UNIVERSIDAD SAN SEBASTIAN

FACULTAD DE MEDICINA Y CIENCIA

SEDE LOS LEONES

THE GAL-8/EGFR AXIS IN THE ANTITUMORAL EFFECTS OF D-PROPRANOLOL ON T98G GLIOBLASTOMA CELLS

Thesis presented to obtain the academic degree of

PhD in Cell Biology and Biomedicine

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Santiago, Chile

2022

Evaluación

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FUNDING

This work was supported by funds of Agencia Nacional de Investigación y Desarrollo of Chilean Government (FONDECYT 1181907 and 1211829, CONICYT Basal Projects AFB-170004 and AFB-170005, ANID/BASAL grants FB210008 and ACE210009).

Tomás Jiménez Miranda was financed by the scholarship for students in doctoral programs of Vicerrectoría de Investigación y Doctorados of Universidad San Sebastián, Chile.

AKNOWLEDGEMENTS

I would like to thank everyone that accompanied and trusted me in this process: my family, friends, tutors, and colleagues. I am grateful for the patience and support that my teachers gave me, who only wished for me to become a better professional and scientist. I will hold on to the lessons learned and display them in my career. I believe in science as a conglomerate effort to describe the phenomena of life and I hope that my contribution will resonate in the future.

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1. ABSTRACT

Glioblastoma (GB) is the most severe type of brain neoplasia, as patients present a lifespan of roughly 12 months. Current treatments only partially diminish GB aggressiveness and do not improve lifespan significantly. The high migratory activity invading secondary regions of the brain is one of the most difficult of its malignant properties to counteract. New therapeutic alternatives are urgently needed. A potential approach to reduce GB malignancy is to simultaneously interfere with the function of different proteins involved in GB pathogenicity. In this thesis we addressed the epidermal growth factor receptor (EGFR) and Galectin-8 (Gal-8).

EGFR is the most commonly altered gene in GB, found overexpressed in 40% to 50% of cases and proposed as a marker of poor prognosis (Li, Liang, Song, Xiang, & Liu, 2018). EGFR variant III (EGFRvIII), a deletion mutant that lacks most of the extracellular domain of the receptor precluding its binding to ligand is one of the most aggressive oncoproteins. EGFR belongs to the ErbB tyrosine kinase family receptor that regulates signaling pathways involved in a variety of cellular processes, such as cell growth, viability, and migration (Ciardiello & Tortora, 2008). Drugs that counteract the activity of EGFR and are currently used in other cancers have little effectiveness in GB.

Our laboratory has shown that the secondary amine D-Propranolol can be used to interfere with EGFR function inducing alterations in its endocytic trafficking behavior that result in its intracellular accumulation. This effect associates with effects against proliferation and viability on different cancer cells and involves a little-known signaling pathway downstream phosphatidic acid (PA). D-Propranolol induces rapid increments of PA, which activates type 4 phosphodiesterases leading to decreased cAMP levels and PKA activity. Direct inhibitors of PKA activity mimic the endocytic effect of D-Propranolol on the EGFR.

As a secondary amine, D-Propranolol may in principle induce lysosomal damage, a condition actively studied as a potential route for the elimination of cancer cells. Damaged endolysosomes are detected by a control system in the

cytosolic compartment constituted by Galectins, which are carbohydrates recognizing proteins, and in this context, Galectin-3 (Gal-3), Galectin-9 (Gal-9) and Galectin-8 (Gal-8), which play different and complementary functions in autophagic removing or repairing of these organelles (Jia et al., 2020). In particular, when endolysosomal membrane present leakages Gal-8 interacts with the lysosome amino acid transporter SLC38A9 and the Ragulator/Rag complex resulting in displacement and inactivation of mTORC1 (Jia et al., 2018). These events serve as a protection mechanism for reestablishing lysosomal function and cell homeostasis. To this date, these intracellular protecting properties of Gal-8 have been investigated in processes of bacterial and viral infections, and tau protein dispersal, remaining unknown in cancer.

Gal-8 is secreted by unconventional mechanisms and its extracellular functions have been associated with multiple pro-tumoral features. Results from our laboratory have described that Gal-8 overexpression in Madin-Darbey Canine Kidney cells (MDCK), a non-cancerous cell type, increases cell proliferation and promotes formation of tumors in xenografts experiments (Oyanadel et al., 2018), while Gal-8 silencing increases apoptosis by 15% in U87 GB cells (Metz et al., 2016). Soluble Gal-8 in the extracellular media induces the migration of U87 GB cells in Transwell assays (Metz et al., 2016). Furthermore, GB cells have been described to manifest aggressive invasion to healthy brain tissue, which leads to recurrence after surgical procedures of tumor removal (Chintala et al., 1999). Preliminary results in collaboration with the laboratory of Dr. Patricia Burgos have shown that D-Propranolol induces Gal-3 recruitment to lysosome-like compartments in HeLa cells, suggesting a potential lysosomal damaging effect of this drug that might contribute to its deleterious effects in cancer cells.

We propose the following hypothesis: *“D-Propranolol decreases viable cell number and invasion of T98G glioblastoma cells interfering with the function of EGFR and Gal-8”*. The general objective is to determine whether D-Propranolol is an effective drug against glioblastoma cells that express malignancy factors such as EGFR and Gal-8. The specific objectives are: 1) To evaluate the effect of

D-Propranolol on the viability of T98G cells, 2) To evaluate the effect of D-Propranolol in lysosomes in T98G cells, 3) To evaluate the impact of Gal-8 expression on T98G cell viability, EGFR signaling and invasion in response to D-Propranolol. Methodology: We used human glioblastoma T98G cells to assess the effects of D-Propranolol on EGFR endocytosis, using indirect immunofluorescence (IFI), immunoblot, and flow cytometry. Viable cell number was evaluated with trypan blue, and proliferation was assessed with EdU thymidine analogue. Lysosomal damage was evaluated by IFI of Gal-3 and Gal-8, as well with transduced Gal-8-ZsGreen in T98G cells, Lysotracker red and Magic red. Gal-8 silencing was achieved by transduction with lentiviral particles containing sh-Gal-8. EGFR and ERK phosphorylation were evaluated by immunoblot. Invasive migration was evaluated by an inverted invasive migration assay.

1.1. Results

1) D-Propranolol decreases viable cell number and proliferation of T98G cells.

2) D-Propranolol induces the endocytosis of EGFR.

3) D-Propranolol induces Gal-3 and Gal-8 recruitment to lysosomes and decreases lysosomal function.

4) Gal-8 overexpression increases cell growth, invasive migration and enhances the signaling response of T98G cells to mitogenic stimulus.

1.2. Conclusions

1) EGFR endocytosis in conjunction with decreased lysosome function appear as combined perturbations implied in the anti-tumoral effect of D-Propranolol.

2) The PA/PDE4/PKA pathway is involved in the viability of T98G cells in D-Propranolol treatments.

3) Gal-8 contributes to the malignancy properties of T98G cells.

2. INTRODUCTION

Glioblastoma (GB) is the most severe brain neoplasia and its current treatment includes a combination of chemotherapy, radiotherapy, and surgical excision of the tumor, which frequently do not increase lifespan and most patients die within 12 months (Louis et al., 2016). The high invasive activity of these cells is one of the most difficult malignant properties to counteract. New therapeutic alternatives are urgently needed. Because the function of EGFR and Gal-8 have been involved in GB pathogenicity, we studied the possibility of interfering with both through the use of D-Propranolol as a potential antitumor drug already tested in other cancers (Barra et al., 2021).

Our laboratory has previously shown that D-Propranolol can be used to interfere with EGFR function inducing alterations in its endocytic trafficking behavior leading in its intracellular accumulation (Shaughnessy et al., 2014) (Barra et al., 2021). In addition, D-Propranolol might also have antitumoral potential through damaging lysosomes similarly to other secondary amines (Stark, Silva, Levin, Machuqueiro, & Assaraf, 2020). Lysosomal membrane damage is being actively researched as a route for eradication of cancer cells. In this context, it is interesting to consider the function of Galectin-8 (Gal-8), which has been associated with GB pathogenesis (Camby et al., 2001). Gal-8 is a member of the galectin family of proteins that bind carbohydrates in glycoproteins and glycolipids in the cytosolic compartment as well as extracellularly after unconventional secretion (Popa, Stewart, & Moreau, 2018). Intracellular Gal-8 participates in the homeostatic response that attempts to remove damaged endolysosomes, while in the extracellular environment Gal-8 confers pro-tumorigenic features associated with cell growth and migration (Oyanadel et al., 2018).

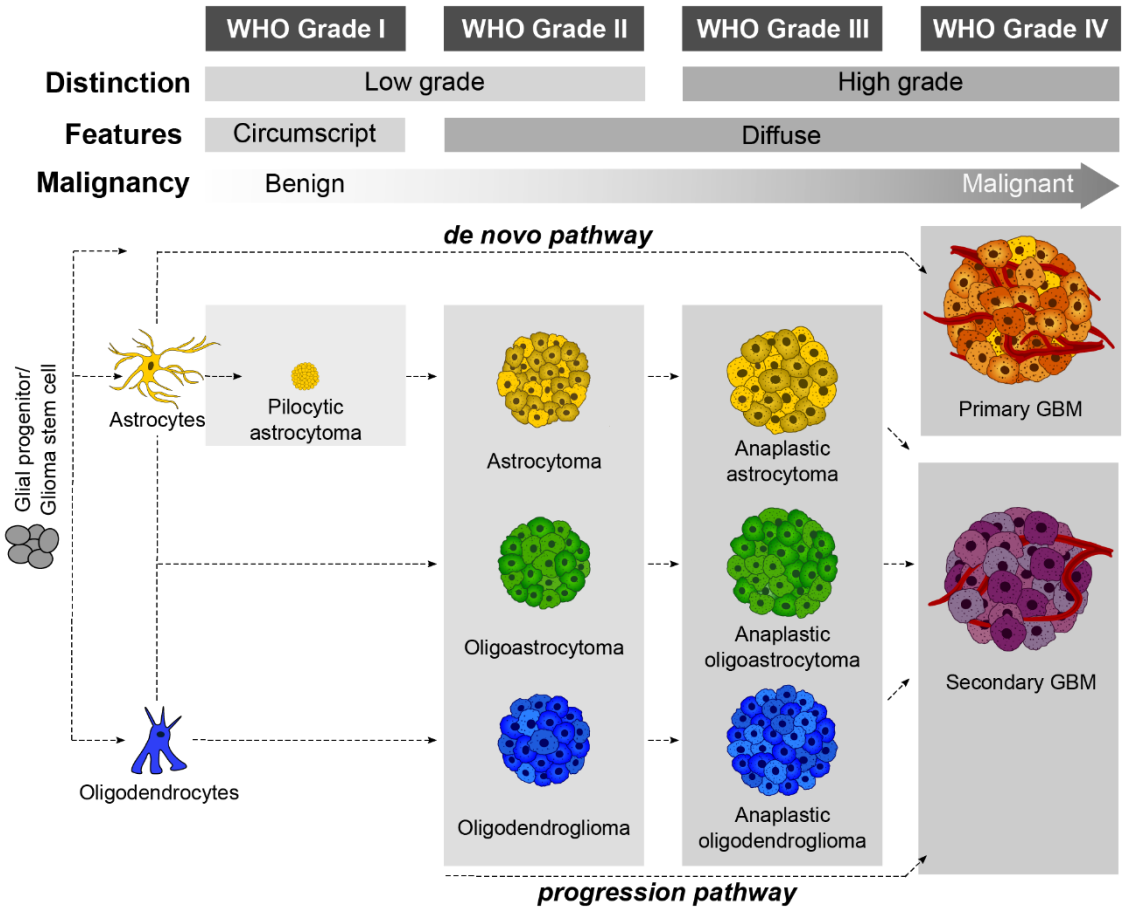
We explore the effects of D-propranolol on the GB cell line that express EGFR and Gal-8, assessing changes in relevant parameters of these proteins, as well as in viable cell number, proliferation, and invasion.

2.1. Glioblastoma is a highly malignant cancer involving EGFR and Gal-8

Glioblastoma (GB) is the most frequent and malign type of astrocytoma accounting for approximately 60% of adult brain tumors (Rock et al., 2012), with a dismal prognosis of median survival of 12 months, despite a multimodal treatment that includes surgical resection, radiotherapy and chemotherapy (Louis et al., 2016). Therefore, new strategies against this cancer are urgently needed.

GB belongs to the group of brain tumors depicted as “gliomas”, which develop for glial cells. GB generates specifically from astrocytes. The international criteria for the nomenclature and diagnosis of GB is defined by the World Health Organization (WHO). This criterion classifies gliomas based on their malignancy from grade I to IV, determined by histopathological parameters. Grade I gliomas are those of low proliferation and which can be treated by surgical procedure, while gliomas from grade II to IV are highly malign and invasive. GB is the most malign of this classification and is therefore assigned as a grade IV glioma (Fig. 1) (Louis et al., 2007); (Jovcevska, Kocevar, & Komel, 2013).

There are two clinical subtypes of GB: primary and secondary GB. Primary GBs are those that generated *de novo*, without malign precursors and without visible lesion progression, and it accounts for 90% of cases. Secondary GBs are arise from the progression of a previous astrocytoma, whether a diffuse astrocytoma (grade II) or an anaplastic astrocytoma (grade III) (Ohgaki & Kleihues, 2013), as depicted in Figure 1 (Tilak, Holborn, New, Lalonde, & Jones, 2021).



Tilak et al., International Journal of Molecular Sciences, 2021.

Figure 1. Glioma grades and origin defined by WHO.

Gliomas can present in four grades and depending on their progression they can be classified as primary or secondary GBs. Primary GBs grow rapidly without pre-existing lesions and their main origin is astrocytes. Secondary GBs develop from the progression of lower-grade gliomas such as astrocytoma, oligodendroglioma or tumors that contain both astrocytes and oligodendrocytes which are named oligoastrocytomas.

Different types of cancer show EGFR dependency on their malignancy and in GB, EGFR gene amplification and overexpression is found in approximately

40% of cases (Hatanpaa, Burma, Zhao, & Habib, 2010). Malignant characteristics of glioblastoma such as an increased cell proliferation, cell invasion, and cell migration have been associated with the exaggerated function of EGFR (Yang et al., 2020) (Mizuguchi, Yamashita, Yokogami, Morishita, & Takeshima, 2019) (Guo et al., 2020). Among diverse EGFR mutants found in GB, EGFR variant III (EGFRvIII) lacks most of the extracellular domain, including the EGF binding pocket, is the most active mutant in glioblastoma (An, Aksoy, Zheng, Fan, & Weiss, 2018). Expression of EGFRvIII is a potent stimulus of cell proliferation, angiogenesis, and invasion of GB (An et al., 2018), exclusively found in tumor tissues and tumor cells (Chen et al., 2019). Even though the EGFR/EGFRvIII system qualifies as a good candidate for targeted therapy in glioblastoma, the current drugs used to counteract EGFR activity do not have the expected efficacy (Lopez-Gines et al., 2021).

Diffuse infiltration of cancer cells into adjacent healthy brain tissue is one of the main complications of surgical procedures (Claes, Idema, & Wesseling, 2007). GB cells have an abnormal regulation of growth factor and integrin signaling pathways, which is reflected in exacerbated proliferation, migration, and invasion (Alifieris & Trafalis, 2015). The different factors that favor the invasion of GB cells include extracellular matrix (ECM) remodeling (Gladson, 1999) and the interaction with non-neoplastic cells, such as glia and neurons (Bougnaud et al., 2016); da Fonseca & Badie, 2013).

Cell migration is a well conserved process that is of importance for different physiological events such as morphogenesis, wound healing, tissue renewal and also pathological ones, e.g., cancer cells spreading. In cell migration, leader cells respond to different extracellular cues which include soluble factors, neighboring cells, and the ECM. The interaction of cells with the ECM occurs mainly through integrins, heterodimeric receptors that exist in at least 24 unique combinations consisting of interacting α-subunits (18 types) and β-subunits (8 types) (Kechagia, Ivaska, & Roca-Cusachs, 2019). Integrins transduce the external stimuli by triggering the clustering of other integrins and the consequent activation of

signaling pathways that lead to cytoskeleton reorganization and morphological polarization that favor cell migration (Mayor & Etienne-Manneville, 2016). Among the different components of the ECM that integrins interact with are the Galectins, a family of glycan binding proteins in which one member, Gal-8, has been described to induce the migration of U87 human GB cells, displaying potent chemoattractant properties (Metz et al., 2016).

2.2. Role of EGFR in cancer

The epidermal growth factor receptor (EGFR) belongs to the ErbB tyrosine kinase family receptor that regulates diverse cellular processes and functions, including cell proliferation, viability, migration, and differentiation (Ciardiello & Tortora, 2008). The function of the EGFR is found exaggerated in a variety of tumors derived from epithelial cells (carcinomas) as well as in glioblastomas (Ciardiello & Tortora, 2008) (Hatanpaa et al., 2010). Overexpression due to gen amplification, activating mutations or autocrine stimulation are among the mechanisms that exacerbate the role of EGFR in cancer progression (Ciardiello & Tortora, 2008). Therefore, tyrosine-kinase inhibitors, such as Erlotinib and Gefitinib, or ligand-binding blocking antibodies such as Cetuximab, constitute the main approach to counteract oncogenic influences of EGFR (Mendelsohn & Baselga, 2000). However, among the main problems of the present treatments that target the EGFR is the low response of many cancers and the resistance developed by cancer cells initially responsive to the available drugs that target the function of EGFR (Yarden & Pines, 2012) (Russo et al., 2015) (Pan & Magge, 2020). Therefore, other alternatives to counteract the oncogenic EGFR role should be explored.

Endocytic trafficking provides mechanisms of regulation that most cancer cells seem to use to enhance EGFR functions crucial for tumor progression (Tomas, Futter, & Eden, 2014). EGFR tyrosine-kinase activation is triggered by ligand binding to EGFR extracellular domain (Shao & Zhu, 2019). Seven ligands have been described, being the epidermal growth factor (EGF) along with transforming growth factor alpha (TGF-α) the most studied (Roepstorff et al.,

2009). Upon ligand binding, the EGFR homodimerizes or hetero-dimerizes with another ErbB family member. This dimerization is crucial for the conformational changes involved in activation of its intracellular tyrosine-kinase domain (Shao & Zhu, 2019). Phosphorylation substrates of the EGFR include the EGFR itself, which is autophosphorylated in several tyrosine residues within its carboxyterminal region (Hsu, Hurwitz, Mervic, & Zilberstein, 1991). The phosphorylated tyrosines serve as recruiting sites for signaling elements of the ERK1/2, PI3K/AKT, phospholipase-C-gamma and STAT3 pathways (Lahusen, Fereshteh, Oh, Wellstein, & Riegel, 2007) (Xie et al., 2010) (D. E. Levy & Darnell, 2002). Activated and Tyr-phosphorylated EGFR also follows different endocytic routes depending on the concentration and kind of ligand (Caldieri, Malabarba, Di Fiore, & Sigismund, 2018) (Sigismund, Avanzato, & Lanzetti, 2018) (Yarden & Pines, 2012). EGF at low concentrations (1-2 ng/ml) induces EGFR endocytosis mainly through the clathrin-dependent route followed by trafficking towards the recycling pathway back to the cell surface thus prolonging the signaling activity of the receptor (Pinilla-Macua, Grassart, Duvvuri, Watkins, & Sorkin, 2017). Recent studies have shown that EGFR activated with non-saturating EGF levels also induce the internalization of the remaining unliganded and inactive monomeric EGFR due to activation of the MAPK p38 (Sigismund et al., 2012). p38 mediates the clathrin-dependent endocytosis of inactive EGFR in response to a variety of stress conditions (Tomas et al., 2014) (Metz et al., 2021). On the contrary, under high but still physiologic ligand concentrations (10-50 ng/ml), the ligand-saturated, activated and dimerized EGFR is endocytosed by both clathrin-dependent and clathrin-independent routes and follows intracellular endocytic routes towards the lysosomal degradation pathway, which avoids its exaggerated signaling (T. Tanaka et al., 2018) (Sigismund et al., 2018).

In cancer cells, alterations of endocytic trafficking has been related with an enhanced EGFR-mediated signaling towards proliferation, survival and invasive migration, all important events in tumor progression (Sigismund et al., 2018). Overexpression and activating mutations of the EGFR, modifications of the endocytic machinery and stimulation with TGF-α can determine endocytic

trafficking pathways that favor and exaggerated activity of the EGFR (Ciardiello & Tortora, 2008) (Tomas et al., 2014) (Roepstorff et al., 2009). For instance, contrasting with the degradation route taken by the receptor under high EGF concentrations, high concentrations of TGF-α promote endocytic recycling rather than degradation of activated EGFR (Francavilla et al., 2016).

Even though EGFR activation occurs at the cell surface by ligand binding or ligand-independent dimerization, it is worth mentioning that internalized EGFR can perform signaling in endosomal compartments, as demonstrated in a study which established a system to evaluate endosome-associated activated EGFR (Wang, Pennock, Chen, & Wang, 2002). Signaling originated from endosomal EGFR can activate AKT but has an attenuated effect on Phospholipase C-γ1 (PLC-γ1) phosphorylation compared to cell surface EGFR activation, indicating differential EGFR signaling from plasma membrane and endosomes (Wang et al., 2002). The physiological consequences of this endosomal signaling are mainly associated with anti-apoptotic effects mediated by the PI3K and AKT pathway (Wang et al., 2002).

2.3. Galectins

Galectins are a family of glycan binding proteins that contain a carbohydrate recognition domain (CRD) with high affinity to β-Galactosides found in glycolipids and proteins with N- or O-glycosylations (Boscher, Dennis, & Nabi, 2011). Galectins are soluble cytosolic proteins that lack a signal peptide for conventional secretion through the exocytic route but are released to the extracellular media by non-conventional mechanisms (Popa et al., 2018). Since 1970, fifteen galectins have been described in mammals, which according to their structure are divided into three groups: prototype, chimeric and tandem repeat. Prototypical galectins (Gal-1, -2, -5, -10, -11, -13 and -15) have a single CRD and can form homodimers. Tandem repeat galectins (Gal-4, -6, -8, -9 y -12) possess two CRDs, one N- terminal and the other C-terminal, with a linker peptide between them, and can dimerize forming tetravalent complexes (Yoshida et al., 2012). There is only one chimeric galectin (Gal-3), which have a CRD on its C-terminal and a non-

lectin recognizing domain through which it can form homomultimers of up to five proteins. The tissue expression of these proteins is variable. For example, only Galectins -1, -2, -4, -7, -8 and -9 have been found expressed in the human brain (Saal et al., 2005).

In the cytosol, some galectins have been described to perform intracellular functions such as pre-mRNA splicing by Gal-1 and Gal-3 (Vyakarnam, Dagher, Wang, & Patterson, 1997). Interestingly, Gal-3, Gal-8 and Gal-9 conform a quality control system that detects damage in endolysosomal compartments that expose glycans and either repair or remove them by autophagy (Chauhan et al., 2016) (Thurston, Wandel, von Muhlinen, Foeglein, & Randow, 2012). This function is addressed in more detail in chapter 2.6.

Once secreted, galectins perform a variety of functions by interacting with cell surface glycoconjugates and ECM proteins (Barondes et al., 1994). Cell processes such as proliferation (Perillo, Marcus, & Baum, 1998) (Hadari et al., 2000) (Rabinovich & Toscano, 2009), differentiation (Rabinovich & Toscano, 2009), migration and apoptosis (Perillo et al., 1998) (Rabinovich & Toscano, 2009) have been found to involve galectins.

The functions of galectins can be redundant or non-redundant, with each galectin exerting different functional effects due to their distinctive affinity and selectivity for glycans (Hirabayashi et al., 2002) (Ideo, Seko, Ishizuka, & Yamashita, 2003) (Cederfur et al., 2008). Furthermore, the role of each galectin can vary according with variations in the glycosylation patterns. The β-galactosides recognized by galectins can change depending in the expression and activity patterns of the glycosyl-transferases in the Golgi complex, in conjunction with the secretion of glycan modifying enzymes, which respond to environmental conditions, including inflammation (Dennis, Nabi, & Demetriou, 2009) (Cerliani, Blidner, Toscano, Croci, & Rabinovich, 2017). For example, the presence of N-acetyl-glucosamine in the extracellular media promotes the ramification of N-glycans on glycoproteins, increasing the binding of Gal-3 (Dennis et al., 2009). On the other hand, sialylation prevents the binding of a

group of galectins (Gal-1, -2 and -3) while increasing the binding of Gal-8 by its N-terminal CRD (Cerliani et al., 2017). Thus, remotion of sialic acid in the cell membrane allows binding of other galectins. Therefore, it is interesting to study the contribution of galectins to different cellular processes and how they might promote complementary or opposite roles based on their specific affinity for different β-galactosides.

2.4. Gal-8 in cancer

Experimental and clinical evidence have shown correlations of galectin expression and tumorigenesis (Barondes et al., 1994); (Liu & Rabinovich, 2005) (Lahm et al., 2004) (Danguy, Camby, & Kiss, 2002) (Lajoie, Goetz, Dennis, & Nabi, 2009), with Gal-8 being one of the most studied lectins in this context. Gal-8 belongs to the tandem repeat type of galectins bearing two CRDs (Ideo et al., 2003). In Gal-8, the N-terminal CRD is unique among other galectins due to its high affinity for sialylated β-Galactosides, such as α2-3-syalilated glycans, while the C-terminal CRD shows high affinity for non sialylated oligosaccharides, such as polylactosamine (Ideo et al., 2003) (Kumar, Frank, & Schwartz-Albiez, 2013). In humans, northern blot analysis show that Gal-8 is one of the most abundant galectins in the organism and is expressed in diverse tissues such as the heart, liver, pancreas, and brain (Bidon-Wagner & Le Pennec, 2002). In mice, it has been described that Gal-8 is expressed in diverse regions of the brain, among them, the choroid plexus, lateral ventriculus, third dorsal ventriculus and the ventral lateral nucleus of the thalamus. In this same study, Gal-8 was detected in the cerebrospinal fluid of patients with different affections, such as, diplopia, headache, vertiginous syndrome, febrile syndrome, and meningitis (Pardo et al., 2017). There is not a clear understanding on how Gal-8 expression is dysregulated in GB and cancer in general, however, some studies have described that increased endogenous Gal-8 expression is followed upon inflammatory conditions. In this context, Gal-8 levels have been found up-regulated in endothelial cells of blood vessels surrounded by perivascular inflammatory infiltrates in samples of lesions of multiple sclerosis patients (Stancic et al., 2011). Therefore, a proinflammatory context, which is common in tumor

microenvironment, could be a possible factor determining an increased Gal-8 expression in cancer cells, however more studies approaching the precise mechanism are required.

Gal-8 has been implied in different functions, including cell adhesion, migration, differentiation, proliferation and apoptosis (Hadari et al., 2000) (Carcamo et al., 2006) (Y. Levy et al., 2001) (Norambuena et al., 2009). Also, Gal-8 expression is increased in different kinds of tumors, and this is correlated with a bad prognosis of certain cancer, such as pancreatic cancer, while an opposite relation seems to occur in colon cancer, in which its malignancy is associated with decreased immunohistochemical detection of Gal-8 (Bidon-Wagner & Le Pennec, 2002). Thus, these studies indicate that Gal-8 expression in tumor cells is organ specific.

Several functional properties related Gal-8 function with cancer. For instance, Gal-8 interacts with integrins at the cell surface impacting upon cell adhesion, migration and in the signaling pathways for the activation of the focal adhesion kinase (FAK) and extracellular regulated kinase (ERK) (Carcamo et al., 2006) (Norambuena et al., 2009). Studies from our laboratory have shown that overexpression of Gal-8 on epithelial cells from no tumoral origin (Madin Darby Canine Kidney, MDCK), promoted epithelial-mesenchymal transition towards a phenotype of migration, invasiveness and hyperproliferation involving the EGFR and proteasomal function, which *in vivo* is reflected in the acquired capability of tumor formation in immunosuppressive mice (Oyanadel et al., 2018). In this same study, it was shown that the overexpression of Gal-8 triggered the transactivation of the EGFR, by the activation of integrin signaling pathways towards the FAK which induces the activating EGFR phosphorylation (Tyr1068). This effect is mediated by extracellular Gal-8 and it is carbohydrate dependent, since the use of lactose reverted the activation of the EGFR. These results highlight the cross regulation of Gal-8 and EGFR, which account for the increased proliferative characteristic of Gal-8 overexpressing MDCK cells (Oyanadel et al., 2018).

In addition, Gal-8 increases endothelial permeability, suggesting a role in metastasis (Zamorano et al., 2019). The activated leukocyte cell adhesion molecule (ALCAM) interacts with Gal-8 and promotes cell adhesion, migration and proliferation, while the silencing of both ALCAM and Gal-8 reduces the growth of triple negative breast cancer cells Gal-8, suggesting a pro-metastatic role in breast cancer cells (Ferragut et al., 2019).

2.5. Gal-8 in GB

A correlation between increased Gal-8 expression and a lower lifespan of GB patients has been suggested from the analysis of a Kaplan Meier curve using the OncoLnc database (Fig. 2). Xenograft experiments of different GB cell lines show that cells residing in the most invasive region of the tumor display high expression of Gal-1, Gal-3 and Gal-8, while *in vitro* these same galectins stimulate the migration of GB cell lines (Camby et al., 2001). The analysis of different cell lines show that cancer cells, including the human GB cell line T98G, display increased Gal-8 expression compared to non-cancer cells such as HEK293-T and HFF-2 fibroblasts (Satelli et al., 2008).

A previous study from our laboratory demonstrated interesting effects of exogenous Gal-8 treatment and silencing in the human GB cell line U87 (Metz et al., 2016). When added to the media, Gal-8 acts as migration chemoattractant as potent as Fetal Bovine Serum (FBS, positive control) in a Transwell assay. It also promoted proliferation by of these cells by 1.5-fold. Both effects are abolished by co-incubation with lactose, indicating that they are mediated by an interaction of Gal-8 with cell surface glycans (Metz et al., 2016). However, when Gal-8 expression is silenced by 60-70% with shRNA, the migratory activity remains unaltered while cell proliferation decreases and apoptosis increases, suggesting a role of Gal-8 in cell survival mechanisms (Metz et al., 2016).

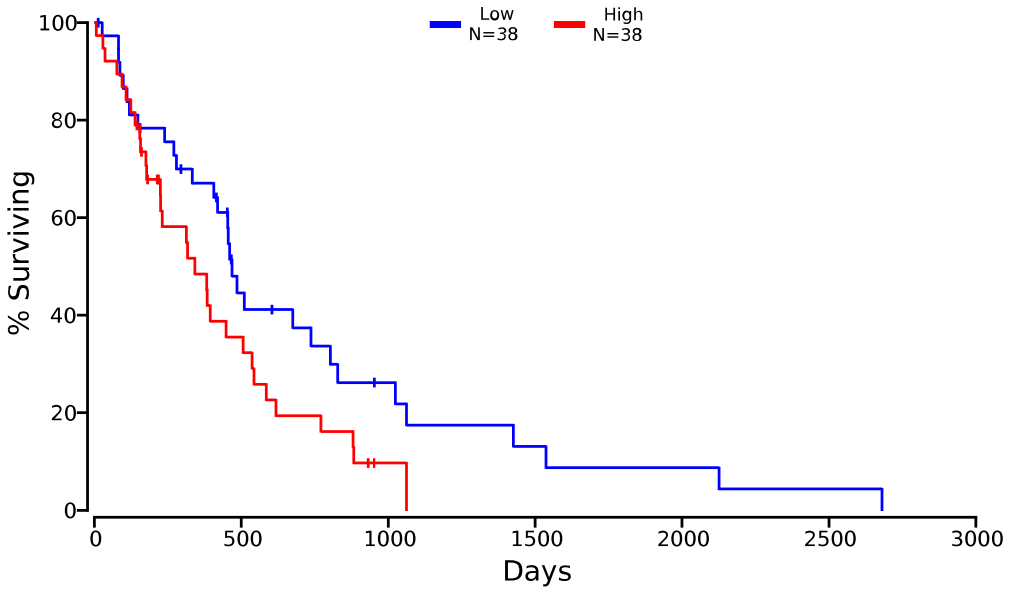


Figure 2. Kaplan-Meier plot of GB patients associated to Gal-8 expression.

Kaplan-Meir plot correlating Gal-8 expression (mRNA) with the survival of GB patients using OncoLnc database from Anaya et al., 2016. To perform the analysis, two groups were generated from the total of available patients based on their Gal-8 expression, a low-level expression group (blue stripe) and a high expression level group (red stripe). The comparison indicates a worst prognosis for patients in the high Gal-8 expression group.

It is unknown whether this protective function of Gal-8 involves its described role in the cytosol, where together with Gal-3 and Gal-9 conform a system that

detect and then repair or remove damaged endolysosomal compartments (Jia et al., 2018).

2.6. Gal-8 on damage recognition of lysosomes

As previously stated, one of the intracellular roles of galectins is their recruitment to damaged endosomes or lysosomes by interacting with key proteins to engage different response mechanisms towards malfunctioning compartments. In this context, Gal-8 is an important coordinator in the activation of autophagy to trigger the degradation of damaged lysosomes (Jia et al., 2018) and endosomes (Fraser et al., 2019). When the lysosome membrane is damaged, the intraluminal glycoconjugates of the membrane proteins are exposed and allows for the recognition by Gal-8. In this damage context, Gal-8 interacts with the amino-acid transporter SLC38A9 in a glycan-dependent manner, demonstrated by using Gal-8 CRD mutants that reduce its glycan affinity. In response to lysosomal arginine or other stimulus, SLC38A9 activates Ragulator, a guanine exchanging factor (GEF) that interacts with Rag A/B, which in its GTP loaded state triggers increased activity from mTORC1. It was also described that when lysosome damage occurs, Gal-8 interacts with RagA/B, displacing mTORC1 from this complex and resulting in its inhibition. The inhibition of mTORC1 activates autophagy to degrade damaged lysosomes. This Gal-8, SLC38A9, RagA/B dynamic response complex has been depicted as GALTOR, which is shown in Figure. 3 (Jia et al., 2018).

Experiments performed on HeLa cells infected with Salmonella have reported the relevance of Gal-8 in the degradation of the bacteria containing endomembranes. In this same study, it was described that Gal-8 interacts with the autophagy receptor NDP52, and cooperate in the degradation of bacteria by xenophagy, a pathogen-specific type of autophagy (Thurston et al., 2012).

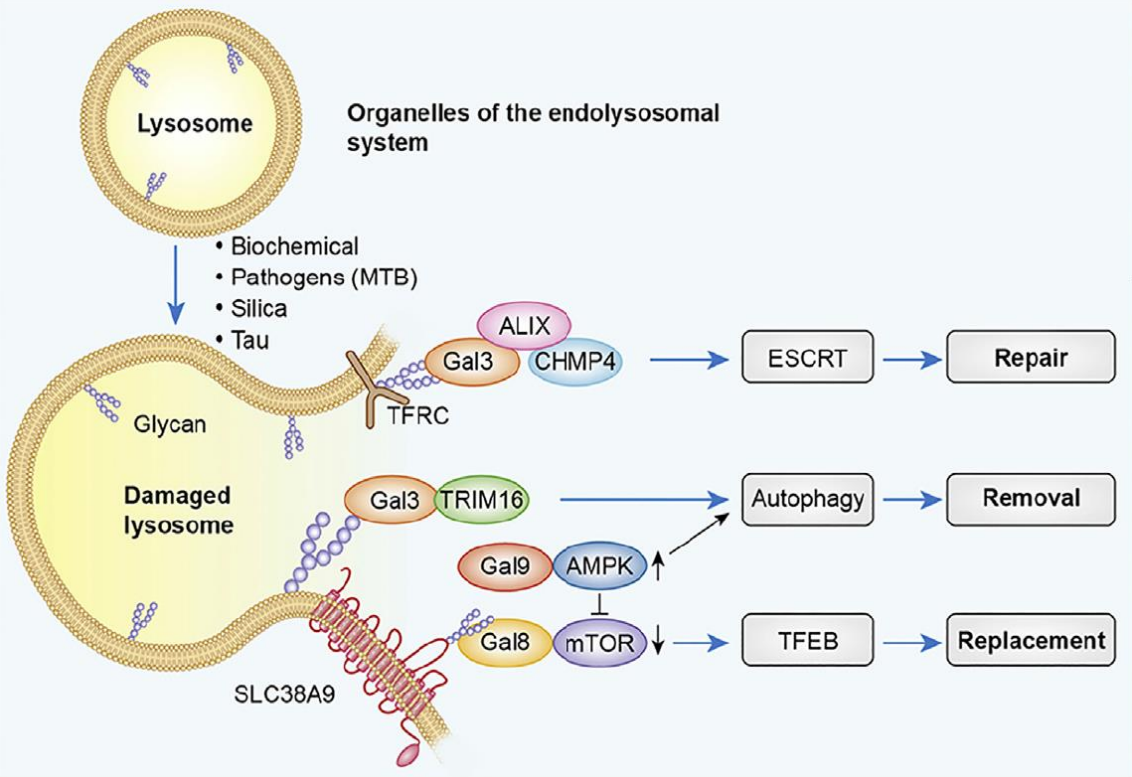
As previously mentioned, glycoconjugate recognition by Gal-8 is necessary for its interaction with SLC38A9. Recent studies demonstrated that remotion of sialic acid from plasma membrane by using neuraminidase results in a decreased aggregate formation of Gal-8, NDP-52 and the autophagy receptor-modulator LC3 when inducing lysosome membrane damage (Hong et al., 2019). This result

indicates the relevance of sialylation in Gal-8 accumulation response and autophagy activation.

The initial response to lysosomal damage is the repair of damaged lysosomes. Experiments performed in HeLa cells describe that lysosomal damage by using the lysosomotropic compound L-leucyl-L-leucine methyl ester (LLOMe), induces the recruitment of TSG101 (ESCRT-I), ALIX and CHMP4A & CHMP4B (ESCRT-III); all proteins of the ESCRT machinery. The ESCRT-III machinery is essential to reestablish healthy lysosomes by membrane repairing, as silencing of CHMP4B notably diminishes this process. The authors determine that this process occurs before the recruitment of Gal-3 (Radulovic et al., 2018). Interestingly, it was later described that Gal-3 is recruited to damaged lysosomes via the interaction of the carbohydrates of the Transferrin Receptor (TfR), and it is important in the recruitment of ALIX, a component of the ESCRT machinery (Fig. 3). The silencing of Gal-3 diminishes the recruitment of ALIX and therefore also the repair of damaged lysosomes (Jia et al., 2020).

In this regard, recent evidence in glial cells showed that Gal-8 plays a relevant role in the regulation of EGFR recycling by coordinating the degradation of damaged endosomes autophagy (Fraser et al., 2019). Gal-8 silencing reduced the activation of EGFR and its downstream signaling towards AKT. Also, this work shows that blocking the degradation of malfunctioning endosomes by autophagy decreases cell viability (Fraser et al., 2019).

Therefore, it is interesting to assess the role of Gal-8 in glioblastoma cells considering that both, intracellular and extracellular roles of Gal-8 can modify the activity of EGFR in cancer, and that Gal-8 intracellular function might promote survival against stress conditions through the autophagy system that removes damaged lysosomes and endosomes.



Jia et al., Developmental cell, 2020.

Figure 3. Galectins control a coordinated lysosomal membrane damage response.
When lysosomal membrane damage occurs, different Galectins coordinate homeostatic responses by recognizing the exposed glycans of lysosomal glycoproteins (and potentially glycolipids). Gal-3 promotes the recruitment of the ESCRT machinery to repair the damaged lysosomal membrane. When lysosomal membrane damage is persistent, Gal-8 triggers the activation of autophagy by displacing mTOR from the Rag/Ragulator/SLC38A9 complex, resulting in its inhibition, and concomitantly Gal-9 activates AMPK furthering favoring the autophagy and removal of damaged lysosomes. Finally, Gal-8 and Gal-9 signaling modulation on the lysosome triggers the activation of the transcription factor TFEB, which leads to the biogenesis of new lysosomes for replacement.

2.7. Lysosomes

Lysosomes are acidic membranous organelles than contain hydrolases that degrade a great portion of the cell macromolecules, and they serve as recycling

compartments for different cell components internalized by autophagy and endocytosis (Ballabio & Bonifacino, 2020). In addition to their degradation functions lysosomes respond and adapt to changes in the environment, as indicated by the discovery that their activity and gene expression are differentially regulated under stress conditions (Sardiello et al., 2009). This regulation is mediated by the coordinated lysosomal enhancement regulation (CLEAR) gene network, that includes the Transcription Factor EB (TFEB), which regulates lysosomal biogenesis by controlling the expression of multiple genes encoding lysosomal components (Palmieri et al., 2011). In normal conditions, TFEB is located in the cytoplasm, while during starvation or lysosomal stress, it is translocated to the nucleus and triggers the transcription of genes involved in lysosomal biogenesis (Sardiello et al., 2009) (Palmieri et al., 2011).

Lysosomes are also involved in the process of autophagy, a dynamic self-degradative process of metabolic wastes, toxic protein aggregates and nonfunctional organelles. These components are first engulfed in a double membrane compartment depicted as phagophore, which then encloses and matures forming an autophagosome. Then, this autophagosome fuses with a lysosome to form an autolysosome, where degradation of the engulfed components occurs (Ravikumar, Moreau, Jahreiss, Puri, & Rubinsztein, 2010).

An increasing body of evidence demonstrate that autophagy and lysosomal activity is increased in advanced cancers. Indeed, it has been reported that autophagy is exacerbated in cancer cells under conditions of decreased nutrients due to a poor vasculature and insufficient blood supply in rapidly growing tumors (Ogier-Denis & Codogno, 2003). In GB tumors, recent studies have described enhanced autophagy activity in tumoral regions with low nutrient supply in response to the lack of glutamine (K. Tanaka et al., 2021).

The expression of Cathepsin-B, a lysosome hydrolase, has been found significantly elevated in GB cell lines that have higher invasiveness compared to lower grade glioma cells, with secretion to the extracellular media and proteolytic

activity on the ECM as a possible mechanism (Konduri et al., 2001) (Mikkelsen et al., 1995).

2.8. Lysosomal damage: treatment against cancer

The essential biological role of lysosomes makes them a relevant target for inducing cell death. Lysosomal membrane permeabilization (LMP), is a phenomenon in which lysosomal components, such as hydrolases, leak into the cytosol with harmful consequences, leading to cell death with necrotic, apoptotic, or apoptotic-like features depending on the magnitude of lysosomal leakage (Aits & Jaattela, 2013).

A variety of drugs can be targeted to trigger LMP leading to cell death as an alternative pathway for selectively killing cancer cells, which are depicted as lysosomotropic drugs (Jensen, Petterson, Halle, Aaberg-Jessen, & Kristensen, 2017). The activity of multiple enzymes lysosomal enzymes is found increased in many cancer tissues compared to non-cancerous tissues (Kirkegaard & Jaattela, 2009). Cancer cells also show altered distribution patterns of lysosomes, associated with changes in expression and activation of dynein and kinesin, which regulate lysosome location. This spatial distribution regulation is involved in cancer cell metastasis and drug resistance (Groth-Pedersen et al., 2012).

These lysosomal changes found in cancer cells have been associated with increased sensitivity towards lysosomotropic compounds, a feature that might provide new opportunities for a therapeutic eradication of cancer cells in a specific and less risky manner (Petersen et al., 2013).

2.9. D-Propranolol as an antitumoral drug

Our laboratory has been working on the possibility of using the endocytic trafficking as a target for pharmacologic intervention in cancer (Norambuena et al., 2010) (Shaughnessy et al., 2014) (Metz et al., 2021). As recently proposed, D-Propranolol might be a potential prototype for an antitumoral drug accomplishing such strategy (Barra et al., 2021). Propranolol is currently prescribed as beta-blocker in the treatment of heart and vascularity diseases, as well as in anxiety disorders (Srinivasan, 2019). Its pharmacologic formulation

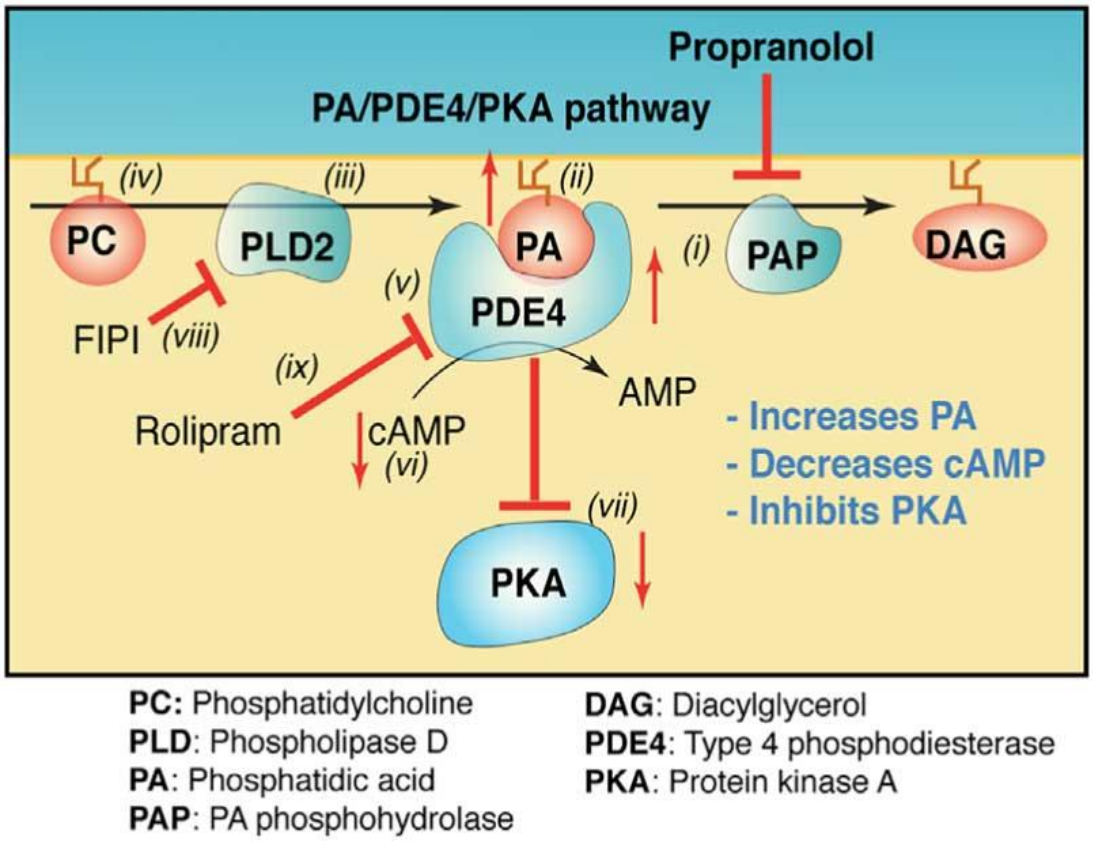
consists of a racemic mixture of the enantiomers Levorotatory(-)-Propranolol (L-Propranolol), bearing the clinically useful properties of beta-blocker, and Dextrorotatory(+)-Propranolol (D-Propranolol), bearing 100 fold less beta-blocker activity (Barrett & Cullum, 1968). Our laboratory reported that both enantiomers induce EGFR endocytosis, and this effect can be deleterious to cells depending on EGFR function (Shaughnessy et al., 2014). Therefore, D-Propranolol can be used to induce EGFR removal from the cell surface through the endocytic trafficking pathway without the inconvenience of a clinically relevant beta blocking activity (Barra et al., 2021).

Pharmacologic induction of EGFR internalization indeed decreases the accessibility of the receptor to external stimuli. In principle, this strategy would decrease the EGFR oncogenic activity even in tumor cells resistant to EGFR inhibitors. In agreement with this assumption, D-Propranolol has been shown to decrease viability of lung tumor cells bearing a mutant oncogenic EGFR (Shaughnessy et al., 2014), resistant to EGFR tyrosine-kinase inhibitors (Barra et al., 2021). D-Propranolol also inhibits the endocytic recycling of both inactive and ligand-activated EGFR, resulting in their accumulation in recycling endosomes, a condition that might result toxic to cells, as suggested (Shaughnessy et al., 2014). Cancer cell lines showing to be sensitive to D-Propranolol include the H1975 non-small cell lung cancer expressing a double mutant $EGFR^{L858R/T790M}$, HeLa cells and MKN45 gastric cell lines transfected to overexpress the EGFR (Shaughnessy et al., 2014).

2.10. The PA/PDE4/PKA pathway in EGFR endocytic trafficking

The pathway triggered by Propranolol in its both enantiomer D- and L-enantiomeric forms starts with the inhibition of phosphatidic acid phosphohydrolase (PAP) activity leading to an increase of phosphatidic acid (PA) levels (Meier et al., 1998). PA is a phospholipid that plays roles as structural components of membranes, as precursor of other phospholipids and phophoinositols and as a signaling element that recruits and activates a variety of proteins bearing a PA binding domain (Tanguy, Wang, Moine, & Vitale, 2019).

The signaling pathway that our laboratory has associated with the endocytic trafficking of EGFR involves PA-mediated activation of type 4 phosphodiesterases (PDE4), the main regulators of cAMP levels (Norambuena et al., 2010). A decrease in the cAMP levels caused by activated PDE4 results in the inhibition of protein kinase A (PKA) inhibition. This PA/PDE4/PKA pathway, depicted in Figure 4, mimics the effects of direct inhibitors of PKA such as H89 and PKI, inducing internalization of EGFR independently of ligand-binding (Norambuena et al., 2010). The EGFR internalization results from an induced endocytosis and a block of the recycling pathway (Norambuena et al., 2010) (Metz et al., 2021). In contrast with the canonical ligand-induced EGFR endocytosis, the endocytosis induced by Propranolol-triggered PA/PDE4/PKA pathway and by PKA inhibitors do not require tyrosine-phosphorylation or ubiquitination of the receptor (Norambuena et al., 2010) (Salazar & Gonzalez, 2002). D-Propranolol mediated EGFR endocytosis involves clathrin-dependent and clathrin-independent pathways, which are respectively induced by the activation of p38 and ERK1/2 downstream the PKA inhibition (Metz et al., 2021).



Metz et al., Traffic, 2021.

Figure 4. The PA/PDE4/PKA pathway regulates the endocytosis of EGFR.
Schematic illustration of the PA/PDE4/PKA pathway modulated by propranolol. Propranolol inhibits the phosphatidic acid phosphohydrolase PAP enzyme (i), increasing the levels of phosphatidic acid (PA) generated by phospholipase D2 (PKD2) (iii) from phophatidylcholine (PC) (iv). PA recruits and activates type 4 phosphodiesterases (PDE4) (v), which decreases the levels of cAMP (vi) and the activity of PKA (vii). The PLD2 inhibitor FIPI (viii) and the PDE4 inhibitor Rolipram are antagonists of the PA/PDE4/PKA pathway and be used to evaluate the role of this pathway in different cell processes such as EGFR endocytosis.

This endocytic step is selective as other receptors are not affected and seems to be due to p38 and ERK-mediated phosphorylations of the EGFR (Metz et al., 2021). However, once endocytosed the EGFR accumulates in perinuclear recycling endosomes reflecting a more generalized inhibition of the recycling

pathway, which extends to other receptors such as the LDLR and TfR (Metz et al., 2021).

2.11. D-Propranolol as a potential lysosomotropic drug

An important property of lysosomotropic drugs is their accumulation in acidic organelles due to their basic and lipophilic features. These drugs can induce pH basification because of proton capturing. Lysosomotropic drugs such as Siramesine, a lipophilic tertiary amine, induces lysosomal dysfunction (Parry et al., 2008). This compound was initially described as σ2 receptor agonist in the treatment for anxiety and depression (Sanchez et al., 1997). It was later described that Siramesine triggers cell death in immortalized and cancer cells (Ostenfeld et al., 2005), associated with lysosome membrane permeabilization and subsequent leak of lysosomal cathepsins and oxidative stress induction (Ostenfeld et al., 2008). Lysosomes damaged by Siramesine are recruited to autophagosomes for degradation through lysophagy, a type of autophagy devoted to dysfunctional lysosomes (Ostenfeld et al., 2008).

Propranolol, as lipophilic secondary amine, shares characteristics with lysosomotropic drugs and has been previously reported to accumulate causing swelling in lysosomes (Cramb, 1986). Preliminary results in collaboration with the laboratory of Dr. Patricia Burgos indicate that D-Propranolol has the potential to induce lysosomal damage, as suggested by the recruitment of Gal-3, a lysosome damage recognition marker, seen in HeLa cells. Whether D-Propranolol induces the same effects in GB cells with impact on cell viability has not been studied.

2.12. Highlights

- EGFR expression and function is enhanced in 50% of GB cases.

- Gal-8 has different potential pro-tumoral roles, evidenced as enhanced proliferation, migration, and degradation of damaged lysosomes.

- Lysosomes are important modulators of cancer progression and therefore serve as an antitumoral target.

- D-Propranolol induces the endocytosis and traffic perturbation of EGFR and has a potential role as a lysosomotropic drug.

2.13. Hypothesis

“D-Propranolol decreases viable cell number and invasion of T98G glioblastoma cells interfering with the function of EGFR and Gal-8”.

2.14. General objective

Determine whether D-Propranolol is an effective drug against glioblastoma cells affecting the malignant factors EGFR and Gal-8.

2.15. Specific objectives

I - Evaluate the effect of D-Propranolol on the viability of T98G cells

1.a) Evaluate the effect of D-Propranolol in EGFR internalization.

1.b) Evaluate the effect of D-Propranolol and the involvement of the PA/PDE4/PKA pathway on cell viability.

1.c) Determine the impact of D-Propranolol on cell proliferation.

II - Evaluate the effect of D-Propranolol in lysosomes in T98G cells.

2.a) Evaluate the recruitment of endogenous Gal-3 to lysosomes in response to D-Propranolol.

2.b) Evaluate the accumulation of endogenous Gal-8 in response to D-Propranolol.

2.c) Evaluate the recruitment of Gal-8-ZsGreen to lysosomes in response to D-Propranolol.

2.d) Assess the effect of D-Propranolol on Cathepsin-B activity.

III - Evaluate the impact of Gal-8 expression on T98G cell viability, EGFR signaling and invasion in response to D-Propranolol

3.a) Evaluate the impact of Gal-8 expression on cell viability in response to D-Propranolol.

3.b) Evaluate EGFR and ERK1/2 signaling in WT and Gal-8 overexpressing T98G cells.

3.c) Evaluate the effect of Gal-8 expression on invasive migration.

3. MATERIALS AND METHODS

3.1. Cell culture

For this study, we used the human glioblastoma cell line T98G which express EGFR and Gal-8.

Cells were maintained with high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and with

antibiotics (100 ug/mL penicillin and 0,1 mg/mL of streptomycin) in a cell incubator with a constant flow of 95% O_2 y 5% CO_2 at 37 °C.

Cell monolayers were detached with 0,17 mg/mL trypsin for 5 minutes and then centrifuged in DMEM 10% FBS culture media at 1000 rpm for 5 minutes. Cell pellet was resuspended with culture media and cells were then counted in a Neubauer chamber and plated for the desired experiment.

3.2. Reagents

For the experiments described in this manuscript we used the following reagents: D-Propranolol (Sigma-Aldrich, P0689), Rolipram (Sigma-Aldrich, R6520), FIPI (F5807), EGF (R&D Systems, #236-EG) and LLOMe (Focus Biomolecules, #10-3220).

3.3. Cell surface EGFR detection by flow cytometry

To label the EGFR of cells under different experimental conditions, we used EGF-Alexa 488 (1:1000 dilution) dissolved in a “binding media” (MEM Hanks; HEPES 0,65 mg/mL; BSA 2 mg/mL) for 45 minutes at 4°C with low agitation. Next, cells were washed twice with cold PBS 1X with 0,1 mM of calcium and 1mM of magnesium (CM) and then incubated with 500µl per well of PBS-EDTA 5mM (pre-warmed at 37°C) for 10 minutes at 37°C. Cells were then gently resuspended from the wells and passed to a 1.5 mL Eppendorf tube. Cells were washed with PBS-FBS 2% and centrifuged at 1,100g for 3 minutes to perform a second wash-centrifuge cycle. Finally, 200 µL of PBS-FBS 2% plus 200 µL of 4% paraformaldehyde (PFA) was added to each sample and then stored in dark at 4°C for flow cytometry analysis the next day. Flow cytometry analysis were performed using the FACS Canto II cytometer and BD FACSDiva™ Software.

3.4. Immunoblot

For immunoblotting, cells were lysed with lysis buffer (HEPES 50mM, NaCl 50 mM, EGTA 1mM, $MgCl_2$ 5 mM, Glycerol 10%, Triton X-100 1%, pH 7.4) for 30 minutes at 4 °C, supplemented with protease inhibitors (PMSF 20mM, Pepstatin 2 µg/ml, Leupeptin 2 µg/ml). Protein samples were loaded in SDS polyacrylamide gel for electrophoretic run and then transferred to a PVDF membrane. We used

the following primary antibodies dissolved in PBS 1X or TBS 1X with Tween 0,1% and BSA 5%: rabbit polyclonal anti-phospho-EGFR (Tyr1068) (Cell Signaling Technology, #3777), rabbit polyclonal anti-EGFR (anti-EGFR984), rabbit monoclonal anti-phospho-ERK1/2 (Cell Signaling Technology, #4370), rabbit polyclonal anti-ERK1/2 (Cell Signaling Technology, #4695), rabbit monoclonal anti-GAPDH (Cell Signaling, #5174). Secondary HRP antibodies were incubated for 1 hour at room temperature and ECL was used for revealing immunoblot membranes.

3.5. Cell surface biotinylation

T98G cells were plated in a six well plate and the day of the experiment they were deprived of FBS for 4 hours and then treated. Cells were washed twice with PBS 1X CM and then cell surface proteins were tagged with 0.5 mg/mL Sulfo-NHS-biotin EZ-Link (Thermo Scientific, #21217) for 30 minutes at 4 °C in agitation. Biotin was then washed three times with PBS 1X-CM and biotin excess was blocked with 1mL of NH_4Cl at 50 mM for 10 minutes, and then washed three times with PBS 1X CM. Cells were then lysed as previously detailed in the immunoblot protocol (Chapter 3.4), and a fraction of this lysate (200 µg) was used for evaluating cell surface biotinylated proteins by incubating with neutravidin-agarose beads for 2 hours at 4°C. Beads were then washed with immunoprecipitation buffer (20 mM HEPES, NaCl 150 mM, glycerol 10%, Triton X-100 0.1%) and then 30 µl of loading buffer 4X with DTT was added and then incubated for 5 minutes at 95°C. Denatured proteins were loaded in SDS acrylamide gel for electrophoretic run and analyzed by immunoblot as previously described.

3.6. Viable cell number assay

To evaluate the viable cell number in our different T98G cell lines, we used 24-well plates and plated 10,000 cells per well the day before the experiment. Cells were plated in triplicate for each condition. Daily intermittent treatments were applied by the following protocol: an initial 1-hour treatment with D-Propranolol, with or without Rolipram or FIPI, next, PBS 1X was added to washout the drugs

and then cells were maintained with DMEM 10% FBS for 5 hours. When this time was elapsed, cells were given another 1-hour treatment, followed by washout and then overnight rest with DMEM 10% FBS. This cycle of treatment was repeated for three days. Next, viable cells were counted using Trypan blue and a Neubauer counting chamber.

3.7. EdU proliferation assay

For cell proliferation assays, we plated 10,000 cells per well in circular cover glasses (Marienfeld, #0111520) in a 24 well plate. We did daily intermittent D-Propranolol as explained in our cell viability assay (Chapter 3.6). The day after the end of treatments, half of the culture media of each cell-containing well was replaced with a 2X EdU (Thermo Fisher Scientific, #C10340) complete medium solution for a final concentration of 10 µM, and then maintained for 2 hours in a cell incubator. Next, cells were fixed with PFA 4% for 15 minutes, and washed twice with a PBS 1X 3% BSA solution. Cells were permeabilized with a PBS 1X 0.5% Triton X-100 solution for 20 minutes, and then washed twice with PBS 1X BSA 3%. We then prepared the Click-iT reaction cocktail as indicated by the manufacturers and incubated the cells for 30 minutes at room temperature protected by light. Cells were then washed once with PBS 1X BSA 3%, and then once with PBS 1X, and incubated for 30 minutes with Hoechst 33342 at room temperature protected with light. Coverslips were then washed twice with PBS 1X and mounted into microscope slides with Fluoromount G and posteriorly analyzed with confocal microscopy.

3.8. Indirect Immunofluorescence

For IFI, we plated 15,000 cells in circular cover glasses (Marienfeld, #0111520) in a 24 well plate and maintained until 80% confluence. After treatments, cells were washed with PBS 1x-CM, and then fixed with PFA 4% or methanol depending on the respective antibody datasheet. After fixation cells were permeated with PBS 1X Triton X-100 0,2% for 15 minutes, then incubated with primary antibody overnight, using the following antibodies: goat polyclonal anti-Gal-8 (R&D Systems, AF1305) 1:200, rat monoclonal anti-Gal-3 (Santa Cruz

Biotechnology, sc-23938) 1:100, mouse monoclonal anti-LAMP-1 (Cell Signaling Technology, #15665) 1:200, mouse monoclonal anti-EGFR (ATCC, #HB-8506) 1:2. Next, cells were washed three times with PBS 1X-CM and secondary fluorescent antibodies were used in 1:500 with Hoechst nucleus tinction used 1:2000 for 30 minutes at 37°C. Finally, cells were washed three times PBS 1X-CM and cover glasses were mounted to microscope slides with Fluoromount-G.

3.9. T98G-Gal-8-ZsGreen cell line generation by cell sorter

To evaluate Gal-8 response by live cell imaging we decided to generate a stable cell line expressing Gal-8 coupled to the green fluorophore protein ZsGreen (Gal-8-ZsGreen). As described, these cells were transduced withlentiviral particles, and then sorted using FACSAria II (Becton Dickinson) cell sorter and then maintained as a stable cell line, depicted as T98G-Gal-8-ZsGreen.

3.10. Lysotracker staining and Gal-8-Zsgreen recruitment experiments

T98G-Gal-8-ZsGreen cells were plated in MatTek glass bottom microwell dishes. In the day of the experiment, cells were incubated for 15 minutes with 50ng/mL of Lysotracker Red (555) in DMEM-Hepes at 37°C. The cells were then washed twice with 1mL of DMEM-Hepes. The treatments were diluted in DMEM-Hepes at the times indicated in the experiments. Live cell imaging was performed with a Leica SP8 confocal microscope, using a 63x oil immersion objective, following a single field of view per experiment with a 30 seconds per frame time resolution. Gal-8-ZsGreen puncta formation over time was followed with live-cell imaging and quantified using the ComDet v.0.5.3 plugin in FIJI to dynamically track the number of particles and their colocalization with Lysotracker red.

3.11. Magic Red staining

For Cathepsin-B activity assays with Magic Red, we used T98G-Gal-8-ZsGreen cells plated in MatTek glass bottom microwell dishes. In the day of the experiment, cells were incubated for 30 minutes with um of Magic Red dissolved in DMEM at 37°C. The cells were then washed twice with 1mL of PBS 1X, and then maintained with DMEM-Hepes to perform live cell imaging. For

quantification, the integrated fluorescence intensity was measured and then normalized to the first frame to report the shift in fluorescence intensity.

3.12. Lentiviral production

For lentiviral production, HEK293T cells were plated and maintained with high glucose DMEM 10% FBS and antibiotic (100ug/mL penicillin and 0,1 mg/mL of streptomycin). With 80-90% confluency, cells were transfected with PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40,000) pH:7.01 1mg/ml. The transfection mix consisted of 220 µl Optimem, 8 µg of interest vector, 8 µg of psPAX2 (lentiviral packaging plasmid), 4 µg VSVG (envelope), and 65 µl of PEImax. The mixture was incubated for 10 mins at room temperature and then was added by dripping on HEK293T cells. Four hours post-transfection, cell media was renewed, and maintained for posterior harvest. After 72 hours of virus production, the virus-rich media was collected by centrifugation, filtered and aliquoted. In the case of PLVX-Gal-8-ZsGreen lentivirus production, the harvested virus-rich media was disposed in an ultracentrifuge tube with 2 mL of TNE buffer supplemented with 25% sucrose, and then ultracentrifuged at 28.000 rpm for 90 minutes using a P28S Hitachi orbital rotor. The enriched pellet was then resuspended in 200µL of RPMI media.

3.13. Lentivirus infection

For lentivirus infection, T98G cells were plated in a 6 well plate and infected when arrived 50% - 60% confluency. Cells were infected with 5 µL or 10 µL of ultracentrifuged virus media (PLVX-Gal-8-ZsGreen) or 500 µL or 1000 µL of non-ultracentrifuged virus media (PLKO-shGal-8). The culture media with lentiviral particles was then washed with PBS 1x 48 hours post infection, and cells were then selected using 2 µg/mL of Puromycin.

3.14. Inverted invasive migration assay

Inverted invasive migration assay was performed following the protocol established by (Hennigan, Hawker, & Ozanne, 1994). For this purpose, Matrigel® was dissolved 1:1 with sterile PBS 1X and 10 µg/mL of Fibronectin (Sigma-Aldrich, #F2006) and then 100 µL of this mix was added to Transwell®

polycarbonate filters of 8 µm and let polymerize for 1 hour. Next, the Transwell insert was inverted, and 80,000 cells were plated at the bottom of the filter and left to adhere for 4 hours. Afterwards, Transwell inserts were set back to their normal position, and DMEM 10% FBS is added to the top of the Matrigel plug, and DMEM without FBS is added to the bottom of the well. The invasion assay is performed for 5 days, with daily D-Propranolol intermittent treatment.

After finishing the assay, Matrigel plugs are washed with PBS 1X, fixed with 4% PFA and stained with 1:2000 Hoechst. Next, we used a Leica TCS SP8 microscope to capture images, using a 10X objective, capturing a multiple-plane Z-stack with 10 µm distance per plane. We then used ImageJ to obtain the integrated fluorescence intensity of the Z-stack. To obtain the invasion index of each condition, we used the following formula: (∑ Integrated fluorescence of invasion/ ∑ Integrated fluorescence of the first 30µm).

3.15. RT-PCR

To evaluate Gal-8 mRNA expression level, we performed RT-PCR using a Bio Rad T100™ thermal cycler with the following primers: CCAGCTTAGGCTGCCATTC (Gal-8 f); AGGCGTGGGTTCAAGTGTAG (Gal-8 r). For normalizing we used human β-actin with the following primers: TGACCCAGATCATGTTTGAG (human β-actin f); TTCTCCTTAATGTCACGCAC (human β-actin r).

4. RESULTS

4.1. D-Propranolol induces EGFR internalization in T98G cells

Our laboratory has described that D-Propranolol induces EGFR internalization in different cancer cell lines (Barra et al., 2021). In this thesis, we wondered whether a similar effect occurred in the human GB cell line T98G. Indirect immunofluorescence of cells preincubated without FBS for 4 hours and then treated with 50 µM or 100 µM of D-Propranolol for 30 minutes showed EGFR redistribution from the plasma membrane to perinuclear endosomes in T98G cells and HeLa cells, which were used as positive control (Fig. 5A). Previous studies demonstrated that the perinuclear endosomes which EGFR accumulates in response to D-Propranolol correspond to recycling endosomes bearing Rab11 (Metz et al., 2021). As another approach, we performed cell-surface biotinylation and found 70% decrease in the mass of EGFR at the plasma membrane when treated with 100 µM of D-Propranolol (Fig. 5B). Lastly, flow cytometry using EGF-

Alexa-488 showed a 20% decrease of cell surface EGFR with 100 µM D-Propranolol treatment and almost 80% decrease after stimulation with 50 ng/mL of EGF (Fig. 5C). These results reproduce the effect of EGFR internalization seen in other cells when treated with D-Propranolol (Shaughnessy et al., 2014) (Barra et al., 2021) (Metz et al., 2021).

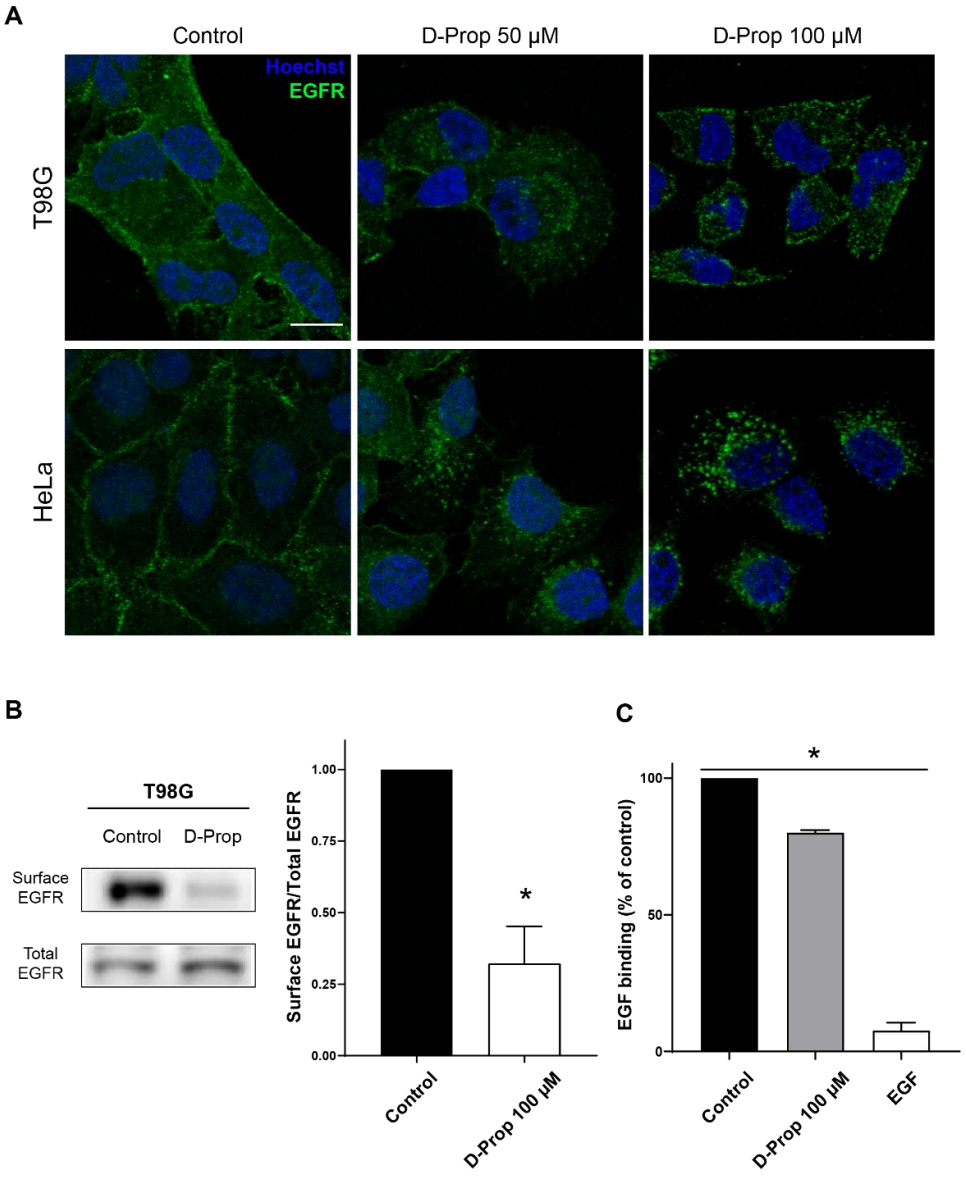


Figure 5. D-Propranolol induces EGFR endocytosis of T98G cells.

(A) T98G and HeLa cells were deprived from FBS for 4 hours and then treated with 50 µM or 100 µM of D-Propranolol for 30 minutes. Indirect immunofluorescence show a redistribution of EGFR from the cell surface to intracellular compartments including perinuclear endosomes. Scale bar= 15 µM. Representative images of N=3. (B) T98G-WT cells treated for 30 minutes with 100 µM of D-Propranolol and then subjected to cell-surface biotinylation assay. D-Propranolol decreased the EGFR mass at the cell surface by 70%, indicating internalization after D-propranolol treatment. N=3, *p=0.05, T-Student test. (C) T98G-WT cells treated for 30 minutes with 100 µM of D-Propranolol or EGF (50 µg/mL) and then incubated with EGF-Alexa-488 for subsequent flow cytometry analysis show a decrease of 20% cell surface EGFR. N=3, *p=0.05, One-Way ANOVA.

4.2. D-Propranolol treatment reduces viable cell number and proliferation

Considering our results of EGFR internalization with D-Propranolol, we asked if this effect is associated with decreased total viable cell number of T98G cells. Cells were plated in a 24 well plate in triplicate for each condition (10,000 cells per well) and treated the next day following the intermittent protocol stablished for other cell lines, which resembles an *in vivo* treatment that considers the 4-5 hours half-life of the drug in blood flow (Fig. 6A) (Shaughnessy et al., 2014) (Barra et al., 2021). The cells were treated twice for one hour with 50 µM D-Propranolol with an interval of 5 hours of complete growth medium between treatments. After three days of D-Propranolol treatment, we found a 65% decrease of viable cell number compared to control conditions, assessed by Trypan blue staining. To evaluate whether this effect associates with internalization of EGFR we studied the PA/PDE4/PKA pathway that induces ligand-free EGFR internalization in several cancer cell models (Shaughnessy et al., 2014) (Barra et al., 2021) (Metz et al., 2021). We used FIPI (75 nM) or Rolipram (30 µM) that respectively counteract the activity of PLD and PDE4 in co-treatments with D-Propranolol. As described in our previous publications (Shaughnessy et al., 2014) (Barra et al., 2021), we found that the use of these antagonists reduce the effect of D-Propranolol on total viable cell number by 50% (Fig. 6A). Thus, we conclude that the negative impact of D-Propranolol on the viable cell number of T98G cells involves the participation of the PA/PDE4/PKA pathway, associated with EGFR endocytosis.

In addition to the effect of D-Propranolol on T98G viable cell number, we evaluated the impact on proliferation rate of T98G cells. We used the thymidine analogue EdU, which is incorporated to the nucleus of actively DNA-synthetizing cells, and therefore can be used as a proliferation read out. Cells subjected to daily intermittent D-Propranolol treatments for three days, as previously explained in Fig. 6A, showed a reduction in the proliferation rate by 20% (Fig. 6B). These results indicate that reduced proliferation is implied in the decreased total cell count (Fig. 6A).

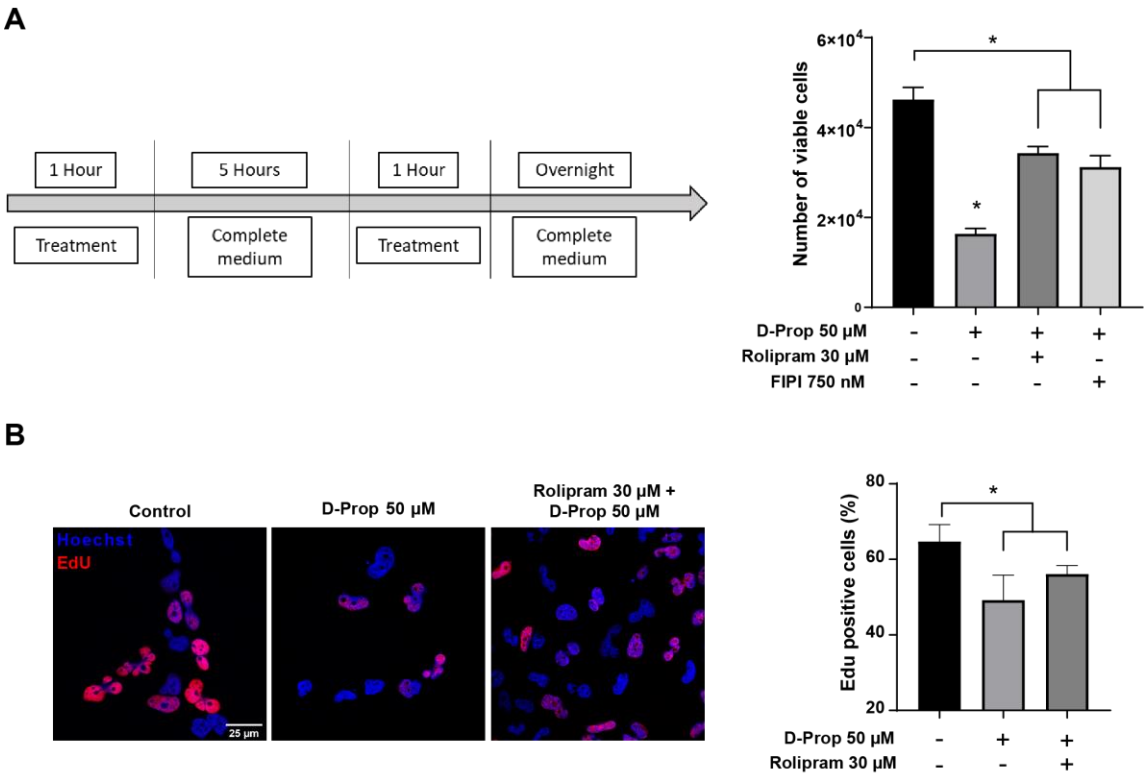


Figure 6. D-Propranolol decreases the viability and proliferation of T98G cells.

(A) T98G cells were treated with D-Propranolol with or without Rolipram 30 µM or FIPI 75 nM as described in the treatment design scheme. This cycle of treatment was repeated for three days and then viable cells were counted by trypan blue staining. Total viable cells were counted in triplicate for each condition. Initial 10,000 cells were plated per condition. N=3, *p=0.05, One-way ANOVA. (B) T98G cells were treated as depicted in Fig. 6A. and then incubated for two hours with

the thymydine analogue, EdU, which is then conjugated with an Alexa fluorophore for evaluation by fluorescence microscopy. DNA-synthetizing cells incorporate EdU and are considered as proliferating cells. The percentage of proliferating cells from the total counted cells was determined. N=3, *p=0.05, One-way ANOVA.

4.3. D-Propranolol induces the accumulation of endogenous Gal-3 and Gal-8

As previously shown, T98G-WT cells show decreased viability in response to D-Propranolol, and it is partially reverted by PA/PDE4/PKA antagonists (Fig. 5A). Therefore, considering the lysosomotropic profile of D-Propranolol (Saha et al., 2020) we wondered if lysosomal perturbations could be a possible explanation to the reduction in T98G cell viability. In this context, recent evidence revealed that Gal-8 present in the cytoplasm participates, together with Gal-3 and Gal-9, in cell-protecting responses to endolysosomal damage (Jia et al., 2018; Jia et al., 2020). Gal-3 and Gal-8 are recruited to damaged endolysosomes that expose glycans to the cytosol, and this accumulation can be observed as an increase of the number of puncta by fluorescence microscopy (Aits et al., 2015). We performed indirect immunofluorescence to detect that accumulation of Gal-3 puncta in our T98G-WT cells when treated with 1mM of LLOMe, 50 µM or 100 µM of D-Propranolol for 1 hour. As expected, we observed LLOMe treatment caused Gal-3 puncta pattern that colocalized with LAMP-1 positive compartments (Fig. 7). Similarly, D-Propranolol treatments (50 µM and 100 µM) caused the same phenotype than LLOMe, strongly suggesting D-Propranolol triggers lysosomal membrane permeabilization (LMP). Quantification analysis confirmed this conclusion, indicating notorious differences in both, number of Gal-3 puncta and colocalization with LAMP-1 compartments, compared to control conditions (Fig. 8).

Next, we asked whether Gal-8 could be sensitive to LMP in response to D-Propranolol. For this, we used an anti-Gal-8 antibody that has been previously used to evaluate endogenous Gal-8 accumulation (Falcon, Noad, McMahon,

Randow, & Goedert, 2018). Indirect immunofluorescence of T98G-WT cells showed that D-propranolol treatment for 1 hour trigger the recruitment of intracellular Gal-8 to punctate membrane structures (Fig. 9), indicative of damaged endolysosomes. These results show that D-Propranolol promotes the recruitment of Gal-3 and Gal-8 suggesting LMP in T98G cells.

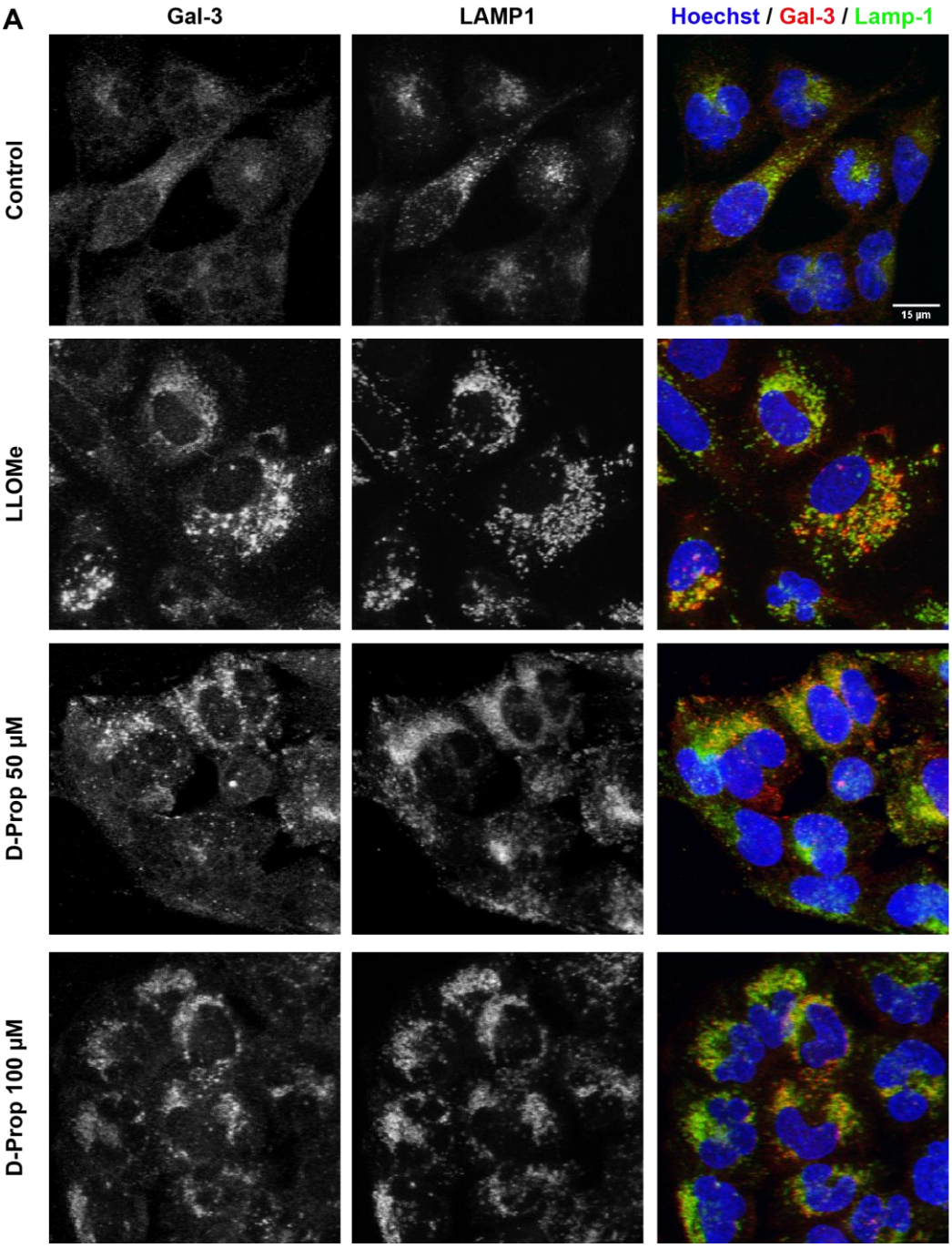


Figure 7. D-Propranolol induces the recruitment of endogenous Gal-3 to lysosome-like compartments.

(A) T98G-WT cells were treated for 1 hour with LLOMe 1 mM, 50 µM or 100 µM of D-Propranolol and then fixed for indirect immunofluorescence for Gal-3 puncta formation evaluation and colocalization with LAMP1 compartments.

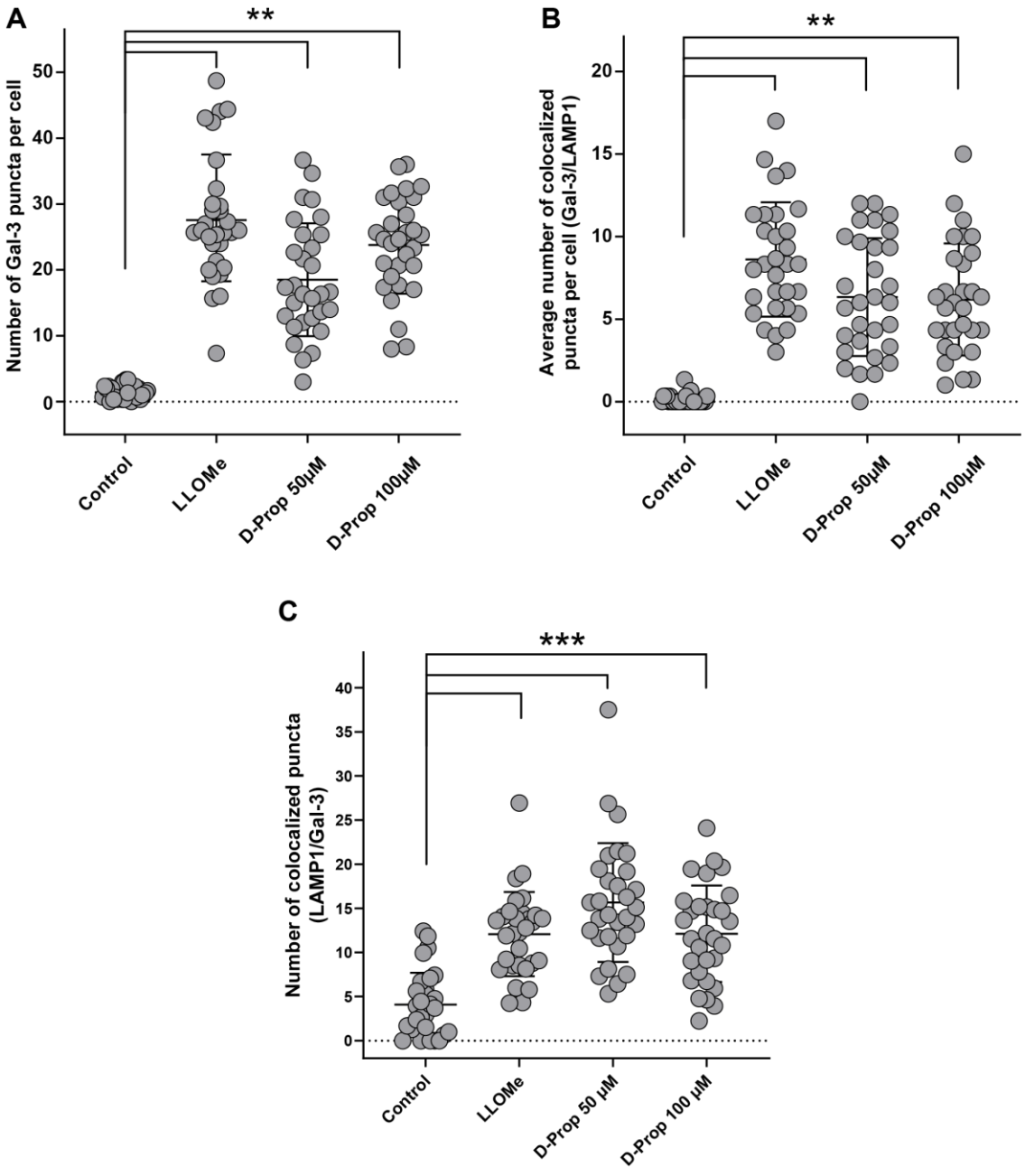


Figure 8. D-Propranolol induces the formation of Gal-3 puncta in lysosome-like compartments.

(A) The number of detectable Gal-3 puncta per cell was evaluated using the ComDet v.0.5.3 plugin in ImageJ. (B) The number of Gal-3 and LAMP-1 colocalized puncta per cell was assessed using the ComDet v.0.5.3 plugin in ImageJ. N=3, 30 cells were counted per condition in each experiment, **p=0,01, Two-Way ANOVA. (C) Colocalization of total LAMP-1 compartments with Gal-3 puncta per cell was evaluated using the ComDet v.0.5.3 plugin in ImageJ. N=3, 30 cells were counted per condition in each experiment, ***p=0,001, Two-Way ANOVA.

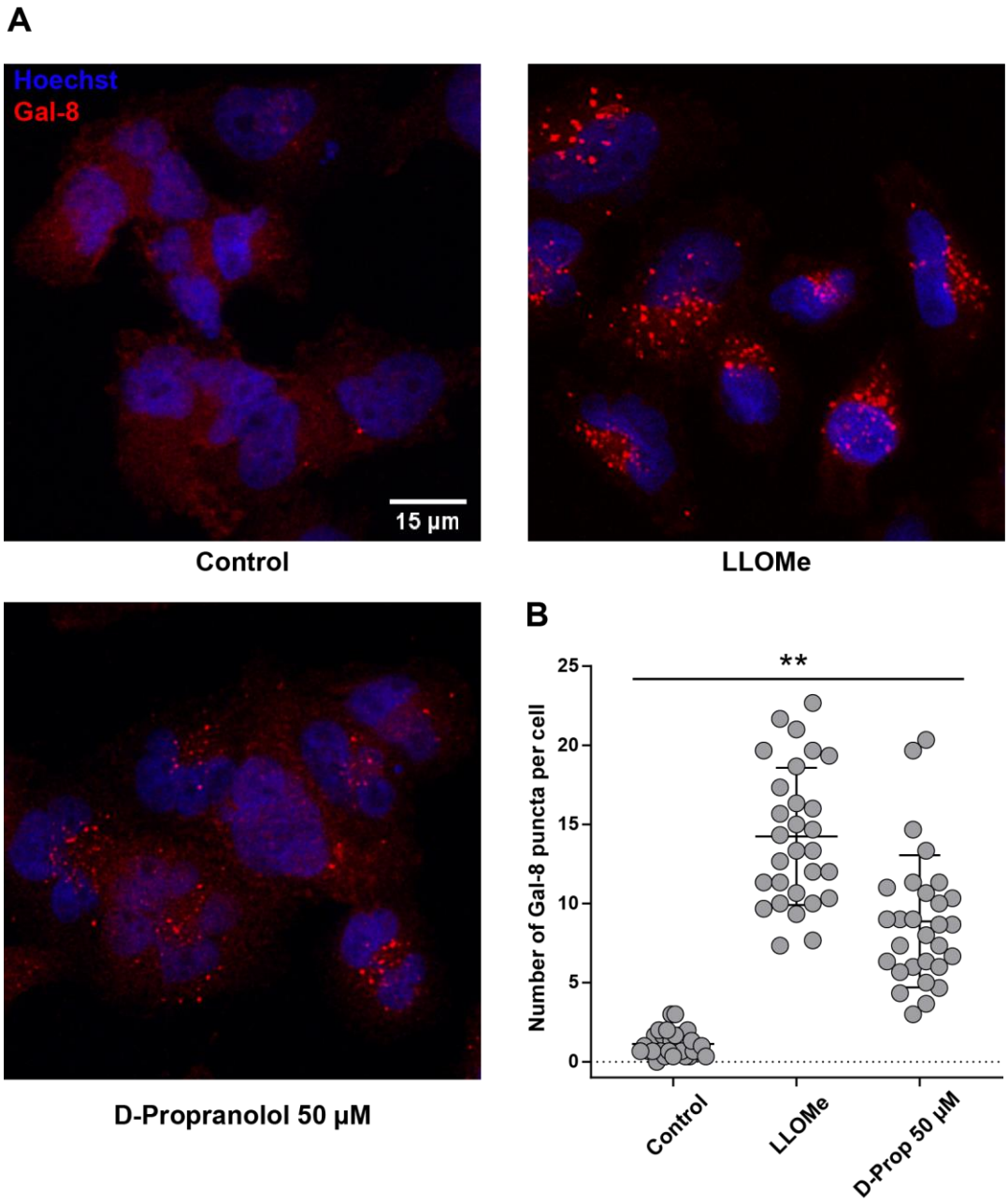


Figure 9. D-Propranolol induces the formation of endogenous Gal-8 puncta.

(A) T98G-WT cells were treated for 1 hour with LLOMe 1 mM or D-Propranolol 50 µM and then fixed for immunofluorescence. (B) The number of detectable Gal-8 puncta per cell was determined using the ComDet v.0.5.3 plugin in ImageJ. N=3, 30 cells were counted per condition in each experiment, **p=0,01, Two-Way ANOVA.

4.4. D-Propranolol induces Gal-8-ZsGreen recruitment to lysosome-like compartments

Next, we sought to evaluate the temporality of Gal-8 recruitment in response to D-Propranolol. For this end, we generated a stable Gal-8 overexpressing T98G cell line by transduction with lentiviral particles containing a pLVX plasmid with the Gal-8 sequence coupled to a ZsGreen fluorophore in its N terminus, cells that were selected by cell sorter (FACSAria II). The cell line generated, referred as T98G-Gal-8-ZsGreen cell line, was used for live cell imaging experiments. Cells were plated in a live cell imaging culture dish and maintained with DMEM-Hepes for the first 10 minutes of the experiment, as a control condition. Treatment with 50 µM of D-Propranolol rapidly induced the redistribution of cytosolic Gal-8-ZsGreen to punctate membrane structures, distribution that remained unaltered in the control condition (Fig. 10B). To determine the acidity of these punctate membrane structures, live T98G-Gal-8-ZsGreen cells were stained with Lysotracker red for 15 minutes, previous to live cell imaging. We observed a rapid increase in colocalization of Gal-8-ZsGreen puncta with Lysotracker red compartments in response to D-Propranolol treatment, as in the first 10 minutes post-treatment this overlapping increased by approximately 20% and maintained rather stable overtime in the remaining 30 minutes post-treatment (Fig. 11B & Fig. 11C). These results strongly indicate that recruitment of Gal-8 triggered by D-Propranolol to acidic punctate membranes occurs in response to the damaged of acidic compartments, very likely corresponding to damaged lysosomes.

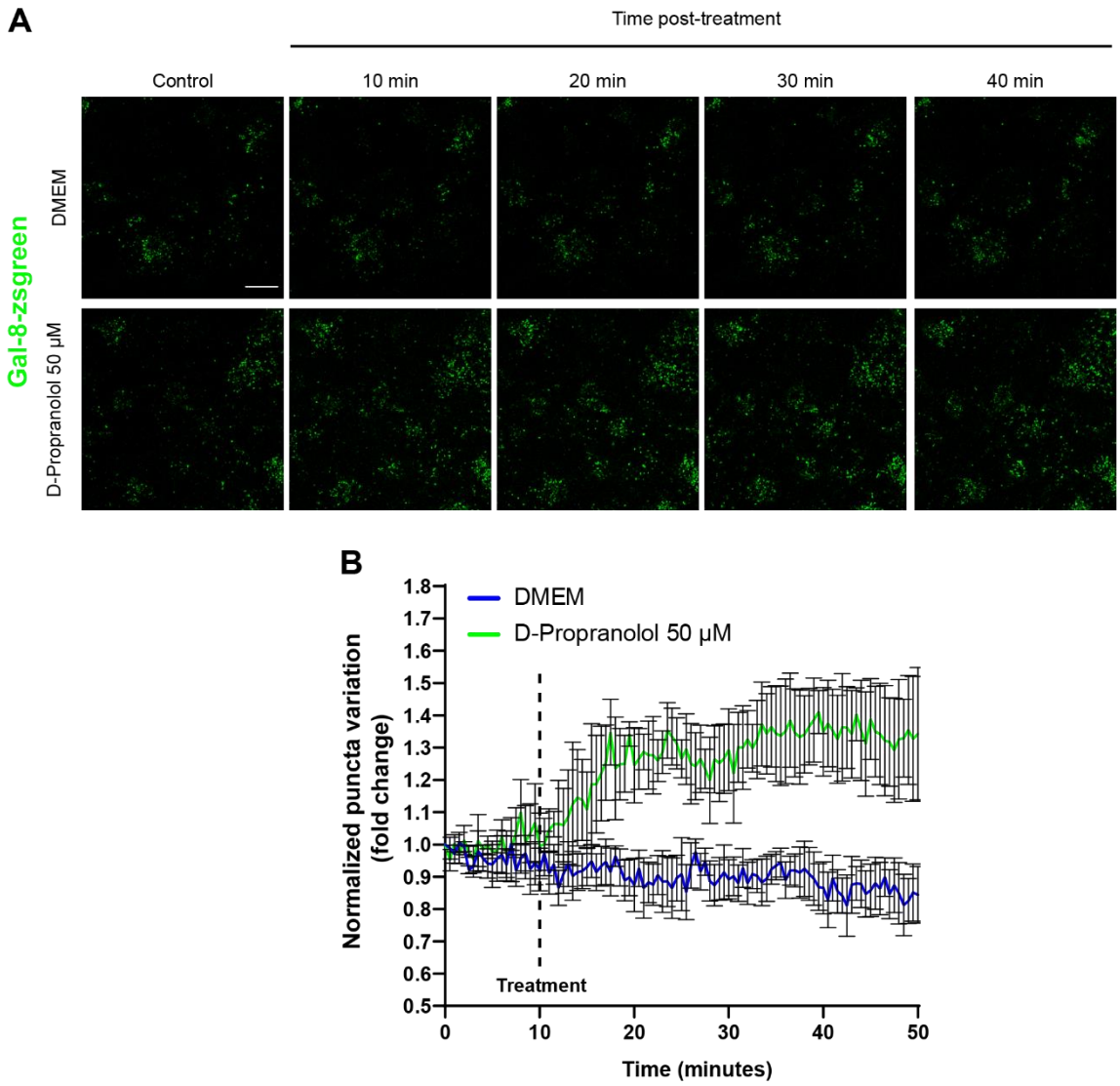


Figure 10. D-Propranolol induces Gal-8-ZsGreen accumulation.

(A) T98G cells were transduced to stably express Gal-8 coupled to the green fluorophore ZsGreen (Gal-8-ZsGreen). These cells were used to evaluate the effect of D-Propranolol on Gal-8 puncta formation by live cell imaging, as a read out for damage of endomembranous compartments. Images were captured during pre- and post-treatment using live-cell imaging confocal microscopy. (B) Gal-8-ZsGreen puncta variation over time was evaluated using ComDet v.0.5.3 plugin in

ImageJ. Scale bar represent 15 µm. Total of N=3; Control, n= 31, 42, 35 and D-Propranolol, n= 32, 36, 30 cells per experiment evaluated.

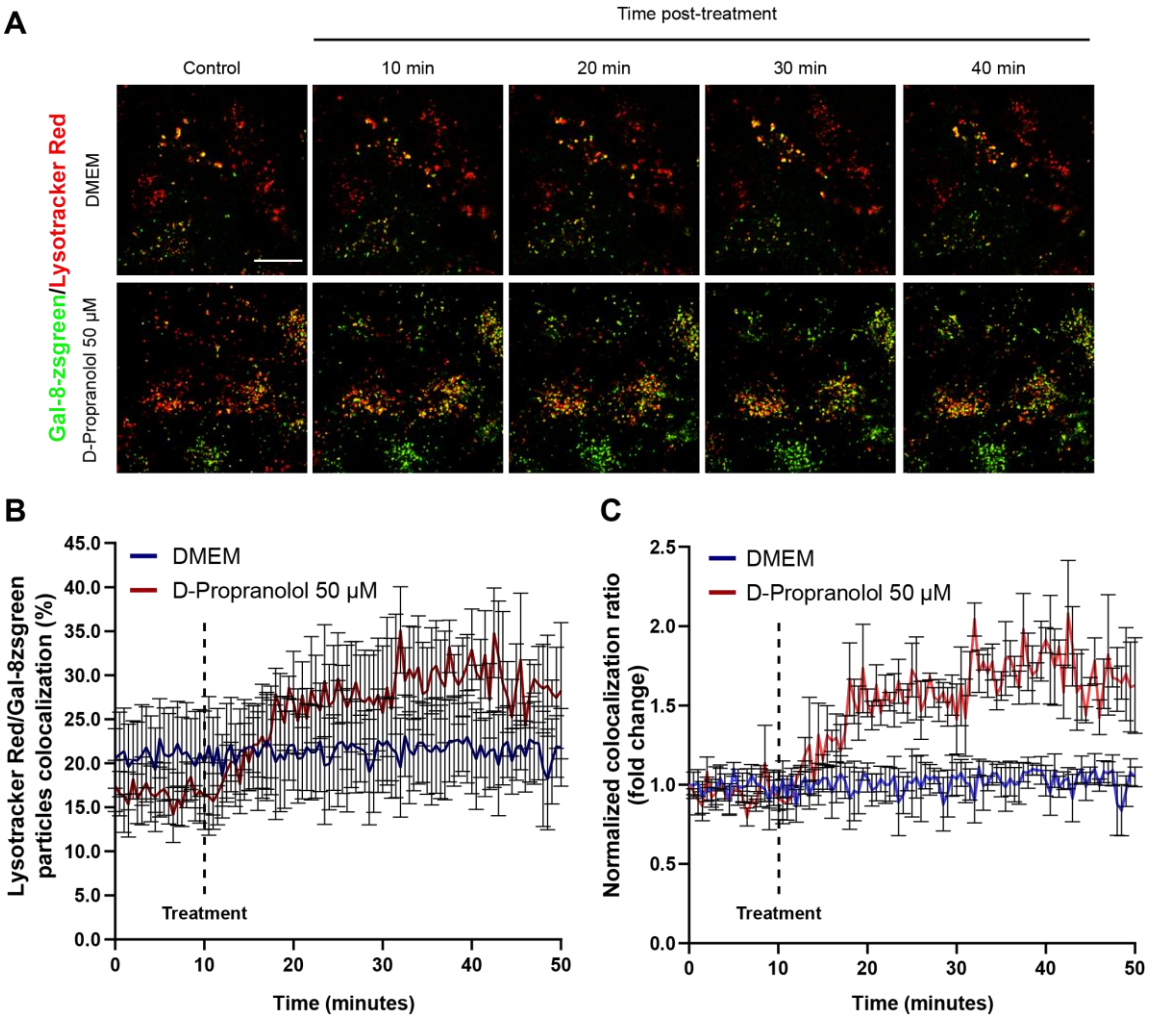


Figure 11. D-Propranolol induces Gal-8 recruitment to lysosome-like compartments.
(A) Colocalization variation over time of Gal-8-Zsgreen to lysosomes was evaluated using the pH sensitive probe Lysotracker Red. Images were captured during pre- and post-treatment using live-cell imaging confocal microscopy. (B) Colocalization percentage of Lysotracker red with Gal-8-ZsGreen puncta was obtained using ComDet v.0.5.3 plugin in ImageJ. (C) Colocalization ratio of Lysotracker red with Gal-8-ZsGreen puncta normalized to frame one (T=0) was obtained from raw

colocalization percentage. Scale bar represent 15 µm. Total of N=3; Control, n= 31, 42, 35 and D-Propranolol, n= 32, 36, 30 cells per experiment evaluated.

4.5. D-Propranolol decreases Cathepsin B activity

Considering the effects of D-Propranolol on Galectin recruitment to lysosome-like compartments, we decided to evaluate the impact of this drug on lysosomal function. We used Magic Red, a fluorophore-coupled peptide that is specifically cleaved by the lysosomal enzyme Cathepsin-B and its fluorescence emission reflects the activity of this enzyme (Boonacker, Elferink, Bardai, Wormmeester, & Van Noorden, 2003). Within minutes, D-Propranolol induced a notorious decay of Cathepsin B activity, which remained altered up to 40 minutes, with a 25% activity decline in comparison to the control condition (Fig. 12B). The decline in Cathepsin B activity occurred in a similar temporality of Gal-8-ZsGreen recruitment to lysosome-like compartments (Fig. 10B & Fig. 11B). This result strongly indicates that D-Propranolol causes lysosomal dysfunction in T98G cells, a phenotype that could contribute to the decreased viability of T98G cells treated with this drug.

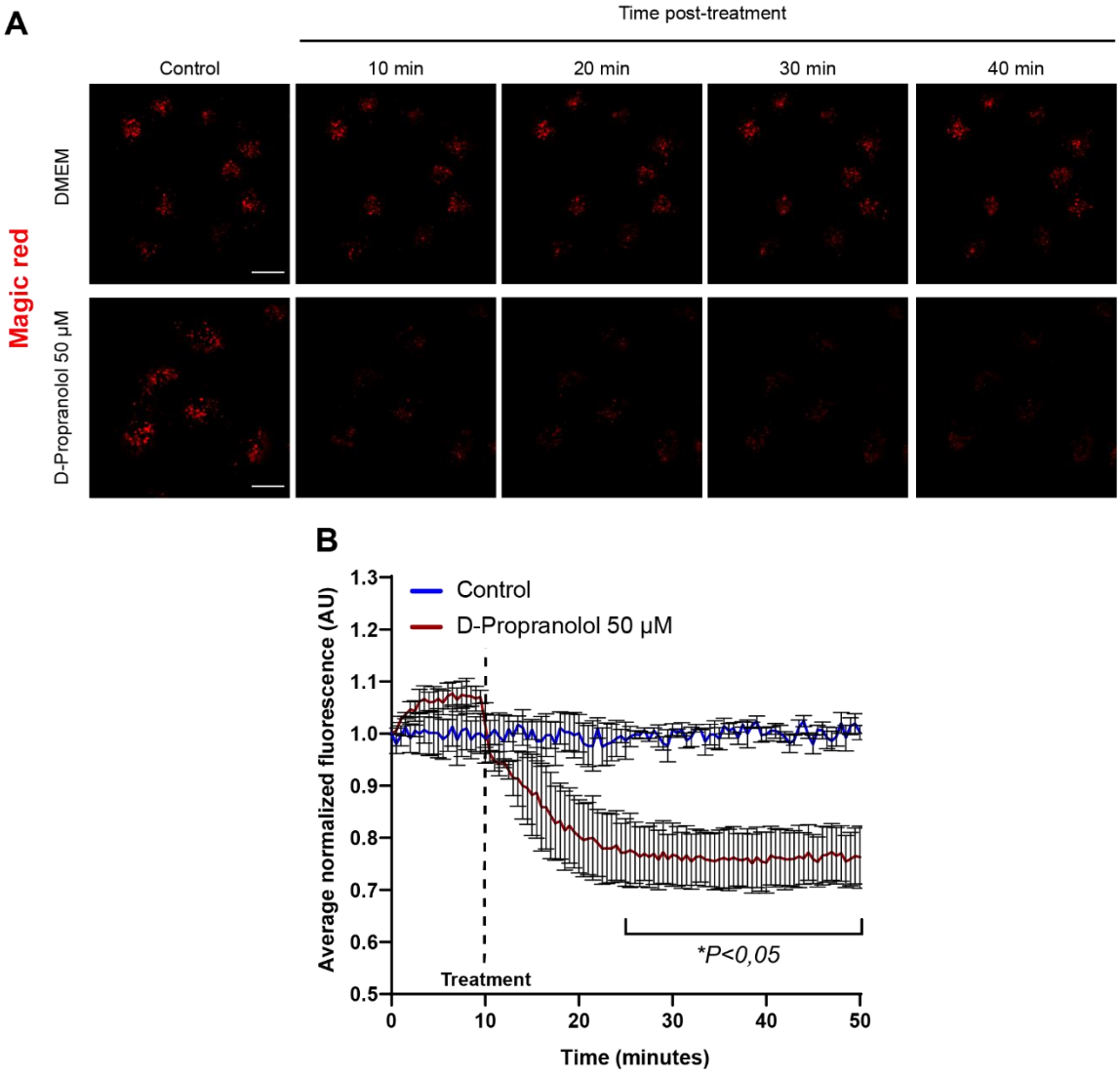


Figure 12. D-Propranolol induces lysosomal dysfunction on T98G-Gal-8-ZsGreen cells.

(A) T98G-Gal-8-ZsGreen cells were pre-incubated with Magic Red, a Cathepsin-B fluorescent substrate. Loss of fluorescence indicate decreased Cathepsin-B activity. Images were captured pre- and post-treatment using live-cell imaging confocal microscopy. Scale bar represent 15 µm. (B) Integrated fluorescence intensity variation over time was evaluated using the ImageJ measure

tool for each frame. Total of N=3; Control, n= 31, 46, 38 and D-Propranolol, n= 33, 30, 29 cells per experiment were evaluated. *p<0.05, Two-Way ANOVA.

4.6. Gal-8 expression determines T98G cell growth

Because recruitment of Gal-8 to lysosome-like compartments with D-Propranolol correlated with either lysosomal damage measured by Gal-3 recruitment and with the decay in lysosomal activity (Cathepsin-B activity), we evaluated the impact of silencing of Gal-8 on the viability of T98G in the presence of D-Propranolol. For this end, we generated a Gal-8 knockdown (KD) T98G cell line, using lentiviral particles encoding two different previously characterized Gal-8 shRNAs, sh #5 and sh #7 (Metz et al., 2016) testing two different dilutions for each shRNA, depicted as C1 or C2. Gal-8 RT-PCR analysis showed sh #7 C1 as the most effective shRNA in T98G cells (Fig. 13B). Thus, stable sh #7 C1 expressing cells were used in further experiments and will be referred as T98G-shGal-8.

To assess the impact of Gal-8 KD on viable cell number in response to D-Propranolol, we used the T98G-shGal-8 cell line and T98G-sh-scramble cells as a respective control. For assessing the effect of high Gal-8 expression, we used the T98G-Gal-8-ZsGreen cell line and T98G-WT as a control of normal Gal-8 level of expression. We plated 10,000 cells in triplicate per condition and maintained them with DMEM supplemented with 10% FBS with or without D-Propranolol, following the previously described intermittent treatment protocol for three days (Fig 6.A), and then viable cell number was evaluated with Trypan blue. Notably, T98G-sh-Gal-8 cells displayed a notorious 60% decrease in cell growth compared to T98G-sh-scramble cells in control conditions (Fig. 14B). On the other hand, T98G-Gal-8-ZsGreen cells showed an approximate 60% increase in viable cell count compared to T98G-WT cells (Fig. 14B).

To better represent the loss on viable cell number of each cell line with D-Propranolol treatment we considered the viable cell number of the control of each cell line as 100% for relative percentual quantification of the treatment conditions.

After the daily intermittent treatments, we found that T98G-shGal-8 cells showed a 10% decrease in cell viability when treated with 50 μM of D-Propranolol compared to T98G-sh-scramble cells, but we did not find significant statistical differences (Fig. 14C). We also did not observe differences in the percentual loss on the viable cell number of T98G-Gal-8-ZsGreen cells compared to T98G-WT cells when treated with 50 μM or 100 μM of D-Propranolol (Fig. 14C). These results demonstrate that Gal-8 expression contributes to cell growth of T98G GB cells, but apparently do not confer improved resistance or susceptibility towards D-Propranolol treatment.

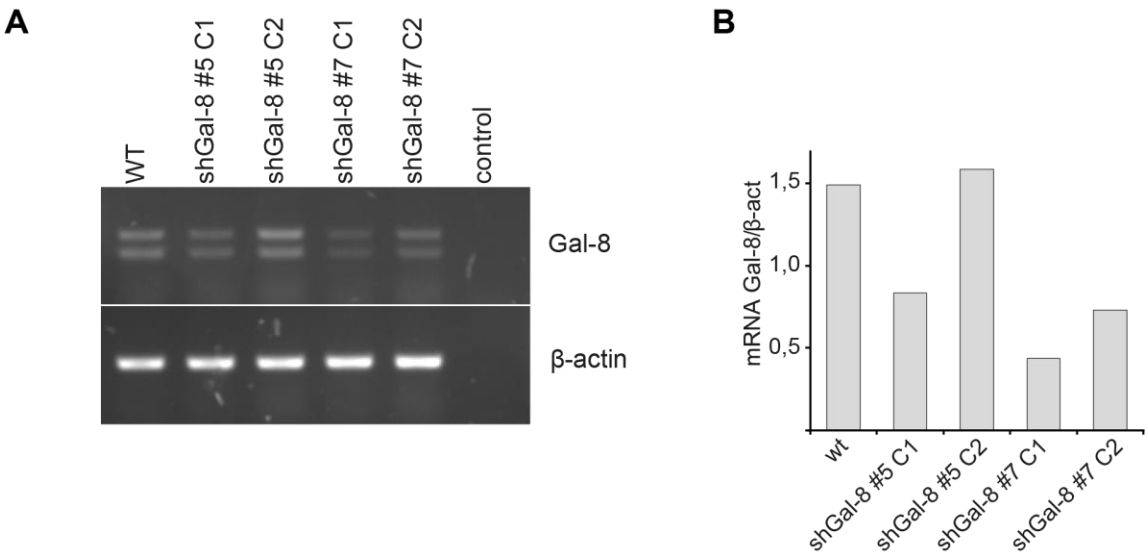


Figure 13. Gal-8 knockdown with shRNA in T98G cells.

(A) T98G cells were transduced with lentiviral particles containing different shRNA for Gal-8 silencing. Cells were selected with puromycin (2 µg/mL) and Gal-8 expression levels were assessed by RT-PCR. (B) Quantification of Gal-8 transcript levels normalized to β-actin indicate that T98G cells transduced with sh#7 C1 have the lowest level of Gal-8 expression and therefore were selected for generating a stable T98G-shGal-8 cell line.

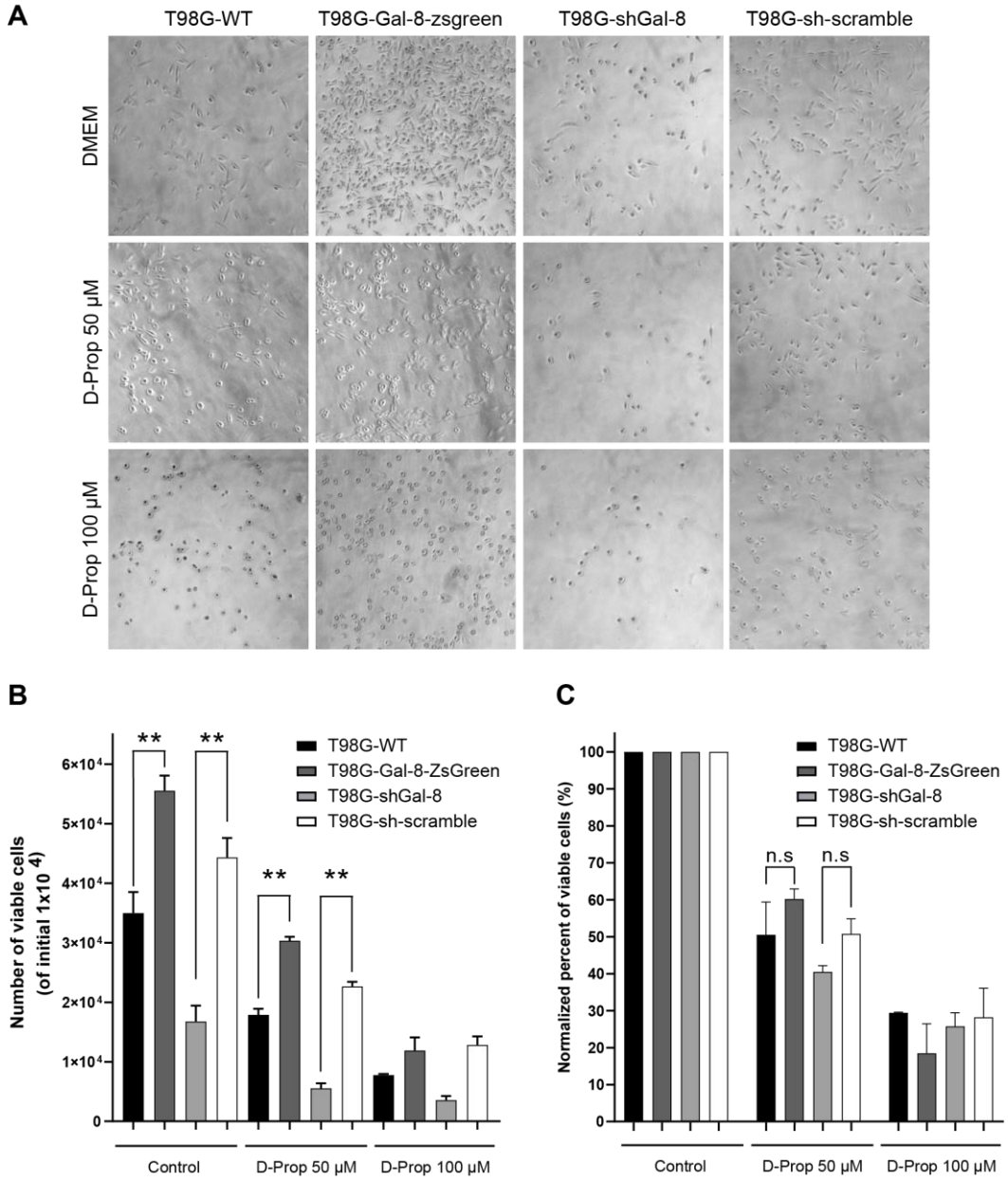


Figure 14. Gal-8 expression is associated with differential growth of T98G cells.

(A) T98G-WT, T98G-Gal-8-ZsGreen and T98G-shGal-8 cells were plated (10,000 cells) and subjected to daily intermittent treatments with 50 µM and 100 µM of D-Propranolol or maintained with DMEM 10% FBS for three days. Representative bright-field microscopy images of cells after three cycles of treatments with D-Propranolol are shown. (B) Total viable cell count with trypan blue after three days from an initial 10,000 cell culture with or without 50 µM or 100 µM D-Propranolol daily intermittent treatment. (C) Percentage of viable cells from each T98G cell line was calculated from their respective untreated condition to evaluate D-Propranolol susceptibility

associated to Gal-8 expression. N=3, cells counted in triplicate per each condition, **p<0.001, Two-way ANOVA.

4.7. Gal-8 overexpression is associated with enhanced signaling response of the EGFR pathway in T98G cells

Based in our observation of differential T98G cell growth associated with Gal-8 expression levels, we decided to evaluate changes in EGFR mitogenic signaling pathways. Our laboratory has previously described that Gal-8 overexpression stimulates pro-tumorigenic features such as increased proliferation and invasion of non-cancerous MDCK cells, involving enhanced EGFR signaling due to its transactivation by Gal-8 (Oyanadel et al., 2018)(Oyanadel et al., 2018). Therefore, we evaluated the autophosphorylation residue Tyr1068 of the EGFR, which is associated with the recruitment of Grb2 and consequent activation of downstream signaling pathways (Rojas, Yao, & Lin, 1996). Also, we assessed the phosphorylation of ERK, which is a downstream target of EGFR involved with cell proliferation. For this end, we used T98G-WT and T98G-Gal-8-ZsGreen cells which were deprived of FBS for 4 hours and treated with EGF (50 ng/mL) in the absence or presence of 50 µM of D-Propranolol for 30 minutes. Cells were then lysed, and total protein extract was obtained to evaluate the phosphorylation of EGFR (pTyr1068) and ERK by immunoblot. We found that treatments with EGF induced a notoriously increased phosphorylation of EGFR (Tyr1068) and ERK in T98G-Gal-8-ZsGreen cells compared to WT cells (Fig. 15B & Fig. 15D). This exacerbated signaling intensity of T98G-Gal-8-ZsGreen cells was also observed when co-treated with 50 µM of D-Propranolol in comparison to WT cells. These results indicate that Gal-8 overexpressing T98G cells display an improved response to mitogenic stimulus with EGF, which correlates with our previous cell count results (Fig. 14A). On the other hand, these observations suggest that Gal-8 overexpression decreases D-Propranolol efficiency in abrogating EGFR downstream signaling compared to WT cells.

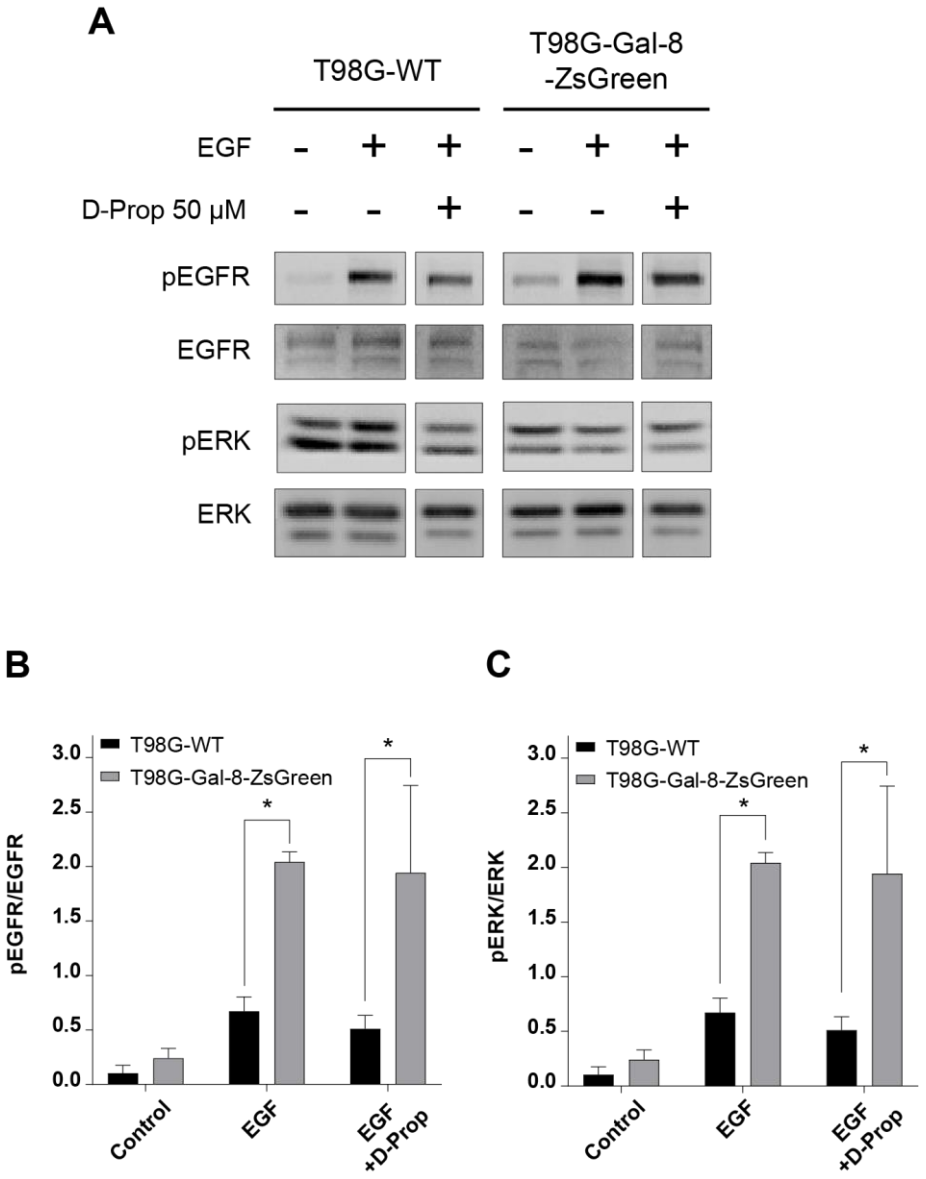


Figure 15. Gal-8 overexpression enhances signaling response of the EGFR pathway in T98G cells.

(A) T98G-WT and T98G-Gal-8-ZsGreen cell were deprived for 4 hours and then treated with EGF (50 ng/mL) in the presence or absence of D-Propranolol (50 µM or 100 µM) for 30 minutes. Cells were then lysed to obtain total protein extract and perform immunoblot. Quantification of phospho-EGFR (Tyr1068) (B) and phospho-ERK (C) normalized to GAPDH and to their relative total protein mass was evaluated. N=3, *p=0,05, Two-way ANOVA.

4.8. Gal-8 overexpression increases T98G cell invasion

Previous experiments from our laboratory have demonstrated that Gal-8 serves as chemoattractant for U87 GB cells in Transwell migration assays (Metz et al., 2016). Considering that our Gal-8 overexpressing cells (T98G-Gal-8-ZsGreen) display increased EGFR and ERK signaling response, which participate in cell migration, we decided to evaluate the impact of Gal-8 expression in the invasion of our generated T98G cell lines. For this end we used the inversed invasive migration assay with Matrigel coated polycarbonate Transwell filters. Cells were plated in Transwell filters and then treated for 5 consecutive days for allowing their invasion through the Matrigel plug. After this period, we found that T98G-Gal-8-ZsGreen had 25% increased invasiveness compared to T98G-WT cells, while T98G-shGal-8 cells showed a 40% lower invasion index compared to T98G-sh-scramble cells (Fig. 15B). These results suggest that Gal-8 expression contributes to the invasive features of T98G cells.

Interestingly, we found that D-Propranolol had no significant effect on T98G-WT cells but reduced the higher invasive migration of T98G-Gal-8-ZsGreen cells near the levels of T98G-WT cells invasion index. T98-shGal-8 cells treated with D-Propranolol showed a tendency for increased invasion compared to untreated T98-shGal-8 cells but is not statistically significant, and the invasion index is lower respect to T98G-sh-scramble cells treated with D-Propranolol. Therefore, the effect of D-Propranolol against invasive migration seems to depend on the Gal-8 level of expression on T98G cells.

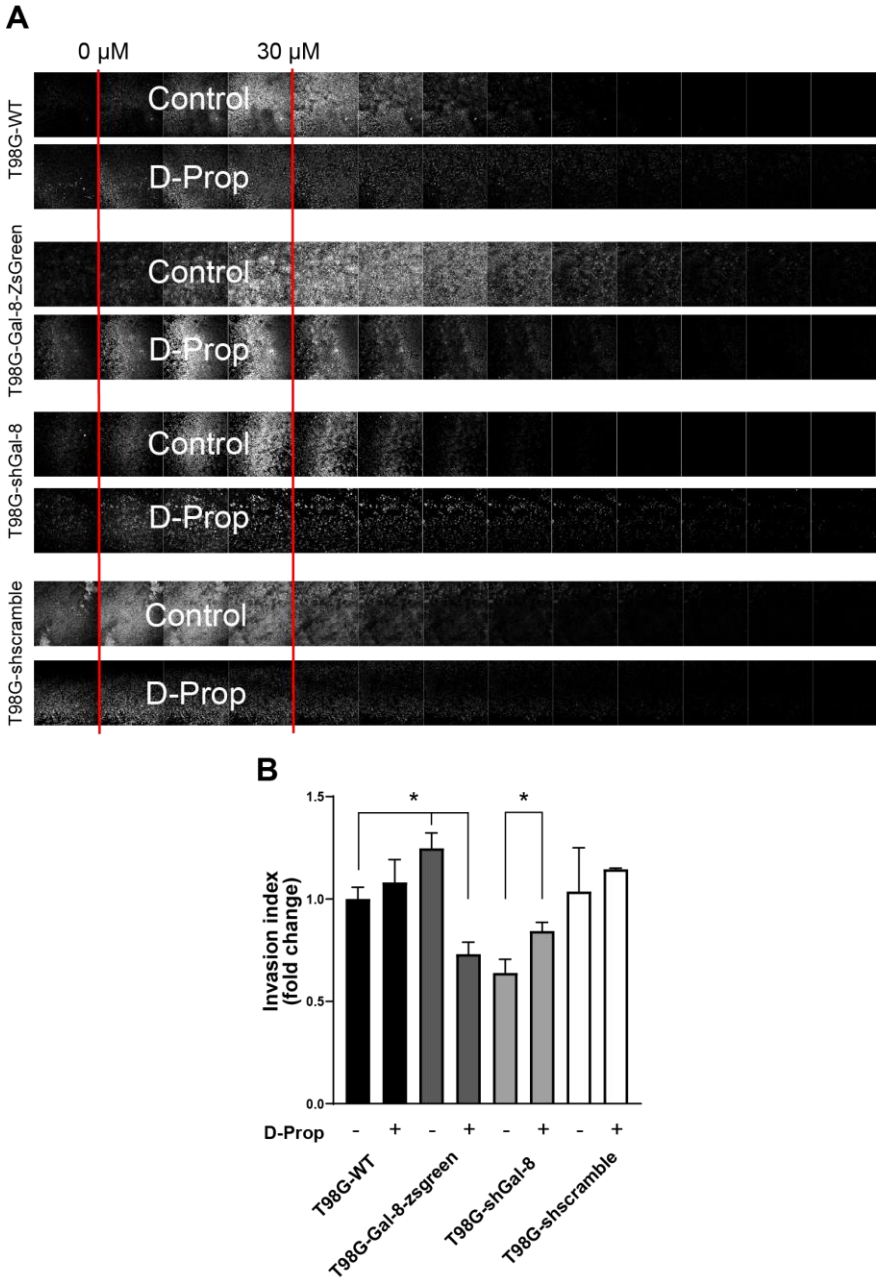


Figure 16. Gal-8 overexpression is associated with increased invasive migration of T98G cells.

(A) T98G-WT, T98G-Gal-8-ZsGreen and T98G-shGal-8 cells were subjected to an inverted invasive migration assay in polycarbonate filters coated with Matrigel plus Fibronectin (2,5 g/mL), with or without daily intermittent D-Propranolol 50 µM treatment using FBS as chemoattractant for 5 days. Filters with Matrigel were fixed with PFA 4% and stained with Hoechst. Confocal sections are shown to depict the invasion extension. (B) The invasion index of each condition was

evaluated by using the formula= ∑ Integrated fluorescence of invasion/ ∑ Integrated fluorescence of the first 30µM. N=3, *p<0.05, Two-way ANOVA.

5. DISCUSSION

5.1. D-Propranolol induces the internalization of EGFR and is associated with decreased viable cell number and proliferation of T98G cells

In this thesis, we explored if D-Propranolol is an effective drug to eradicate human GB cells, using the T98G human GB cell line. Considering the relevant role of EGFR traffic perturbation by D-Propranolol in determining cell survival and proliferation reported in our previous studies (Shaughnessy et al., 2014) (Barra et al., 2021), we first decided to evaluate if D-Propranolol induces the internalization of EGFR in T98G cells. Our immunofluorescence experiments showed that the use of D-Propranolol at 50 µM and 100 µM induced the internalization of EGFR in T98G cells, as the distribution of the receptor decreased from the plasma membrane concomitant with the formation of a puncta pattern (Fig. 5A) that most likely correspond to recycling endosomes as described in other cell lines (Metz et al., 2021). Also, we found that D-Propranolol used at the IC50 described for HeLa cells (100 µM) (Shaughnessy et al., 2014) induced approximately 20% internalization of EGFR in T98G cells in our flow cytometry experiments (Fig. 6C). Moreover, cell surface biotinylation assays showed a 70% decrease in cell surface EGFR when treated with D-Propranolol 100 µM (Fig. 6B). This difference on the magnitude of the read-out internalization could be accounted for differences in the protocol of each technique. Our cell surface biotinylation assays were performed entirely at 4°C while our flow cytometry protocol contemplated a step to detach live cells at 37°C after being incubated with EGF-Alexa-488, temperature at which endocytic traffic is still active, and could decrease the available cell surface EGFR to read-out. We conclude from these different experimental approaches that D-Propranolol induces the endocytosis of EGFR in T98G cells.

Endocytosis plays a major role in the regulation of EGFR function and its detection of extracellular stimuli, and has been found frequently altered in cancer cells (Roepstorff, Grovdal, Grandal, Lerdrup, & van Deurs, 2008). Therefore, the

endocytic pathway has been considered a potential target to counteract the oncogenic influence of EGFR in cells that overexpress this protein and therefore have a high reliance on its function (Shaughnessy et al., 2014) (Barra et al., 2021). The activation of the PA/PDE4/PKA pathway, which is achieved with D-Propranolol as well as with Propranolol (the racemic mixture of D- and L-Propranolol), leads to EGFR endocytosis through clathrin-dependent and clathrin-independent mechanisms (Norambuena et al., 2010) (Shaughnessy et al., 2014) (Barra et al., 2021) (Metz et al., 2021). The internalization of the EGFR induced by this PA/PDE4/PKA pathway involves an increased endocytosis from the cell surface and an arrest at recycling endosomes due to a block of the recycling pathway (Norambuena et al., 2010) (Metz et al., 2021). These effects are accompanied by a reduction of the EGFR downstream activation of signaling pathways such as ERK and AKT (Barra et al., 2021), which have been involved in proliferation and survival processes (Lavoie, Gagnon, & Therrien, 2020) (Lien, Dibble, & Toker, 2017), respectively. In cancer cells, EGFR activation promotes ERK and AKT sustained signaling implied in the enhancement of cell proliferation, survival, and invasive migration (Schmid, 2017).

Our results show that an intermittent daily-dose treatment with D-Propranolol diminishes around 65% population of viable cells compared to the control group (Fig. 6A). This is congruent with decreased viability of HeLa cervical carcinoma, MKN45 gastric cancer and H1975 lung carcinoma cells (Shaughnessy et al., 2014) treated with D-Propranolol. Next, we found that co-treatment of D-Propranolol with Rolipram or FIPI, partially reverted the decreased viable cell number, showing a loss of 30% of viable cells compared to control condition, indicating an involvement of the PA/PDE4/PKA pathway in this effect, which has been previously demonstrated to be regulate the endocytic traffic of the EGFR (Shaughnessy et al., 2014) (Barra et al., 2021) (Metz et al., 2021). Our EdU incorporation assays show that daily intermittent treatment with D-Propranolol reduces the proliferation of T98G cells by 20% compared to untreated cells (Fig. 6B), suggesting that the decreased total viable cell count involves decreased proliferation rate. Therefore, we can stipulate that the decreased viable cell

number of T98G cells with D-Propranolol is associated with EGFR recycling perturbation by modulation of the PA/PDE4/PKA pathway, which likely implies attenuation of signaling pathways associated with cell proliferation (Fig. 17B).

5.2. D-Propranolol induces lysosomal damage and galectins recruitment

Amphipathic cationic compounds such as Desipramine have been shown to induce lysosomal membrane damage (Kallunki, Olsen, & Jaattela, 2013). Considering that D-Propranolol is also an amphipathic cationic molecule (Sozzani et al., 1992), we decided to explore the effect of this drug on the lysosome, a relevant organelle in cancer pathogenesis. Lysosomes are critical for cell nutrition, owed to their supplement of degradative enzymes, as well as protein and organelle homeostasis through autophagy (Ravikumar et al., 2010). Damaged endolysosomal compartments are detected and either repaired or removed by the system involving the complementary functions of Gal-3, Gal-8 and Gal-9 (Jia et al., 2018).

Gal-3 recruitment to endolysosomes is currently used to assess the damage of these organelles in response to lysosomotropic drugs, as this recruitment reflects the exposure of glycans that normally decorate the luminal face of their membrane (Aits et al., 2015). Therefore, we first tested whether D-Propranolol induces the puncta distribution of Gal-3 that is typical of endolysosomal damage. We indeed found evidence that D-Propranolol induces membrane damage of lysosome-like compartments in 1-hour treatments, based on the accumulation of Gal-3 puncta decorating LAMP-1 compartments, a similar pattern that we observed when we used the lysosomal membrane damaging compound LLOMe (Fig. 7).

We next focused on Gal-8, which is also recruited to lysosomal membranes under lysosomal damaging conditions. We found that treatments with D-Propranolol promoted the formation of distinctive endogenous Gal-8, but in a lower degree than with LLOMe (Fig. 9). To further characterize Gal-8 response, we performed fluorescence imaging experiments in live cells to assess the kinetics of the recruitment process, and for this end we generated a stable Gal-8-

ZsGreen expressing T98G cell line. We found that D-Propranolol induces the recruitment and accumulation of Gal-8-ZsGreen to lysosome-like compartment further suggesting endomembrane permeabilization (Fig. 10). Our results show a rapid Gal-8-ZsGreen puncta accumulation with D-Propranolol treatments, reaching a plateau at 20 minutes (Fig. 10B), suggesting a saturation limit of Gal-8 accumulation. Colocalization of Gal-8-ZsGreen with Lysotracker-positive compartments also increases gradually over time in response to D-Propranolol and reaches a plateau (Fig. 11B & Fig. 11C). These results reflect the recruitment and accumulation of Gal-8-ZsGreen to endolysosomal compartments in response to D-Propranolol, indicating that this compound could be inducing lysosomal membrane permeabilization.

A consequence of Gal-8 recruitment to the lysosome is the subsequent displacement of mTORC1 from the lysosome, resulting in its inhibition. mTOR is a serine/threonine protein kinase that corresponds to the catalytic subunit of two different complexes, mTORC1 and mTORC2. mTORC1 is composed of mTOR, Raptor (regulatory protein associated with mTOR) and mLST8 (mammalian lethal with Sec13 protein 8) (D. H. Kim et al., 2002) (Hara et al., 2002). Active mTORC1 is recruited to lysosomes by the family of heterodimeric Rag GTPases (Rag A/B, Rag C/D) which are in their active state in the presence of amino acids (Bar-Peled, Schweitzer, Zoncu, & Sabatini, 2012). Specifically, it has been described that in presence of guanine, the lysosomal membrane amino-acid transporter SLC38A9 activates Ragulator, a guanine exchanging factor (GEF) that activates Rag A/B by GTP loading (Saxton & Sabatini, 2017) (Bar-Peled et al., 2012). On the lysosome, mTORC1 integrates signals coming from nutrient availability and activate different downstream effectors, such as p70S6 Kinase 1 (S6K1) which in turn promote protein synthesis by activating various substrates that trigger mRNA translation initiation (Holz, Ballif, Gygi, & Blenis, 2005). Indeed, mTORC1 is a relevant complex in the regulation of cell growth and proliferation by sensing different metabolic cues (J. Kim & Guan, 2019). Also, activated mTORC1 induces an inhibitory phosphorylation of ULK1 (S757), an essential kinase involved in the assembly of the initiation autophagy complex that promotes autophagosome

formation, and thus mTORC1 acts as an autophagy repressor (Kim, Kundu, Viollet, & Guan, 2011).

As previously stated, Gal-8 is in dynamic complexes with mTORC1 (Jia et al., 2018). In resting state, Gal-8 interacts with mTORC1 and Raptor (Jia et al., 2018). However, under lysosomal damage, Gal-8 increases its interaction with SLC38A9 and GTP loaded Rag A/B, as its association with mTORC1 decreases, which leads to the translocation of active mTORC1 from the lysosome to the cytoplasm, where it becomes inactive (Jia et al., 2018). This Gal-8 containing complex has been depicted as the GALTOR system, and its formation leads to the activation of autophagy by mTORC1 inhibition to favor the degradation of damaged lysosomes (Jia et al., 2018). Considering the impact of mTORC1 activity on the regulation of cell proliferation, the evaluation of the activity of the GALTOR system is an interesting target to determine its implication in response to D-Propranolol in the context of our obtained results of decreased viability and proliferation in T98G cells.

mTORC1 activity can also be modulated by phosphatidic acid (PA), which has been previously described as an activator of mTORC1 (Fang et al., 2001). As mentioned in this manuscript, D-Propranolol induces EGFR endocytosis by the PA/PDE4/PKA pathway (Norambuena et al., 2010) (Metz et al., 2021). As an inhibitor of PAP enzymes, D-Propranolol treatment leads to an increase in PA levels followed by PA-mediated activation of PDE4, which decreases the levels of cAMP and PKA activity (Norambuena et al., 2010). Inhibition of this pathway with FIPI or Rolipram counteracts the effect of propranolol on EGFR endocytosis (Norambuena et al., 2010) (Metz et al., 2021). Interestingly, PA has been shown to enhance the activity of mTOR by direct interaction with its FRB domain (Fang, Vilella-Bach, Bachmann, Flanigan, & Chen, 2001). Elevating the levels of PA in the cell leads to increased S6K1 phosphorylation, one of the downstream kinases of the mTOR signaling pathway (Fang et al., 2001). PA is not only relevant for direct activation of mTOR, as it has been recently found that PA drives the translocation of mTORC1 to the lysosomes where it interacts with active Rag A/B

GTPase and promoting both locking and activation of mTORC1 in the lysosome (Frias et al., 2020). It is noteworthy that one of the main acidic phospholipids of the lysosomal membrane is PA, which has been involved in LMP (Zhao et al., 2012). In initial phases of LMP, caspase-8 cleaves Bid, a pro-apoptotic member of the Bcl-2 family that induces the permeabilization of the outer mitochondrial membrane. Then, PA mediates the recruitment of tBid to the lysosomal membrane where it homo-oligomerizes and induces the formation of lipidic pores (Zhao et al., 2012). PA dependency of lysosome membrane susceptibility is an interesting topic for future exploration. Whether D-Propranolol increases the levels of PA in lysosomes is unknown.

Our Magic Red Cathepsin-B activity assays show a rapid decrease of Cathepsin-B activity with D-Propranolol treatment (Fig. 12B). The loss of Cathepsin-B function has been described as an indicator of lysosomal dysfunction, as its inhibition results in decreased lysosomal degradation of different proteins such as EGFR (Cermak et al., 2016). Gal-8 recruitment to lysosome-like compartments suggests that lysosome membrane permeabilization is the most likely possibility to explain the D-Propranolol effect on Cathepsin-B activity decline. It is also noteworthy that the most pronounced decrease on the activity occurs in the first 10 minutes post-treatment, which is congruent with temporality of Gal-8-ZsGreen accumulation and recruitment in response to D-Propranolol.

In GB, the stability of the lysosomal membrane has been suggested as a relevant target for eradicating tumor cells (Le Joncour et al., 2019). Mammary-derived growth inhibitor (MDGI) has been reported as a biomarker for invasive GB tumors with expression levels correlated with the histological grade of the tumor (Hyvonen et al., 2014). Silencing of MDGI results in altered lipid composition of the lysosome, triggered lysosomal membrane permeabilization (LMP) and reduced GB cells viability (Le Joncour et al., 2019). This suggest that MDGI provides lysosomal membrane stability by regulating the trafficking of polyunsaturated fatty acids to the lysosome (Le Joncour et al., 2019). Other

studies focused on pancreatic ductal adenocarcinoma (PDA) pointed to Myoferlin, a member of the Ferlin family of membrane repair factors (Gupta et al., 2021). Myoferlin silencing in these highly malignant cells results in lysosomal dysfunction and impaired *in vivo* xenografts tumor growth associated with lysosome membrane permeabilization (Gupta et al., 2021). PDA cells redirect Myoferlin from the plasma membrane to the lysosomal membrane, thus revealing a novel mechanism of membrane stabilization (Gupta et al., 2021).

Lysosome-membrane permeabilization is hazardous to cells not only due to lysosome loss of function but also because of the consequential release of protons, reactive oxygen species and cathepsins into the cytosol (Boya & Kroemer, 2008). Cathepsins are hydrolases that can trigger Lysosomal cell death (LCD) and can also activate the NLRP3 inflammasome leading to an inflammatory response and pyroptosis (Boya & Kroemer, 2008). Several compounds have been reported to induce LMP, such as Siramesine (Parry et al., 2008). The lysosomotropic drug Siramesine triggers cell death in tumor cells due to the release of lysosomal cathepsins and induction of oxidative stress (Ostenfeld et al., 2005) (Ostenfeld et al., 2008). Indeed, alterations of lysosomal function can very likely contribute to the effect of D-Propranolol on reduced T98G viable cell number, and further experiments to demonstrate cathepsin leakage and the activation of pro-apoptotic targets in response to this drug are required to confirm lysosomal-dependent cell death.

The precise mechanism of how D-Propranolol affects endolysosomal compartments remains unknown. A common feature of lysosomotropic drugs is the induction of lysosome membrane permeabilization in addition to besides their sequestration within the lysosome. These agents promote the fluidization of lysosomal membrane with consequences in the passive diffusion of lipid-soluble molecules. Recent studies using fluorescent dyes to evaluate the effect of weak basic lysosomotropic drugs on lysosomal membrane and intraluminal space volume with confocal microscopy, revealed increased size of the lysosomal membrane, suggesting a direct intercalation of these lysosomotropic drugs into

the lysosomal membrane (Stark et al., 2020). As a weak basic amine, D-Propranolol is likely to induce a similar effect on the permeabilization of the lysosomal membrane.

5.3. Contribution of Gal-8 expression to T98G viable cell number

During our experiments with T98G-Gal-8-ZsGreen cells we noticed a higher cell grow rate compared to WT cells. In comparison, the total viable cell count of these cells increased by 50% (Fig. 14B), indicating that Gal-8 overexpression can stimulate proliferation of T98G human GB cells. On contrast, the knock-down of Gal-8 in T98G cells decreased the total viable cell number by roughly 50%, further suggesting the impact of Gal-8 on cell growth (Fig. 14B). The expression of Gal-8 has been reported in several carcinomas, as it is one the most widely expressed galectins in cancer (Elola et al., 2014). An increased Gal-8 expression has been found in breast cancer relative to healthy tissue (Danguy et al., 2001). Previous studies have described the expression of Galectins 1, -2, -4, -7, -8 and -9 in the human brain (Saal et al., 2005), with Gal-3 and Gal-8 being the most abundantly expressed in human GB cell lines (Lahm et al., 2001). Whether Gal-8 levels vary according with GBM pathogenicity is unknown. However, immunohistochemical evaluation of nude mice brain xenografted with the human GB cell line U373 demonstrated higher Gal-8 expression in the cells residing in the invasive region the tumor, suggesting a role of Gal-8 in invasion (Camby et al., 2001).

Furthermore, in the non-tumoral MDCK cells, overexpression of Gal-8 has been shown to stimulate cell proliferation and migration involving the activation of integrin-mediated signaling towards FAK and transactivation of the EGFR, due to the secretion of Gal-8 to the extracellular media. MDCK cells overexpressing Gal-8 also undergo epithelial-mesenchymal transition and become tumorigenic when tested as xenografts in immunodeficient mice (Oyanadel et al., 2018). Our immunoblot experiments showed no significant differences in the phosphorylation of EGFR (Tyr1068) and ERK in basal conditions in our T98G-Gal-8-ZsGreen cells compared to WT cells. However, we observed that the signaling response of T98G-Gal-8-ZsGreen cells to the addition of EGF was exacerbated compared to

WT cells, in both EGFR (Tyr1068) and ERK (Fig. 15B & Fig. 15D). Considering that the total mass of EGFR was similar in both cell lines, the possibility of increased EGFR availability in the cell surface by Gal-8 mediated EGFR endocytic traffic regulation is an interesting theory. Indeed, previous studies have shown that Gal-8 is relevant for maintaining an optimal recycling of EGFR by targeting non-functional early endosomes for degradation by autophagy and therefore maintaining a pool of viable endosomes (Fraser et al., 2019). In fact, the knock-out of Gal-8 results in decreased EGF-induced phosphorylation of EGFR and ERK, highlighting the relevance of Gal-8 in EGFR trafficking (Fraser et al., 2019). If Gal-8 expression is involved in the regulation of EGFR trafficking in T98G cells and the implication of this modulation on cell proliferation are still to be determined (Fig. 17A).

As previously mentioned on this manuscript, the intracellular role of Gal-8 on the removal of damaged lysosomes and its impact on the viability of GB cells has not been previously studied. Considering our results of D-Propranolol on lysosomal function, we wondered if Gal-8 expression could have an impact in the viable cell number in D-Propranolol treatments, and for this end we used our different T98G cell lines. Interestingly, we did not find any significant differences in the normalized percentage of viable cell number (Fig. 14C). However, we did observe a tendency of decreased viable cell number in T98G-sh-Gal-8 cells compared to the other cell lines in D-Propranolol treatments (Fig. 14C). An increased repetition of this experiment (N) could serve to confirm the significancy of this tendency. No evident difference in the viable cell number was appreciated among T98G-WT and T98G-Gal-8-ZsGreen cells in D-Propranolol treatments (Fig. 14C). Altogether, these results indicate that Gal-8 has no impact in determining viable cell number in response to D-Propranolol.

5.4. Gal-8 overexpression promotes T98G cells invasive migration

Considering the effect of Gal-8 on the cell growth of T98G cells, we decided to explore the impact of the expression of this protein in cell invasion, a relevant malignancy feature in GB. We found that T98G-Gal-8-ZsGreen cells display a

notorious higher invasion index compared to both T98G-WT and T98G-shGal-8 cells (Fig. 16B). In this context, our laboratory has previously reported that exogenously added Gal-8 to the media increases the migration of U87 GB cells in Transwell assays (Metz et al., 2016). In concordance, Gal-8 has a relevant role in promoting cell adhesion on different types of cancer and non-cancer cells, which requires sugar interaction of extracellular Gal-8 with integrins, triggering signaling cascades implied in cell migration (Y. Levy et al., 2001). Having this in consideration, it is likely that higher levels of Gal-8 secreted from T98G Gal-8 overexpressing cells stimulates their invasive migration, however this still needs to be validated.

An interesting observation is the distinctive impact of D-Propranolol in the invasion of T98G-Gal-8-ZsGreen cells compared to WT cells. T98G-WT cells are practically not affected, while the increased invasive migration observed by T98G-Gal-8-ZsGreen cells can be reduced to the levels of WT cells under D-Propranolol treatment. In this regard, previous studies have shown that tumors from U373 human GB cells xenografted to the brain of nude mice display differential Gal-8 expression in specific regions of the tumor (Camby et al., 2001). It was determined by immunohistochemical analysis that in the invasive area of the tumor, U373 cells have increased Gal-8 levels compared to the cells of the central region of the tumor (Camby et al., 2001). Therefore, our Gal-8 overexpressing T98G cells could be resembling the expression profile of invasive cells of a GB tumor. This suggests a Gal-8 reliance of GB cells to acquire a more invasive phenotype, and therefore, alterations on the distribution and function of this galectin could have a more significant impact on Gal-8 overexpressing cells that on WT cells. As previously exposed in this manuscript, D-Propranolol treatment triggers the accumulation of Gal-8 to damaged lysosome-like compartments, and it is then reasonable to propose that the additional Gal-8 protein level in T98G-Gal-8-ZsGreen cells is destined to this intracellular response when treated with D-Propranolol and result in decreased release to the extracellular media (Fig. 17B). Further experiments are needed to be support this hypothesis and evaluate the impact in cell invasion.

Our laboratory has described that extracellular Gal-8 promotes indirect integrin-mediated transactivation of EGFR by FAK (Oyanadel et al., 2018). EGFR plays a crucial role in cell migration, as treatments with EGF induce tyrosine dephosphorylation of FAK in different cancer lines (Lu, Jiang, Blume-Jensen, & Hunter, 2001). This dephosphorylation induces FAK downregulation, inducing refractile morphological changes that lead to detachment to the ECM and increased cell migration (Lu et al., 2001). Moreover, recent studies have demonstrated that EGFR function determines the migration of T98G GB cells, which displayed increased migration in response to EGF, but this effect was abolished with the use of the tyrosine kinase inhibitor Erlotinib (Pudelek et al., 2020). Based in the increased signaling response with EGF in T98G-Gal-8-ZsGreen cells, it is plausible that Gal-8 could be modulating EGFR recycling and its availability at the cell membrane, as this function of Gal-8 has been previously described (Fraser et al., 2019) and discussed in this manuscript. Indeed, enhanced EGFR and integrin recycling could account for the basal increased migration and invasion of T98G-Gal-8-ZsGreen cells, and the perturbation of this recycling by D-Propranolol can justify the notorious decrease in the invasion index of these Gal-8 overexpressing cells (Fig. 17A).

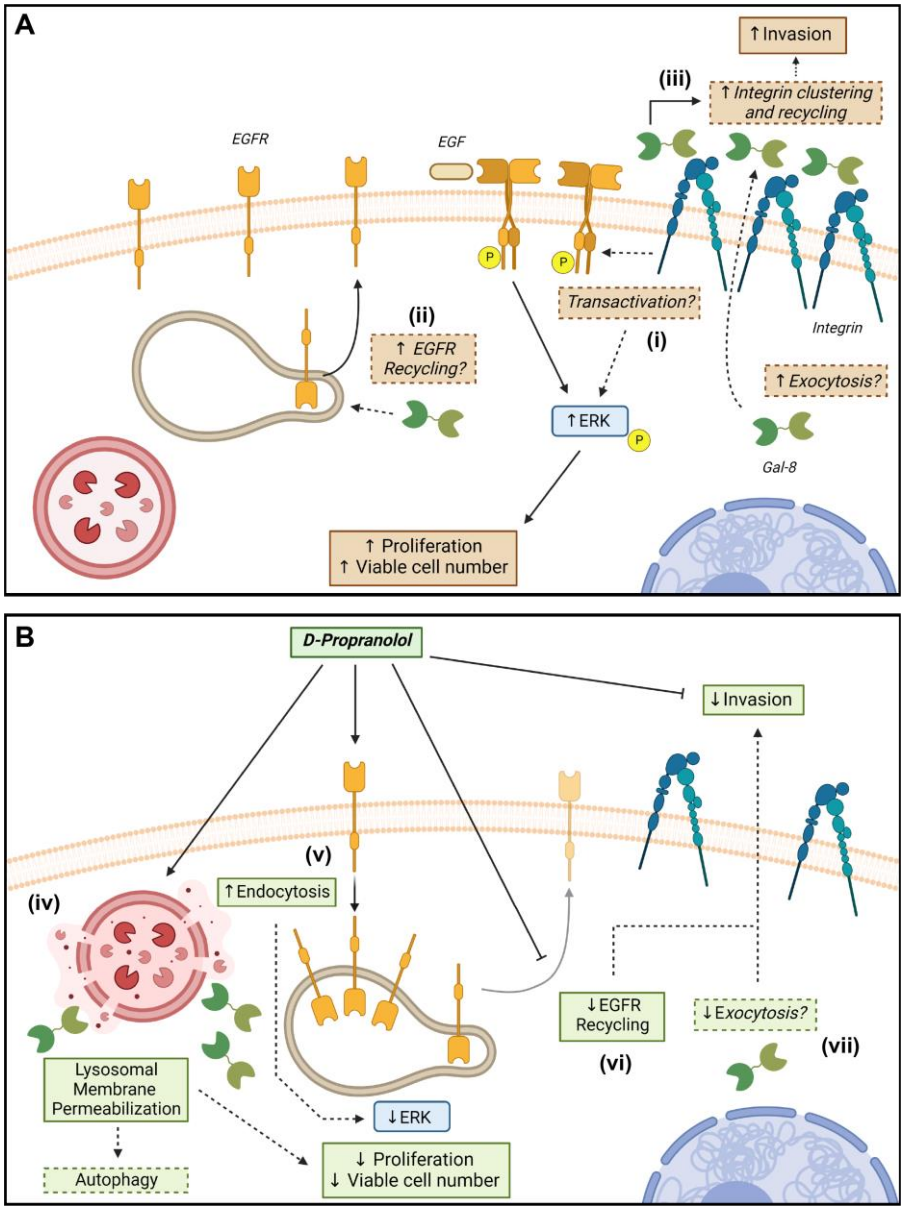


Figure 17. D-Propranolol interferes with Gal-8 and EGFR functions in T98G GB cells.

(A) Gal-8 increases T98G cells proliferation by the following proposed mechanisms: (i) integrin activation by extracellular Gal-8 and consequent EGFR transactivation (ii) increased EGFR recycling towards the plasma membrane, enhancing the sensing of mitogenic factors and downstream signaling (ERK). Enhanced cell invasion could be explained by increased Gal-8 exocytosis triggering integrin clustering and recycling (iii). (B) D-Propranolol decreases viable cell number by (iv) lysosomal membrane permeabilization (v) reduced cell surface EGFR by endocytosis and recycling perturbation, leading to decreased EGFR activation, and downstream signaling. Diminished cell invasion by D-Propranolol could be due to decreased EGFR migration related signaling (vi) and lower Gal-8 levels in the extracellular media as a consequence from removal of damaged lysosomes (vii).

6. CONCLUSIONS

- D-Propranolol decreases viability and proliferation of T98G cells.
- D-Propranolol affects T98G viability by a mechanism involving EGFR internalization and the PA/PDE4/PKA pathway.
- D-Propranolol triggers dysfunction of lysosomes in T98G cells, accompanied by lysosome membrane permeabilization and the recruitment of Gal-8 y Gal-3.
- An increase in Gal-8 expression enhances T98G cell growth and invasion.

7. PROYECTIONS

- Evaluate the secretion of Gal-8 in T98G-Gal-8-ZsGreen cells in comparison to WT cells, in presence and absence of D-Propranolol.
- Determine the impact of Gal-8 expression on EGFR and integrin recycling.
- Evaluate Gal-8 mediated EGFR transactivation by integrin.
- Evaluate cathepsin leakage to the cytosol and lysosomal cell death by D-Propranolol.

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