsychotics may have anticancer properties.

Among the drugs effective in both cell lines, PIM stood out, with numerous studies supporting its potential as an anticancer agent. Its antiproliferative effects have been demonstrated in various tumor cell lines, including neuroblastoma cells [35,36], which is consistent with our results. Additionally, CLZ induced cell death in SH-SY5Y at concentrations of 30 μ M and higher after a 72 hour incubation period, as demonstrated in two studies [76,77]. This finding is consistent with our results, where CLZ exhibited cytotoxic effects in both SH-SY5Y and A172 cells. Notably, CLZ was the only drug among the six tested that showed anticancer effects in both cell lines, highlighting its potent cytotoxic properties for the treatment of brain tumors.

Regarding RIS, some studies have shown that it has neurotoxic and neuroprotective activities in neuroblastoma cells [78]. However, in our study, RIS did not exhibit neurotoxic effects in SH-SY5Y cells. Instead, it promoted proliferation in glioblastoma cells, which is in line with previous findings [79]. On the contrary, OLZ did not show any toxicity in any of the cell lines studied, which contradicts previous studies that reported significant neuroprotective effects on SH-SY5Y cells [78,80] and decreased the viability in A172 cells, achieving notable antiproliferative effects with an IC₅₀ of 27.9 μ M [81]. This divergence could be due to differences in experimental conditions, such as variations in drug concentrations, incubation periods, or cell line handling.

Furthermore, we evaluated the impact of the amino acid Tyr on both cell lines to understand its effects. Our results showed that Tyr was not toxic to the cells. Although there were some small, insignificant decreases in cell viability, these were likely due to the generation of tyrosyl radicals by oxidation, which can cause cellular oxidative damage at higher concentrations of Tyr, as described in our previous study [72]. These results were somewhat expected, given that Tyr is an amino acid naturally produced by our bodies and that cells, particularly SH-SY5Y cells, can express enzymes involved in Tyr synthesis [82].

In our evaluation of the combined effects of the repurposed drugs more effective seen above with Tyr, our results showed a more pronounced reduction in cell viability for the combination of CLZ with Tyr in SH-SY5Y cells compared to CLZ alone. This suggests a possible synergistic effect between CLZ and Tyr, which was confirmed by synergistic studies at 72 hours, demonstrating that Tyr increased the effectiveness of CLZ in reducing cell viability. However, further studies into the mechanisms involved in this synergism could elucidate the pathways involved in this interaction. The other two drugs, DOM and PIM, exhibited less toxicity when combined with Tyr than when tested individually, indicating better outcomes for these drugs when not combined with Tyr in SH-SY5Y cells. This suggests that combining these two drugs with Tyr may mitigate their cytotoxic effects, possibly through competitive interactions or changes in metabolic pathways. The observed differences between the three drugs may be attributed to the lower concentrations used for testing DOM and PIM in combination compared to the concentrations used for CLZ. Higher concentrations of DOM and PIM could produce different results, therefore testing different concentrations later could confirm if they effectively produce the same results. Regarding the A172 cell line combinations, our findings indicated that the combination of CLZ with Tyr produced better results at a higher concentration of CLZ (50 μ M). However, at the lowest concentration, CLZ was more effective alone than in combination with Tyr, suggesting that the presence of Tyr could reduce CLZ's effectiveness at lower dosages. Synergistic studies

confirmed these results, showing pronounced synergism between CLZ and Tyr at 24 hours, especially at higher CLZ concentrations. This suggests a dose-dependent synergistic effect, becoming more evident at higher CLZ concentrations in A172 cells. For RIS, the combination with Tyr didn't show increased toxicity compared to RIS alone in the A172 cells, indicating a lack of synergism. Synergistic studies confirmed this, showing no synergistic effect between the two compounds at any combinations or time. These findings suggest that combining RIS with Tyr may not improve therapeutics outcomes and that RIS's efficacy may be best used independently.

When assessing the impact of the repurposed drugs with H₂O₂, our findings revealed that, among the three drugs tested in SH-SY5Y cells, only CLZ showed a slight decrease in cell viability compared to the drug alone, but the reduction was not considerable. However, synergistic studies indicated a high synergism between CLZ and H₂O₂, with synergism values close to 0, suggesting a strong synergism effect. As previously mentioned, H₂O₂ induces cellular stress, demonstrating that this repurposed drug works well even under stressful conditions. According to this research, the combination of CLZ and H₂O₂ can be a powerful therapeutic strategy, especially in situations of cellular stress. In the A172 cells, our results indicated that neither CLZ nor RIS caused significant decreases in cell viability compared to the drugs alone. Notably, CLZ, the most promising drug in combination, exhibited higher cytotoxic when combined with Tyr rather than with the H₂O₂. From these results, we concluded that the repurposed drugs don't perform the same under oxidative stress. When combined with H₂O₂, an oxidative stress inducer, the drugs were less cytotoxic, suggesting that A172 cells become more resistant to the drugs. This reduced efficacy under oxidative stress suggests that excessive oxidative stress can modify cells behavior, potentially serving as a mechanism used by tumor cells to escape treatment.

When evaluating the effects of the repurposed with H₂O₂ and Tyr in the SH-SY5Y cell line, we observed that the addition of Tyr did not significantly impact cell viability compared to the drugs alone with H₂O₂. However, when comparing the drugs with Tyr alone, we noted a greater decrease in cell viability when H₂O₂ was added to the combination of the drug and Tyr. This indicates that the combination of all three compounds produced a more substantial reduction in cell viability and increased toxicity. These results were confirmed by synergy studies, which showed significant synergism at 72 hours for the combination with CLZ and PIM and at 24 hours for the combination with DOM, all with highly significant values close to 0. Therefore, in the presence of Tyr, oxidative stress does not significantly impact the SH-SY5Y cells response and behavior to the drug treatment, unlike the response seen with the drug and H₂O₂ alone. This suggest that Tyr may mitigate the

protective effect of oxidative stress on SH-SY5Y cell viability, enhancing the cytotoxic effects of the drugs. In the A172 cell line, our results revealed no accentuated changes in cell viability when comparing the combination of drugs alone with H₂O₂. This was corroborated by synergistic studies, which showed that the synergistic effects observed over 24 hours were quite high, indicating that the synergism between these compounds was not very strong. When Tyr was added to the combination of drugs and H₂O₂ the effects observed with and the drugs alone were also mitigated due to the oxidative stress. This indicates that the A172 cells become more resistant to the administered compounds under oxidative stress. These findings emphasize the differential impact of oxidative stress on drug efficacy in different cell lines, implying that cell-specific factors may influence the response to combined drug and oxidative stress therapies. Understanding these interactions on a molecular level could guide the development of more effective treatments.

After a period of 48 hours, the extracellular concentration of Tyr combined with CLZ varied between the two cell lines, suggesting that these cells depend on Tyr differently for their growth and development. However, comparing cell viability values with the Tyr concentration obtained in the extracellular medium reveals similar behavior in both cell lines. When cell viability is close to 100%, meaning there is no significant decrease in viability, Tyr is completely consumed by the cells, resulting in no detectable Tyr in the extracellular medium. However, when cell viability decreases significantly, the Tyr concentration also decreases substantially, indicating partial but not complete consumption by the cells. Nevertheless, an exception was observed in the case of CLZ 25 µM + H₂O₂ 132 µM + Tyr 500 µM, where cell viability decreased significantly, but Tyr concentration increased, suggesting a small production of Tyr by the SH-SY5Y cells. These results indicate that Tyr concentration varies inconsistently and is not strongly dependent on cell viability values or the specific cell lines tested. These findings indicate a complex interaction between Tyr, CLZ and H₂O₂ in A172 cells, which differs from the responses observed in SH-SY5Y cells. This suggest that cell type-specific mechanism may influence the metabolism and extracellular concentration of Tyr. Moreover, it highlights that more aggressive cells, such as glioblastoma cells, can still increase Tyr levels even under conditions of oxidative stress, as was observed in the combination of CLZ 25 µM + H₂O₂ 132 µM + Tyr 500 µM.

Overall, our results highlight the potential anticancer properties of certain drugs targeting dopamine receptors, particularly antipsychotics, in neuro-oncology, while also highlighting their behavior in combination with an amino acid and under conditions of cellular stress.

Conclusion and Future Perspectives

In conclusion, several of the antipsychotics studied demonstrated cytotoxic effects on both neuroblastoma and glioblastoma cell lines, suggesting their potential as anticancer treatments. Among the six repurposed drugs, CLZ showed the most promising results on both cell lines. Its superior efficacy may be attributed to its higher affinity for D4 receptors compared to D2 receptors. In contrast, the other repurposed drugs primarily targeted dopaminergic D2 or serotonergic receptors, probably contributing to their reduced effectiveness. Therefore, we conclude that the tested cell lines likely have a higher prevalence of D4 receptors. However, further studies are needed to precisely determine which dopamine receptors are presented in these and other tumor cells. Additionally, this research emphasizes the importance of receptor affinity in the development of targeted anticancer therapies.

Furthermore, our work demonstrated that the outcomes of repurposed drug combinations with Tyr and/or H₂O₂ vary depending on the drug and cell line. The most favorable results were observed with CLZ combined with Tyr in both cell lines, and with H₂O₂ only in neuroblastoma cells. On the contrary, combinations of the repurposed drugs with Tyr and H₂O₂ in SH-SY5Y cells exhibited synergistic among the three compounds.

This study also highlighted the impact of oxidative stress on the consumption of Tyr by the cells when combined with CLZ, suggesting that cells prioritize the use of available extracellular nutrients to try to mitigate stress. This emphasizes the importance of considering cellular and body stress in therapeutic contexts.

Future work should investigate these repurposed drugs on various tumor cell lines to explore their potential anticancer effects beyond neuro-oncology. Additionally, future studies should aim to validate the most promising results through *in silico* and subsequent *in vivo* tests to confirm their applicability in the treatment of oncology patients with brain tumors. Furthermore, these repurposed drugs could be tested in combination with reference drugs for brain tumors to determine if they enhance efficacy and improve therapeutic outcomes.

Refereces

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