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Early Pharmacological Profiling of $ATB^{0,+}$ Inhibitors

Caracterización farmacológica temprana de inhibidores de $ATB^{0,+}$

Trabajo de Fin de Grado

Modalidad: Proyecto de investigación

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La presente propuesta de investigación ha sido redactada por la alumna Ithaisa Medina Alonso durante el curso académico 2019-2020 y bajo la dirección del Dr. José M. Padrón Carrillo. El trabajo forma parte de la línea de investigación *Diseño, descubrimiento y evaluación de fármacos anticancerígenos.*

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Fdo. José M. Padrón

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ABSTRACT

Cancer cells experiment an overexpression of amino acid transporters to support the increasing demand of amino acids. $ATB^{0,+}$ transporter up-regulation has been identified in multiple tumors. This transporter constitutes a possible selective therapeutic target to treat cancer. Two families of molecules, naphtol-derived Betti bases and glutamic acid based dipeptides seems to behave as $ATB^{0,+}$ inhibitors. This project aims to explore the interaction between the $ATB^{0,+}$ transporter and the mentioned compounds. To do this we will evaluate their interaction with $ATB^{0,+}$ through computational approaches. We will also realize a colony formation analysis and a cell cycle analysis. Functional expression studies will be carried out in mammalian cells and electrophysiological tests will be realized on *Xenopus laevis*. Finally, autophagy and expression levels of the CHOP and ASNS genes will be analyzed as a read out of amino acid starvation.

Keywords: $ATB^{0,+}$, SCL6A14, amino acid transporter, antitumor activity, glutamic acid based dipeptides, Betti bases

RESUMEN

Las células cancerígenas experimentan un aumento de expresión de los transportadores de aminoácidos para abastecer el incremento en la demanda de los mismos. Se ha identificado una sobreexpresión del transportador $ATB^{0,+}$ en múltiples tipos tumorales. Este transportador de aminoácidos constituye una posible diana terapéutica selectiva para tratar el cáncer. Dos grupos de moléculas, las Betti bases derivadas de naftol y los dipéptidos a base de ácido glutámico, podrían comportarse como inhibidores de $ATB^{0,+}$. Este proyecto pretende explorar el tipo de interacción existente entre dichos compuestos y el transportador $ATB^{0,+}$. Para ello se evaluará la interacción de ambos grupos de moléculas con $ATB^{0,+}$ mediante aproximaciones computacionales. Se realizarán también un análisis de formación de colonias y un análisis del ciclo celular. Se llevarán a cabo estudios de expresión funcional en células de mamífero y ensayos electrofisiológicos en *Xenopus laevis*. Finalmente, se analizarán la autofagia y los niveles de expresión de los genes CHOP y ASNS como indicadores de la inanición de aminoácidos.

INTRODUCTION

It is well known that metabolic activities are altered in cancer cells relative to normal cells (Deberardinis & Chandel, 2016). Most of the reprogrammed activities allow cells to grow and proliferate at pathologically elevated levels. Among other needs, the demand of amino acids boosts enormously in cancer. Amino acids play an essential role in cellular function and survival. Not only they are obligatory building blocks for proteins synthesis, but also precursors for secondary metabolites and bioactive molecules. Even those amino acids that can be synthetized endogenously become essential for cancer cells, because the amino acid requirement exceeds the synthesis capacity. Such phenomenon becomes particularly evident for glutamine, so that in the absence of an external influx of this amino acid, cancer cells cannot proliferate. This unique feature of tumor cells has been named as "glutamine addiction" (Wise & Thompson, 2011).

Since amino acids cannot pass through cell membranes on their own, mammalian cells express multitude of amino acid transporters in their plasma membranes. Among the known amino acid transporters (approximately 430), $ATB^{0,+}$ is the only one with a very broad substrate specificity. This transporter is classified into the gene superfamily solute carrier (SLC) and it is also referred to as SCL6A14 (Solute Carrier Family 6 Member 14). $ATB^{0,+}$ transports 18 of the 20 proteinogenic amino acids, recognizing neutral as well as cationic ones and thus, comprise all essential amino acids (Sikder et al., 2017). Previous studies reported that $ATB^{0,+}$ can also transport various D-amino acids (Hatanaka et al., 2002). The carrier uses the Na^+ and Cl^- gradient, and membrane potential to internalize the amino acids into cells (Sikder et al., 2017).

In order to support the increasing demand of amino acids, cancer cells experiment an overexpression of amino acid transporters. Tumors must up-regulate those amino acid transporters to attend their metabolic demands. $ATB^{0,+}$ seems to be the most relevant transporter to promote tumor growth due to its broad substrate specificity, a high concentrative capacity from three different energy sources (gradient of Na^+ and Cl^-, and membrane potential), and coupling to mTOR signaling (Bhutia et al., 2014). In fact, $ATB^{0,+}$ up-regulation has been identified in multiple tumors. It was first demonstrated in colorectal cancer (Gupta et al., 2005), and later it was corroborated also for cervical cancer (Gupta et al., 2006), ER-positive breast cancer (Karunakaran et al., 2011) and pancreatic cancer (Coothankandaswamy et al., 2016). In contrast, healthy cells express this transporter at low

levels. These conditions make $ATB^{0,+}$ transporter a potential selective target for cancer treatment as a way to induce amino acid starvation without causing remarkably effects in normal cells.

Hypothetically, in tumor cells that primarily rely on $ATB^{0,+}$ for their amino acid supply, blocking its function either with small molecules or monoclonal antibodies would be able to stop cancer growth (Fernandes & Padrón, 2019). The inhibition of this transporter would decrease amino acid, lipid, and nucleotide synthesis; restrain mTOR signaling by blocking angiogenesis; and suppress the action of other transporters such as SLC7A5 (LAT 1) and SLC7A11 (Bhutia et al., 2014). These effects are shown schematically in Figure 1.

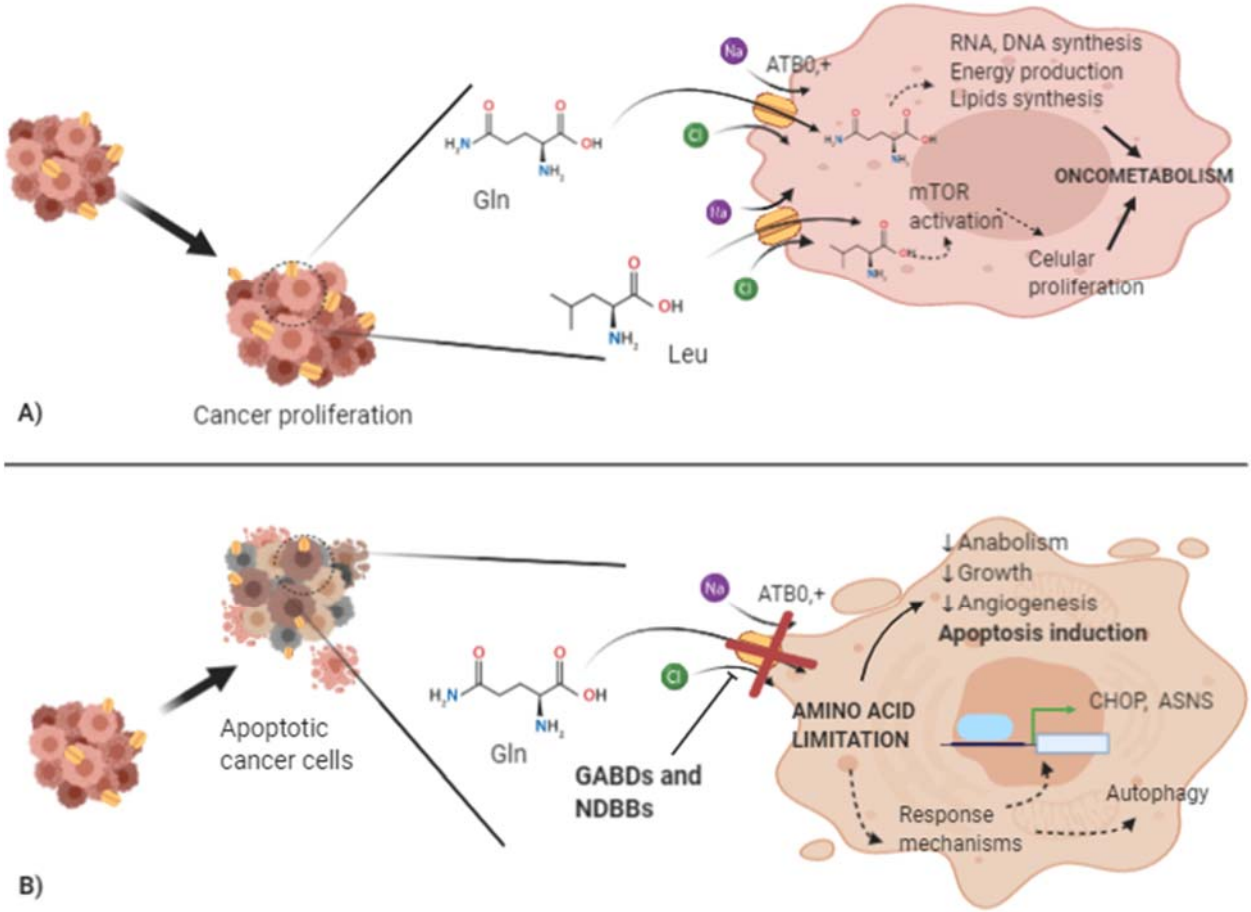

Figure 1. Potential consequences of $ATB^{0,+}$ blockade in tumor cells. **A)** Current tumor progression without $ATB^{0,+}$ inhibitors. **B)** Expected tumor growth inhibition in the presence of GABDs and NDBBs

At present, a single compound has been identified as selective blocker of the $ATB^{0,+}$ amino acid transporter: α-methyl-DL-tryptophan (α-MT). α-MT arrest tumor growth in a

$ATB^{0,+}$ dependent manner. In vitro experiments showed that this inhibitor produced amino acid starvation specifically in $ATB^{0,+}$ overexpressing cancer cells, inducing autophagy and apoptosis without having detrimental effects in normal cells (Karunakaran et al., 2008).

Recently, two families of compounds were reported as potential $ATB^{0,+}$ inhibitors ($iATB^{0,+}$). The compounds showed *in vitro* anti-proliferative activity against different cancer cell lines. On the one hand, glutamic acid-based dipeptides (GABDs) were able to inhibit all cancer solid tumors tested although tryptophan dipeptides showed no detectable activity against HBL-100 cells (Silveira-Dorta et al., 2015). The observed difference was attributed to $ATB^{0,+}$ transporter, since the expression of $ATB^{0,+}$ in HBL-100 cells is undetectable while the rest of cell lines tested overexpress the amino acid transporter. On the other hand, the anti-proliferative activity of naphthol-derived Betti bases (NDBBs) was studied *in vitro* with positive results against several cancer cell lines. Moreover, the possible mode of interaction of the NDBBs with $ATB^{0,+}$ was explored computationally through docking methods. Obtained docking scores were comparable to those of α-MT (Puerta et al., 2019). These results might support the hypothesis that both GABDs and NDBBs behave as tryptophan mimetics and block the transporter $ATB^{0,+}$. The most remarkable anti-proliferative results previously obtained of the mentioned compounds are shown in Table 1.

Table 1. Anti-proliferative activity (GI50 in μM) of selected NDBBs and GABDs against breast cancer cells

Compound		HBL-100 (TNBC)	MDA-MB-453 (HER2+)	MCF-7 (ER+)	T-47D (ER+)
GABDs	9g	>100	-	-	33±2.5
	10g	>100	-	-	36±7.8
NDBBs	14c	>100	94±11	63±11	13±3.1
	14d	>100	42±7.0	53±12	55±9.1
	14n	82±25	96±9.9	>100	13±3.3
	14o	76±27	59±9.1	56±10	8.5±0.7

Data obtained from Silveira-Dorta et al., 2015 and Puerta et al., 2019 studies. TBNC = Triple Negative Breast Cancer; HER2 = Human Epidermal growth factor Receptor 2; ER = Estrogen Receptor.

Strategies to limit nutrients required for tumor growth using nutrient degrading enzymes have previously been suggested for cancer therapy. The best clinical examples of

exploiting cancer nutrient dependencies have been reported for blood cancers with successful results (Garcia-Bermudez et al., 2020). To the best of our knowledge, $ATB^{0,+}$ represents the first nutrient amino acid transporter under investigation to be exploited as a pharmacological target for cancer treatment, and may usher in a new era of cancer medicine. Both, GABDs and NDBBs show potential to provide us chemical entities which could lead to the discovery of new chemotherapeutic agents. It seems reasonable to consider that researching those groups of compounds might serve as a starting point to develop $iATB^{0,+}$.

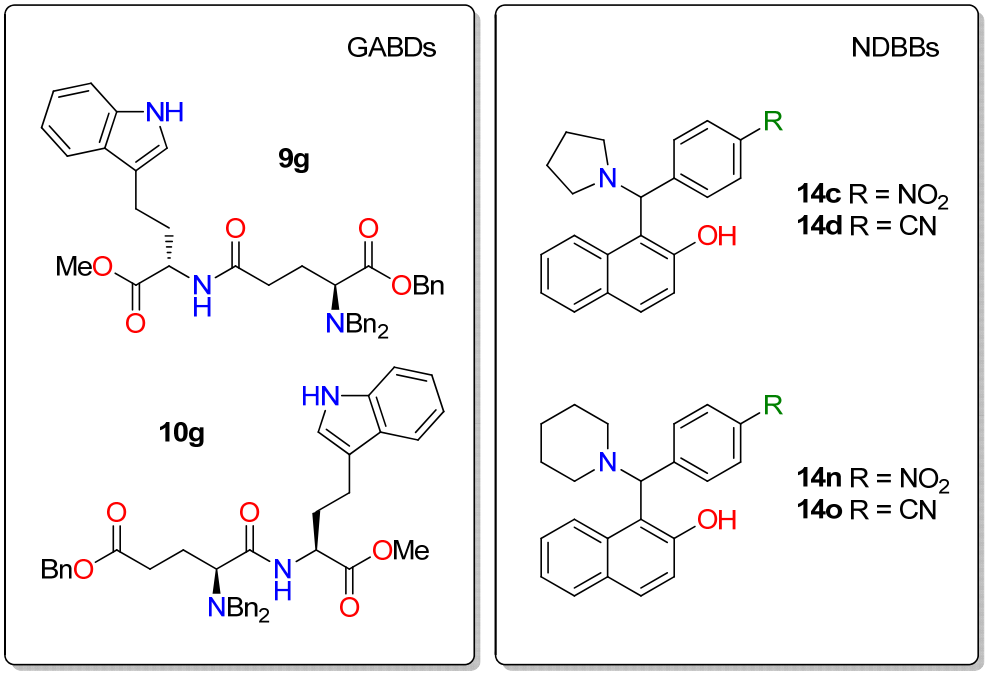

Figure 2. Chemical structure of potential $iATB^{0,+}$. Left: GABDs. Right: NDBBs.

HYPOTHESIS

The results of previous studies lead us to consider glutamic acid-based dipetides and naphthol derived Betti bases as possible new sources of pharmacological entities for the treatment of solid tumors. We speculate that their mechanism of action consist in the inhibition of cell growth through disruption of the cellular uptake of amino acids behaving as tryptophan mimetics, thus blocking the $ATB^{0,+}$ transporter.

OBJECTIVES

Our main objective is to study the interaction of the amino acid transporter $ATB^{0,+}$ with compounds that showed antiproliferative activity in cells. In particular, we will focus on GABDs and NDBBs.

The following specific objectives are proposed:

1. To explore computationally the interaction of both subsets of compounds with the amino acid transporter $ATB^{0,+}$.
    1.1. To develop a structural model of the transporter.
    1.2. To realize interaction predictions between the proposed compounds and the transporter through docking methods.

2. To carry out cell-based experiments to evaluate the capacity to interact with $ATB^{0,+}$.
    2.1. To monitor compounds' capacity to inhibit cell uptake of amino acids.
    2.2. To evaluate the ability of GABDs and NDBBs to produce amino acid starvation in cells.
    2.3. To elucidate the type of interaction, if any, that takes place between the compounds and the transporter through interpretation of the bioassays results.

MATERIALS AND METHODS

Chemical entities

Among the battery of pre-tested compounds, we have selected those that are most likely to be iATB$^{0,+}$ (Figure 2). Referring to Betti bases, those are the pyrrolidine derivatives **14d-c** and, to a lesser extent the piperidine siblings **14n-o** (Puerta et al., 2019). On the subject of glutamic acid based dipeptides, tryptophan dipeptides **9g** and **10g** seems to be the best candidates (Silveira-Dorta et al., 2015). Commercial chemicals will be used without further purification.

Cell lines and culture

We will select human cell lines that show different expression levels of ATB$^{0,+}$: ·Human retinal pigment epithelial cells (HRPE), human fibroblast cells (BJ-hTERT) (ATB$^{0,+}$ basal expression) and the human cancer cell lines HCT116 (colon cancer with ATB$^{0,+}$ basal expression), MCF-7 (breast cancer, ATB$^{0,+}$ positive), HBL-100 (breast cancer, ATB$^{0,+}$ negative), LS174T (colon cancer, ATB$^{0,+}$ positive), BxPC3 (pancreatic cancer, ATB$^{0,+}$ positive), and AsPC-1 (pancreatic cancer, ATB$^{0,+}$ positive.

Cells will be grown in RPMI-1640 medium containing 5% fetal bovine serum, 1 mM glutamine and antibiotics (100 U/mL of penicillin and 0.1 mg/mL of streptomycin). In addition, Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum will be used in the colony formation assay. Cells will be preserved in a 37ºC, at a 95% humidified atmosphere in 60 mm cell culture dishes in growth medium and maintained at low passages.

Computational studies

a) Homology modeling of ATB$^{0,+}$

The 3D structure of the ATB$^{0,+}$ transporter is not available in Protein Data Bank. Therefore, we will carry out a homology modeling procedure as described recently (Palazzolo et al., 2019). The protein sequence will be retrieved from Uniprot database and

used to blast into Protein Data Bank. We will employ the Clustal Omega Software for multiple alignments.

The TM prediction program TM TMHMM will be draw on to determine the topology of $ATB^{0,+}$ as well as PROTTER will be used to build a secondary structure prediction. Selected 3D structures will be optimized and refined through the MOE Structure Preparation Module of the Molecular Operating Environment 2018. Required modifications at crystal structure will be done using the MOE Homology Model tool.

b) Equilibration and Cluster Extrapolation

Molecular dynamics simulations and frame clustering procedures will be carried out with the Schrödinger Small Molecule Drug Discovery Suite. The Desmond System Builder Tool will be utilized to place de *apo*-model into a membrane bilayer. Protein orientation will be set up according to the OPM server, which provides spatial arrangements of membrane proteins. The system will be submitted to a MD simulation using Desmond Molecular Dynamics tool. Periodic boundary conditions (PBCs) will be set. The Desmond Trajectory Frame Clustering Tool will be used to select the most representative frame. The OPLS (Optimized Potentials for Liquid Simulations) force field will be applied in the MD simulation and in the frame clusterization procedures.

c) Molecular docking

We will explore GABDs and NDBBs interaction in Glide v8.2 docking software. Open Babel application will be used to convert the files into the adequate format. Tested ligands will be obtained using the HyperChem software. We will apply ¨Standard precision¨ protocol in Glide with OPLS force field and post-docking minimization

Biological evaluation

Colony formation assay

The colony formation assay will be performed as another index of the anti-proliferative activity of the compounds as an adaptation of the method described previously (Karunakaran et al., 2008; Rafehi et al., 2011).

Single cell suspensions will be prepared by trypsinization of cultured flasks containing cells. We will detach cells by pipetting up and down carefully against the wall. The cell lines will be seeded onto 6 well plates in DMEM (~10,000 cells per well). Cells

will be counted automatically employing the cytometer Moxi Z Counter. On the next day, cells will be exposed to GADBs and NDBBs for 2 weeks at diverse doses. Medium is removed carefully from each plate and cells will be washed with PBS and fixed in 100% (v/v) methanol for 30 min, followed by staining with crystal violet in distilled water (dH_2O) for 30-60 minutes. The dye excess will be washed with dH_2O and wells will be dried. Digital images of the colonies will be obtained by a camera and the colony counting will be carried out using ImageJ.

Cell cycle analysis

Since GABDs and NDBBs have demonstrated to suppress cell growth (Puerta et al., 2019; Silveira-Dorta et al., 2015), we will investigate the influence of those treatments on cell cycle to determine which stage is affected. For this purpose we will follow the methods described in the literature (Karunakaran et al., 2008).

Before running cell cycle analysis, we will harvest the cells carefully extracting the medium and cells following the corresponding trypsinization. After washing in PBS we will fix for 2 h with 70% ethanol at 4ºC. We will centrifuge ethanol suspended cells for 5 minutes and decant ethanol. Then we must wash twice in PBS and introduce samples into a centrifuge being careful to avoid cell loss when discarding the supernatant.

Cells will be cultured in 6-well plates in RPMI-1640 medium culture medium in the presence or absence of the compounds. Cells will be treated with 0.1% sodium citrate, 1 mg/mL RNase A and 50 µg/mL propidium iodide, keeping in the dark at room temperature for 30 min. We will submit the samples to FACS analysis and measure the forward scatter (FS) and side scatter (SS) to identify single cells with a cytometer. Emission will be measured using the long-pass 600 or 610 nm filter. We will finally classify the cells for their cycle stage. Protocol adapted from (Pozarowski & Darzynkiewicz, 2004).

Molecular cloning of $ATB^{0,+}$ cDNA

$ATB^{0,+}$ will be cloned using standard techniques as previously described for its use in function expression system assays (Nakanishi et al., 2001). A cDNA library will be constructed using poly (A) + RNA isolated from mammary tumor cell line (MCF-7). The SuperScript plasmid system will be employed for this purpose. The cDNA probe for screening the library will be prepared by RT-PCR using specific primers for the human

$ATB^{0,+}$ cDNA reported in GenBank (access nº. AF151978). The RT-PCR product will be sequenced for confirmation of its identity. Screening will be done under high stringency conditions. The longest positive clone will be used for functional analysis.

Inhibition of amino acid uptake

a) Functional expression of $ATB^{0,+}$ in mammalian cells

The ability of the different products to inhibit $ATB^{0,+}$ mediated amino acid transport will be assessed in mammalian cells. For this purpose, we will apply the vaccinia virus expression technique as reported before (Srinivas et al., 2007). This procedure involves infection of the cells with a recombinant vaccinia virus carrying the gene for T7 RNA polymerase, and a lipofectin-mediated transfection of the cells with plasmid DNA. Radiolabelled glycine (10 µM total glycine: 0.05 µM radiolabelled glycine; 9.95 µM unlabelled glycine) will be used as the substrate for $ATB^{0,+}$ in cDNA-transfected cells. The transport buffer will be 25 mM Hepes/Tris (pH 7.5) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose.

Transport will be measured during 30 minutes at 37 ºC. Then, the transport buffer will be aspirated followed by 3 washes with 2mL of ice-cold transport buffer. Cells will be lysed with SDS in NaOH and we will determine radioactivity.

Statistical analysis will be done by Student's t-test and $P < 0.05$ will be considered significant. The IC_{50} (concentration of inhibitor needed to cause 50% inhibition) values will be calculated by fitting the resulting data to the equation:

$$y = \frac{y' + a \times b}{b + x}$$

Where y is the uptake in presence of the inhibitor, $y'$is uptake in absence of a maximal concentration of the inhibitor, a is uptake in absence of the inhibitor, b is concentration of the inhibitor needed for 50% inhibition, and x is concentration of the inhibitor.

b) Xenopus laevis oocytes expression system

$ATB^{0,+}$ mediated transport of amino acids and its blockade by NDBBs and GABDs will be studied using the *Xenopus laevis* heterologous expression system (Nakanishi et al.,

2001). Capped cRNA from the cloned human $ATB^{0,+}$ cDNA will be synthesized using the mMESSAGE mMACHINE kit. Mature oocytes from *X. laevis* will be isolated by treatment with collagenase A (1.6 mg/mL), manually defolliculated, and maintained at 18°C in modified Barth's medium supplemented with 10 mg/mL gentamycin as described before (Hatanaka et al., 2002; Srinivas et al., 2007). On the following day, oocytes will be injected with 50 ng of $ATB^{0,+}$ cRNA. Electrophysiological studies will be performed by the two-microelectrode voltage-clamp method.

Oocytes are perfused with a NaCl-containing buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 3 mM Hepes, 3 mM MES, and 3 mM Tris, pH 7.5), followed by the same buffer containing different NDBBs or GABDs. The transport of glycine dependence on Na^+ and Cl^- will be evaluated by comparing the induced currents in presence and in absence of these ions.

Statistical analysis will be done by Student's *t*-test and $P < 0.05$ will be considered significant. In the analysis of the saturation the $K_{0,5}$ kinetic parameter will be calculated by fitting the values of the substrate induced currents to the Michaelis Menten equation describing a single saturable transport system.

Amino acid deprivation

a) CHOP and ASNS

The levels of C/EBP-homologous protein (CHOP) and asparagine synthetase (ASNS) will be monitored in presence of NDBBs and GABDs as described before (Karunakaran et al., 2011). ASNS and CHOP are important sensors of amino acid nutrition in mammalian cells and their expression is enhanced during amino acid deprivation (Bhutia et al., 2014; Fafournoux et al., 2000). The expression of the both genes will be analyzed using real time PCR as well as qPCR.

RNA will be isolated from cells using the Trizol method according to standard protocols (Rio et al., 2010). After the RNA is isolated, its concentration will be measured using a Nanodrop ND-1000 system, followed by cDNA synthesis using a high capacity cDNA synthesis kit. We will measure relative mRNA levels using the StepOnePlus real-time PCR system. Specific primers for ASNS and CHOP will be design trough the Primer BLAST tool from NCBI data base. Thermal cycling condition will be; pre-incubation at 94

ºC for 4 min, followed by 20 cycles of 94 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 40 s. PCR products will be run on agarose gels followed by ethidium bromide staining.

b) Analysis of autophagy

Autophagy will be analyzed as an indirect indicator of amino acid starvation. In order to survive in the absence of exogenous amino acid influx, cells engage in autophagic degradation of intracellular macromolecules and organelles as an alternative source of amino acids (Bhutia et al., 2014). The localization of LC3 will be analyzed as beforehand (Karunakaran et al., 2011; Zhang et al., 2017). Briefly, cells will be plated on a coverslip to reach 50% confluence, and transfection of these cells will be done with 1 µg of the plasmid using FuGENE transfection reagent. The cells will then be maintained in culture for 24 h to reach 80 – 90% confluence prior to treatment with NDBBs and GABDs. At the end of the treatment with the tested compounds, the culture medium will be aspired from each well and gently wash the cells twice with PBS at room temperature. Cells will be fixed with 4% paraformaldehyde for 15 minutes and we will wash 3 times with PBS at room temperature.

Then, we will incubate the cells with serum in Triton-X PBS for 1h at room temperature to block unspecific binding of antibodies. We will diluted the desired concentration of primary antibody in antibody dilution buffer and incubate the cells in primary antibody at 4ºC. The cells will be washed 3 times (10 min each one). The cells will be incubated in diluted secondary antibody in a humidify chamber and repeat washes. Then we will rinse the coverslips in water and mount on glass microscope slides. Samples must be properly stored at 4ºC in the dark until we analyze them. Finally, we will examine them by a fluorescence microscopy using the computerized software ImageJ.

IMPLEMENTATION

Computational studies

a) Homology modeling of ATB$^{0,+}$

We will obtain the sequence of human ATB$^{0,+}$ (ID= Q9UN76) from open Uniprot database. Later, we will search a protein Blast for a human ATB$^{0,+}$ homolog to choose the main template. We will produce a multiple alignment among the NSS (Neurotransmiter Sodium Simporter) family. A second local blast will be necessary to identify an adequate template to fix the EL2 gap, so we will repeat the procedures locally.

The topology of ATB$^{0,+}$ will be predicted. Selected 3D structures will first be optimized and refined by correcting crystallographic-related errors. Then, the selected portion of the second template will be placed carefully in the corresponding position. Any required modifications will be done in the crystal structures at the point of connection between the two templates, in order to have flexibility to shape their junction in the chimeric model of ATB$^{0,+}$. Intermediate models will be submitted to energy minimization to release internal constraints. Finally, the top-scoring model will be submitted to energy minimization until the root mean square gradient reached >0.5 kcal/mol.

b) Equilibration and cluster extrapolation

In this section of the computational modeling we will carry out molecular dynamic simulations and frame clustering procedures. The protein model will be placed and properly oriented into a membrane bilayer system. This system will be solvated with water molecules and we will add chloride ions, in sodium chloride form, that neutralize the exceeding positive charge until reach a 0.15 M concentration. Next, it would be energy-minimized to relax the assembly and remove collisions between the different components in their new configuration.

The system will undergo a 500 ns MD simulation in order to produce an equilibrated model. For each cluster we will select the most representative frame. Distances between clusters will be computed from the root mean square derivation (RMSD) matrix with respect to the first frame of the simulation.

c) Molecular docking

We will explore GABDs and NDBBs interaction in Glide to evaluate their ability to bind the ATB$^{0,+}$ channel. The most significant frame from the most populated cluster will

be selected as the reference structure model for molecular docking procedures. Three pivotal ions (one Cl^- and two Na^+) will be transferred to the $ATB^{0,+}$ equilibrated model. The ions-$ATB^{0,+}$ 3D structure will be energy minimized to fix structural issues in the ion binding domain.

GABDs and NDBBs will be obtained and optimized, as well as α-MT, which will be evaluated for comparison purposes. Since the NDBBs under consideration are racemic mixtures we will analyze independently both enantiomer series. The scores of docking interactions of the tested compounds will be expressed as energy measurements (kcal/mol). We will pay special attention for those ligands with best docking scores when we carry out the biological tests.

Biological evaluation

Colony formation assay

We will study the antiproliferative effects of GABDs and NDBBs using the colony formation assay. In this test, we will expose HBL-100, BJ-hTERT, MCF-7, HCT116, LS174T, BxPC3 and AsPC-1 cell lines to the compounds at two different drug concentrations, the so-called low dose and high dose. The doses will be selected according to previous results obtained in the group. Colony formation assays will be run for a period of 2 weeks, changing medium every 2 days. The quantification of colony formation assays from scanned well plate images will be expressed as percentage of control plates. So that only the colonies are detected, we will adjust threshold to reduce levels of non-specific background and we must ensure that all cell colonies have been correctly registered. We will check for differences in activity between compounds and cell lines, paying special attention to the $ATB^{0,+}$ expression profile of the cell line.

Cell cycle analysis

In order to study the effect on cell cycle, HBL-100, BJ-hTERT, MCF-7, HCT116, LS174T, BxPC3 and AsPC-1 cell lines will be submitted to a treatment of a fixed concentration of the tested compounds. The concentration will be decided according to GI_{50} values previously obtained for each compound. Treatment incubations will last for 24, 48 and 72 h. $ATB^{0,+}$ negative cell lines will serve as a negative control. We will treat cells with RNase A (to ensure that RNA is not stained) and propidium iodide, and submit them to

FACS analysis. The amount of DNA in these cells, detected as chromosome content by propidium iodide binding, will be used to identify cells in various stages of cell cycle: G_1/G_0, 2N; S, 2–4 N; G_2/M, 4N (where N is the normal chromosome content). We will look at the relative percentage of cells in every cycle phase.

Inhibition of amino acid uptake

a) Functional expression of $ATB^{0,+}$ in mammalian cells

The interaction of GABDs and NDBBs with the transporter will be assessed by monitoring the ability to inhibit radiolabelled glycine transport. The transport function of $ATB^{0,+}$ will be measured in HRPE cells to which the vaccinia virus expression system will be applied. The cDNA will be inserted under the control of T7 promoter in the plasmid.

This test in HRPE cells will be repeated three times with three independent transfections and transport measurements will be made in duplicate in each experiment. We will compare between the glycine uptake of cells transfected with vector alone and cells transfected with $ATB^{0,+}$ cDNA to evaluate the relative levels of glycine uptake. The constitutive activity of HRPE cells will be subtracted for transport activity measured in cDNA- transfected cells to calculate cDNA-specific activity. In absence of inhibitors the transport will be taken as 100%.

The influence on the kinetic parameters of $ATB^{0,+}$ will be investigated by analyzing the saturation kinetics of the amino acid transport in the absence and presence of the compounds. The competitive nature of the inhibition of $ATB^{0,+}$ mediated glycine uptake by NDBBs and GABDs could be confirmed by two different approaches: analysis of saturation kinetics of glycine uptake in the absence (control, 100%) and presence of a fixed concentration of the compound and analysis of glycine uptake at two different concentrations in the presence of increasing concentrations of NDBBs and GABDs. Concentration cyphers will be based in the GI_{50} values of the compound against the cell line.

b) Xenopus laevis oocyte expression system

We will study the ability to inhibit amino acid uptake of GABDs and NDBBs through the *Xenopus laevis* expression system. Mature *Xenopus* oocytes will be collected and isolated from frogs under anesthesia. Oocytes will be injected with $ATB^{0,+}$ cRNA and water-injected oocytes will serve as controls. The oocytes will be subjected to

electrophysiological studies 6 days after cRNA injection. The membrane potential will be clamped at -50mV.

Oocytes will be perfused with NDBBs and GABDs while the induced current is monitored. We will check current dependence of Na^+ and Cl^- ions, vital for $ATB^{0,+}$ function (Nakanishi et al., 2001). The differences between the steady-state currents measured in the presence and absence of substrates will be considered as the substrate induced currents. Experiments will be repeated at least three times with separate oocytes.

Amino acid deprivation

The tests outlined in this section should be performed once we have already confirm the investigational compounds are $iATB^{0,+}$ according to the results of the previous assays.

a) CHOP and ASNS

We will monitor the expressions of ASNS and CHOP as a read out for amino acid nutritional status of the cells. In this experiment, HBL-100, BJ-hTERT, MCF-7, HCT116, LS174T, BxPC3 and AsPC-1 cell lines will be treated with two diverse concentrations of the selected compounds in presence of amino acids. The doses will be selected according to previous results of antiproliferative activity. The incubation with GABDs and NDBBs will be performed for duration periods of 48 and 72 h, called short and long period, respectively. The samples will be measured in triplicates. The quantification of the genes expression will be read as RNA amount and mRNA relative levels. We will check for changes in ASNS and CHOP expression according to $ATB^{0,+}$ status along the battery of different cell lines.

b) Analysis of autophagy

For studies related to autophagy, GFP-LC3 plasmid will be transfected into HBL-100, BJ-hTERT, MCF-7, HCT116, LS174T, BxPC3 and AsPC-1 cell lines, followed by treatment with the NDBBs and GABDs indicated. We will monitor the localization pattern of LC3 with and without adding the tested compounds and in presence of amino acids. Every cell line will be treated for 48 h with each one of the compounds independently or in combination with the autophagy inhibitor 3-methyladenine. We will incubate cells in primary and secondary antibodies. The cells undergoing autophagy would exhibit punctate fluorescent signals for LC3 when examined at fluorescent microscope. The quantification of fluorescent signals from scanned images will be expressed as percentage of cells with punctate GFP-LC3.

EXPECTED IMPACT

This project is expected to have an impact over the scientific knowledge, specifically in the oncology field. Likewise, we can expect a series of results from the different experimental settings described above. Docking studies should give an initial idea about the interactions that could take place among the small molecule inhibitors (NDBBs and GABDs) and the transporter, and it will allow making predictions about what could happen in cells. With virtual model simulations we have the opportunity to explore the coupling mechanisms between $ATB^{0,+}$ protein and its ligands without external factors. In first place, we look forward to achieve a computational model similar to the one we based on, so that our model constitutes the closest to reality as possible. We will explore docking scores of the different amino acids and α-MT and compare them to previous scores obtained at similar models to corroborate it is well constructed. For example, we hope to get a docking score around –6.3 kcal/mol for α-MT (Palazzolo et al., 2019).

In consonance with our hypothesis, we expect that NDBBs and GABDs could display docking interaction energies comparable to that of α-MT. If these assumptions become true, we would corroborate the previous docking results of NDBBs (Puerta et al., 2019) and it will suppose the first docking study run on GABDs. Moreover, we expect that products which have more interaction with $ATB^{0,+}$ turn out to be the products with more inhibition activity. Apart from that, it seems reasonable to forecast no selectivity between the enantiomer series of Betty bases due to the permissive substrate specificity of the $ATB^{0,+}$ transporter.

The colony formation assays should provide a first impression of the potency of the compounds and their selectivity toward the diverse cancer cell lines. We should be able to observe an inhibition of the colony formation in those cell lines that overexpress the transporter. Therefore, the four cell lines that are malignant and $ATB^{0,+}$ positive (MCF-7, LS174T, BxPC3, and AsPC-1) are expected to show a remarkably affection by the products in the clonogenic assay. In contrast, $ATB^{0,+}$ negative cell lines like HBL-100 should not experiment any change in their ability to form colonies at the same conditions. On their behalf, BJ-hTERT and HCT116 cell lines, which present a basal expression of $ATB^{0,+}$, ought to have little or no effects provoked by NDBBs and GABDs. Summarizing, we expect that GABDs and NDBBs suppress colony formation in an $ATB^{0,+}$ dependent manner.

Cell cycle analysis should reveal how GABDs and NDBBs affect the progression of different cell lines through cell cycle, especially depending on their $ATB^{0,+}$ profile. It is expected to show that the treatment has no effect on cell cycle in HBL-100, since it does not express $ATB^{0,+}$. Similarly, the products should have no effect on cell cycle in BJ-hTERT and HCT116 cell lines, whose $ATB^{0,+}$ expression levels are very low. Conversely, we expect to observe some affection on the cell cycle progression in $ATB^{0,+}$ positive cell lines (MCF-7, LS174T, BxPC3, and AsPC-1). When we analyze the data it should display that the relative percentage of cells in the different cycle phases favors one of the phases over the rest. This would indicate that cells are arrested at a particular stage when treated with the tested compounds.

Concerning the functional expression of $ATB^{0,+}$ in mammalian system, our objective is to inquire if GABDs and NDBBs are capable to inhibit amino acid uptake through $ATB^{0,+}$. Hence, we expect seeing that the glycine transport is inhibited in a products dependent manner. We will be able to confirm a competitive nature of the inhibition if relative levels of glycine uptake show a significant suppression of the transport on transfected cells in presence of GABDs and NDBBs. We should also observe that the inhibition potency increases with the doses. In contrast, those cells transfected with the vector alone would experiment a normal uptake of glycine, huge lower than $ATB^{0,+}$ transfected cells, and the addition of the products should not affect them.

The electrophysiological tests in *Xenopus laevis* expression system should bring us a second tracer of the compounds ability to produce the inhibition of amino acid influx. In this test we will monitor the transport of any given compound into oocytes by inward currents induced by that compound. At first, we would expect the currents to be undetectable in presence of GABDs and NDBBs so that we can affirm these products are $iATB^{0,+}$. Nevertheless, in case that functional expression in HRPE results indicate inhibition of glycine uptake and we still observe inducible currents in this test; it could indicate that our compounds are actually transportable substrates of $ATB^{0,+}$. Repeating the assay with the potential $iATB^{0,+}$ but in absence of amino acids or any other known substrate of the transporter could settle any doubts. Further, it must exist no detectable currents in absence of Na^+ or Cl^- otherwise we would not be measuring $ATB^{0,+}$ dependent currents.

Monitoring the levels of ASNS and CHOP mRNAs should help answering if GABDs and NDBBs cause amino acid deprivation in $ATB^{0,+}$ positive cancer cells. We expect that treatments with GABDs and NDBBs increase the levels of these mRNAs in

MCF-7, LS174T, BxPC3, and AsPC-1 cells. High levels of expression for CHOP and ASNS will indicate us that amino acid deprivation is actually taking place in treated $ATB^{0,+}$ positive cells. In malignant cells with no $ATB^{0,+}$ expression (HBL-100) and non-malignant cell lines (BJ-hTERT) you would expect to find basal levels of CHOP and ASNS mRNAs.

Referring to analysis of autophagy, this test should provide indirect evidences of amino acid nutritional status over the diverse cell lines. We expect to find that treatment of $ATB^{0,+}$ positive cell lines with GABDs and NDBBs induce autophagy as a proof of amino acid starvation. In these terms, we expect to identify punctate localization of LC3 in immunecytochemical analysis for MCF-7, LS174T, BxPC3, and AsPC-1 treated cells. Under identical conditions HBL-100, BJ-hTERT and HCT116 should not undergo autophagy, because they present low or not expression levels of $ATB^{0,+}$. The results of autophagy induction analysis ought to be in consonance with CHOP and ASNS mRNAs evaluation, since both measure amino acids deprivation.

As a whole, results of the multiple assays and their interpretation will allow us to elucidate what kind of interaction takes place between GABDs and NDBBs and the $ATB^{0,+}$ transporter. Altogether, we expect that resulting data of computational studies and biological tests will correlate each other and lead us into the same considerations. In case results support our hypothesis that these products behave as $ATB^{0,+}$ blockers, further studies will be necessary. In summary, this study would pave the way for the development of $ATB^{0,+}$ inhibitors with anticancer activity.

CONCLUSIONS

Starting from the evidence that GABDs and NDBBs show potential to provide us the basis for possible new chemotherapeutic agents, we intend to follow such evidence with the project here presented. To the best of our knowledge, there is no other line of research in the scientific landscape that proposes other molecules as potential iATB$^{0,+}$, apart from α-MT. The implementation of this project would contribute to the knowledge of this amino acid transporter, as well as the kind of interactions it establishes with non-amino acidic molecules.

Through the procedures exposed above we will be able to understand the interactions that take place between GABDs and NDBBs with the transporter ATB$^{0,+}$. However, we have encountered a lack of methodology that enables the evaluation of the activity of ATB$^{0,+}$ outside cells.

Since ATB$^{0,+}$ seems to be a selective target, the identification of GABDs or NDBBs as iATB$^{0,+}$ could represent a great input to cancer treatment. The next step would involve the identification and optimization of a lead. Employing the computational model, we could construct, carry out *in silico* studies and select those structure modifications that improve the docking scores. Then, we could evaluate the selected compounds to successive derivative rounds.

CONCLUSIONES

Partiendo del hecho de que los GABDs y los NDDBs muestran potencial para aportarnos la base para posibles nuevos agentes quimioterápicos, pretendemos seguir esta pista con el presente proyecto. Hasta donde sabemos, no existe otra línea de investigación en la que se propongan otras moléculas como potenciales iATB$^{0,+}$, aparte del ya conocido α-MT. La implementación de este proyecto contribuiría al conocimiento científico sobre este transportador de aminoácidos, así como al de las interacciones que establece con moléculas no aminoacídicas.

Mediante los procedimientos descritos con anterioridad podremos interpretar el tipo de interacción que se da entre los GABDs y NDBBs con el transportador ATB$^{0,+}$. Sin

embargo, nos hemos topado con una falta de metodología que nos permita la evaluación de la actividad de $ATB^{0,+}$ fuera de la célula.

Como $ATB^{0,+}$ parece constituir una diana selectiva, la determinación de estas moléculas como $iATB^{0,+}$ podría suponer una gran aportación al campo del tratamiento oncológico. El próximo paso implicaría la identificación y optimización de un cabeza de serie. Empleando el modelo computacional que se construirá en este proyecto, podríamos realizar estudios *in silico* y seleccionar aquellas modificaciones estructurales que mejoren las puntuaciones de docking. Entonces, someteríamos los compuestos seleccionados a rondas de derivatización sucesivas.

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