Instituto Universitario de
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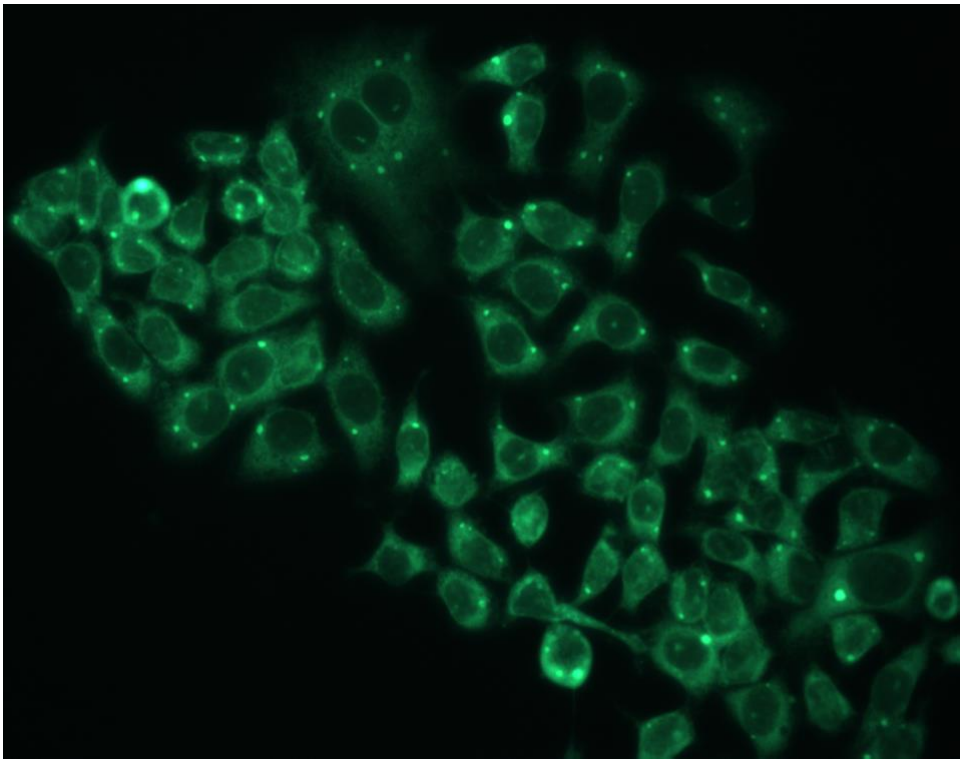
Universidad de La Laguna

TRABAJO DE FIN DE GRADO

Early Pharmacological Profiling of SLC6A14 inhibitors



Patricia Fumero Cruz

Tutorizado por Dr. José M. Padrón y Adrián Puerta

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La presente memoria de investigación ha sido realizada por Patricia Fumero Cruz durante el curso académico 2020-2021 en las instalaciones del Instituto Universitario de Bio-Orgánica "Antonio González" (IUBO-AG) y bajo la dirección del Dr. José M. Padrón y de Adrián Puerta. El trabajo forma parte de la línea de investigación *Diseño, descubrimiento y evaluación de fármacos anticancerígenos.*

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TABLE OF CONTENTS

ABSTRACT

One of the hallmarks of cancer is its deregulated metabolism. Cancer cells achieve their required increased amount of energy by using different methods. One of them is overexpressing amino acids transporters, such as SLC6A14 transporter. At present, there is only one reported inhibitor of the protein, α-methyltryptophan, which shows very low potency. The lack of a method to detect SLC6A14 inhibitors might explain the scarcity of available inhibitors. In this study, we tested four candidate fluorescent probes in an effort to develop a method to detect small molecule inhibitors of SLC6A14. Colony formation assay, chemosensitivity testing and fluorescence microscopy are used to detect their effects. Results showed that the probes are not good substrates for the method.

RESUMEN

Uno de los sellos distintivos del cáncer es su metabolismo desregulado. Las células cancerosas logran satisfacer sus necesidades energéticas aumentadas mediante el uso de diferentes métodos. Uno de ellos es la sobreexpresión de transportadores de aminoácidos tales como el transportador SLC6A14. En la actualidad, solo se conoce un inhibidor de la proteína, el α-metiltriptófano, que muestra una potencia muy baja. La falta de un método para detectar inhibidores de SLC6A14 podría explicar la escasez de inhibidores disponibles. En este estudio, probamos cuatro sondas fluorescentes candidatas con el objetivo de intentar desarrollar un método para detectar moléculas pequeñas inhibidoras de SLC6A14. Se utilizaron ensayos de formación de colonias, pruebas de quimiosensibilidad y microscopía de fluorescencia para detectar sus efectos. Los resultados mostraron que las sondas no son buenos sustratos para el método.

INTRODUCTION

The fundamental goal of anticancer drug discovery is to kill or to reprogram malignant cells while minimizing adverse effects on normal cells. However, achieving this goal is not easy, as cancer is composed of a very large number of molecularly and phenotypically distinct diseases, often occurring within the same patient or even within the same tumor [1].

In the past three decades, target-based drug discovery (TDD) –in which a defined molecular target with an important role in the pathology to be studied is established– has been the dominant approach to drug discovery in the pharmaceutical industry, driven by the advances in molecular biology and genomics [2]. Nevertheless, in recent years there has been an increased interest in phenotypic drug discovery (PDD) approaches. PDD, unlike TDD, is based on the evaluation of the phenotypic responses of certain compounds on a cell screen, identifying those biologically active compounds. In this case, knowledge of the molecular target is not essential from the first instance. Thus, target focus is the underlying attribute that differentiates phenotypic drug development from target-based discovery. In this project, based on PDD strategy, potentially active molecules to produce starvation in breast cancer cells were tested.

Cancer cells have an increased demand for glucose and amino acids to support their higher rate of growth and proliferation [3]. One of the ways they are supplied with the huge amount of energy they need is through amino acids transporters. In humans, there are approximately 400 transporters for various nutrients, metabolites, and drugs that are classified into 52 gene families. These transporters are known as *Solute Carriers* (SLC) [4, 5].

Solute carrier family 6 member (SLC6A14), known as amino acid transporter $B^{0,+}$ ($ATB^{0,+}$), transports all amino acids with exception of the acidic ones, aspartate and glutamate [6]. SLC6A14 is highly expressed in many human cancers of epithelial origin, including cervical, colorectal, pancreatic and breast cancer [7]. In contradistinction to the other amino acid transporters of its family, SLC6A14 has three different driving forces that participate in energizing it: membrane potential, and Na^+ and Cl^- gradients [8]. Accurately, it transports one molecule of amino acid in a symport with 2 Na^+ and 1 Cl^-, using as well the transmembrane potential [9] (Figure 1). These mechanisms allow

SLC6A14 to concentrate its amino acid substrates inside the cells more than 1000-fold compared to the extracellular medium. Furthermore, both L and D amino acids are substrates [4].

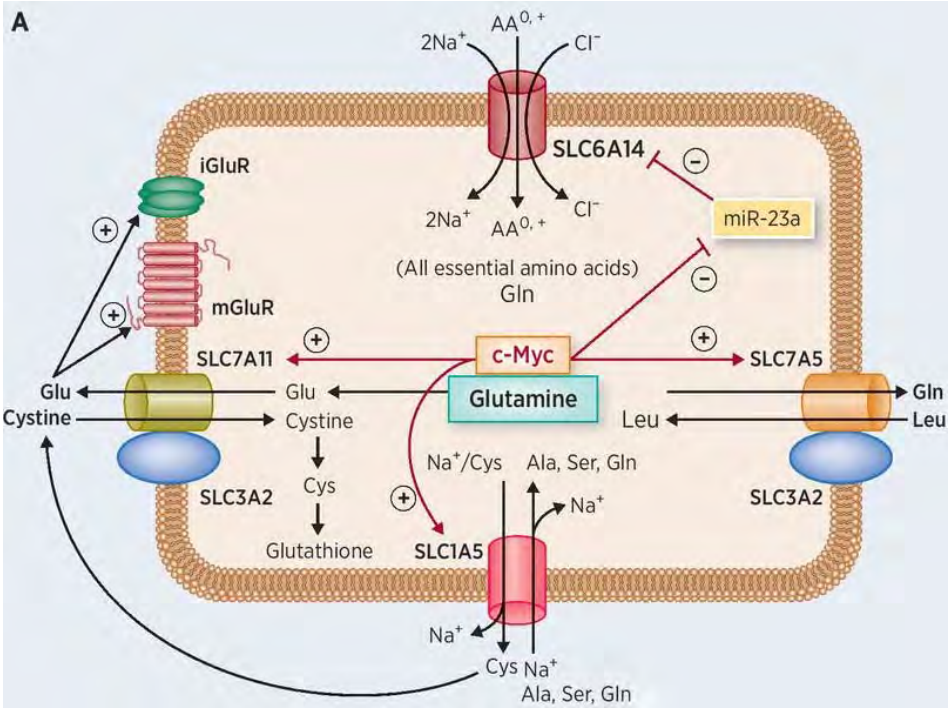


Figure 1. Amino acid transporters that are upregulated in cancer and their functional inter-relationship. *Adapted from [9].*

All of the abovementioned characteristics of SLC6A14 make it an attractive target for cancer treatment. SLC6A14 could be a delivery system for anticancer drugs in the form of amino acid-based prodrugs [10]. Nowadays, the only known blocker of this transporter is α-methyl-DL-tryptophan (α-MT). The treatment of colon cancer cells with α-MT induces amino acid deprivation, decreases mTOR activity, increases autophagy, promotes apoptosis and suppresses cell proliferation and invasion [11]. Although α-MT does not affect healthy cells, its activity needs high concentrations (millimolar). In our research group, different tryptophan derivatives were synthesized as SLC6A14 inhibitors candidates: glutamic acid-based dipeptides (GABDs) [12] and naphthol-derived Betti bases (NDBBs) [13]. However, the lack of a method to test SLC6A14 inhibition did not allow to confirm this extent.

In this work, a set of four fluorescent tryptophan derivatives were assayed *in vitro* using breast cancer cell lines in order to establish their scope for a SLC6A14 inhibition assay.

HYPOTHESIS

SLC6A14 is an amino acid transporter which is overexpressed in tumor cells [6, 7, 14]. This transporter plays a relevant role in cancer progression as it provides tumor cells with the amount of amino acids they need to supply their increased metabolic needs. Inhibitors of SLC6A14, by depriving the susceptible cells of their required amino acids, could be a new source of pharmacological entities for the treatment of some types of cancer, such as breast or pancreatic cancer.

OBJECTIVES

The main objective of this work is to develop methods to test the capacity of SLC6A14 inhibitors to prevent cell growth in MCF7 tumor cell line. To achieve this goal, we considered the next specific objectives:

1. To run a literature search to gather different methodologies used to detect small molecules inhibitors of amino acid transporters.

2. To develop experimental methods to test the ability of SLC6A14 inhibitors to prevent cell growth in tumor cells, including:
   a. The assessment of SLC6A14 transporters inhibition using fluorescence assays.
   b. The quantification of cell growth inhibition through *in vitro* studies.

3. To check if SLC6A14 inhibitors are P-glycoprotein substrates, in order to compare cellular behavior differences between resistant and non-resistant cell lines.

MATERIALS AND METHODS

Literature search

Three accessible and well-known databases were looked for scientific literature: PubMed, Scopus, and Google Scholar. The terms chosen for the search were: "SLC6A14", "SLC6A14 inhibitors", "Small molecule methods", "Breast cancer" and "MCF7". Article selection process was based on how close the title and abstract were to the field.

Chemical entities

The fluorescent probes BLB0013, BLB0014, BLB0015 and BLB0016 (Figure 2) were used in this study and they are available at our research group.

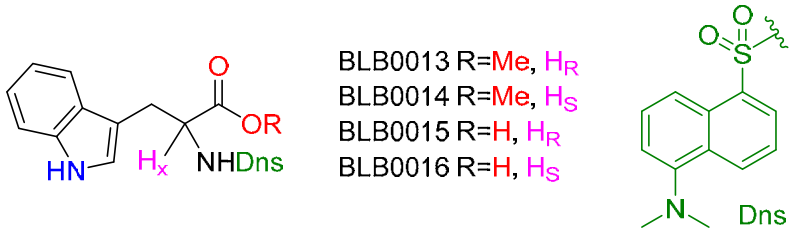


Figure 2. Chemical structure of fluorescent probes based on dansylated

Cell lines and culture

Human cell lines showing a positive expression of SLC6A14 [15, 16] were selected for the assays. Specifically, MCF7 ER^+ breast cancer cell line was used as a study model. MCF7 with an induced resistance based on the overproduction of a drug efflux pump called P-glycoprotein (PGP) was also used. These cells were kindly provided by Dr. Godefridus J. Peters (Cancer Center Amsterdam, Vrije Universiteit, Amsterdam, The Netherlands).

Cells were grown in RPMI-1640 medium containing 5% fetal bovine serum (FBS), 1 mM glutamine and antibiotics (100 U/mL of penicillin and 0.1 mg/mL of streptomycin) and were preserved at 37ºC in a 95% humidified atmosphere using 60 mm cell culture dishes and maintained at low passages.

Thawing the selected cell lines

First of all, a 1.5 mL cryogenization vial of each cell line (MCF7 and MCF7/PGP) were thawed from an –80ºC freezer. To do this, the vials were taken outside the freezer and placed in a recipient with ice to prevent a sudden change in temperature. After a few minutes, once thawed, the freezing media (DMSO 10% in FBS) was diluted 1:10 in RPMI-1640 and centrifuged using Allegra™ X-12R Centrifuge at 940 rpm, 4ºC, for 5-6 minutes. Then, supernatant was discarded and the pellet was resuspended in culture media. Finally, the sample was seeded in 60 mm cell culture dishes and preserved in the conditions explained above.

Clonogenic Assay: Colony formation assay

Both MCF7 and MCF7/PGP cells were initially trypsinized, resuspended in medium and quantified using Neubauer Chamber (Zuzi®). Then, cells were seeded in 6 well plates (~500 cells/well) and, 24 hours later, the different BLBs were added in duplicate at 1.5 µM for BLB0013, 15 µM for BLB0014, and 100 µM for BLB0015 and BLB0016 based on their growth inhibition properties. After eight days of culture, samples were fixed with 750 µL of absolute MeOH for 10 minutes and stained with 750 µL of crystal violet at 1% for 5 minutes. Eventually, colonies were quantified using AutoCellSeg software.

Chemosensitivity testing

Chemosensitivity test was performed using the SRB assay of the National Cancer Institute (NCI, USA) with slight modifications [17]. SRB is a bright pink aminoxanthene dye with two sulfonic groups. Under mildly conditions, SRB binds to protein basic amino acid residues in TCA-fixed cells to provide a sensitive index of cellular protein content [18].

To test the effect of P-gp overexpression in the antiproliferativity activity of the compounds, MCF7 and its P-gp overexpressing variant (MCF7/PGP) in the presence or absence of verapamil (a known P-gp inhibitor) were selected. The cell culture medium containing verapamil was prepared at a final concentration of 10 µM verapamil. Cells were counted with Moxi Z and seeded in 96-well plates at a density between 2,500 cells/well. After 24 hours, pure compounds were added. Each agent was initially dissolved in DMSO at 400 times the desired final maximum test concentration of DMSO (0.25%

v/v, negative control). Compounds were tested in triplicates at different decimal dilutions in the range 0.01–100 μM.

Drug incubation times were 48 hours, after which time cells were precipitated with 25 μL ice-cold 50% (w/v) TCA and fixed for 60 minutes at 4ºC. Then, 25 μL of a SRB solution (0.4% v/v in 1% of acetic acid) were added and stained for 15 minutes protected from light. After the staining procedure, SRB excess was removed and plates were quickly rinsed four times with 1% acetic acid to remove unbound dye. After being rinsed, the cultures were air dried until no standing moisture was visible. Bound dye was solubilized with 10 mM unbuffered Tris base (pH 10.5) for 5 minutes on a gyratory shaker. The optical density (OD) of each well was measured at 530 nm, using Bio Tek's (Winooski, USA) PowerWave XS Absorbance Microplate Reader. Values were corrected for background OD from wells only containing medium. The percentage growth (PG) were calculated with respect to untreated control cells (C) at each of the drug concentration levels based on the differences in OD at the start (T_0) and end of drug exposure (T), according to NCl formulas. With these calculations, a PG value of 0 corresponds to the number of cells present at the start of the drug exposure, while negative PG values denote net cell kill.

- $PG = 100 \times [(T-T_0) / (C-C_0)]$ If $T > T_0$
- $PG= 100 \times [(T-T_0) / (T_0)]$ If $T \leq T_0$

The effect was defined as percentage of 50% growth inhibition (GI_{50}), which represent the concentration at which PG is +50.

Cellular uptake assay

Approximately 50,000 cells/well of MCF7 and MCF7/PGP were seeded in 6 wells plates onto coverslips previously added to each well. After 24 hours, compounds in concentrations of 40 μM (BLB0013 and BLB0014) and 100 μM (BLB0015 and BLB0016) were added. Then, 24 hours later, wells were fixed with *p*-formaldehyde (PFA) at 4% in PBS for 20 minutes, protected from light, at room temperature and preserved in 500 μL of PBS. In the end, coverslips were taken outside the wells and mounted on slides with glycerol at 50%. Photographs were taken at random locations under Leica DM 4000 B® fluorescence microscope with a filter of 340-380 nm excitation range. Untreated cells were used as the negative control.

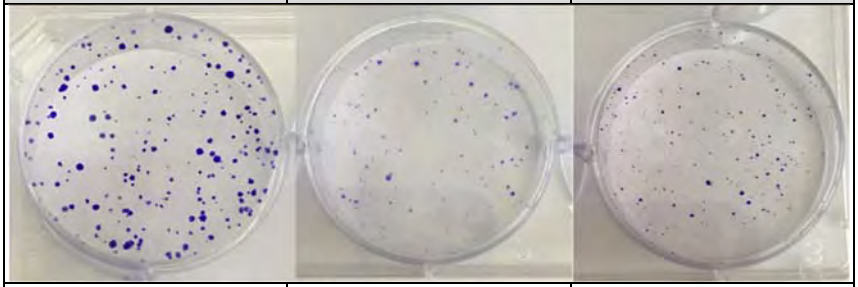
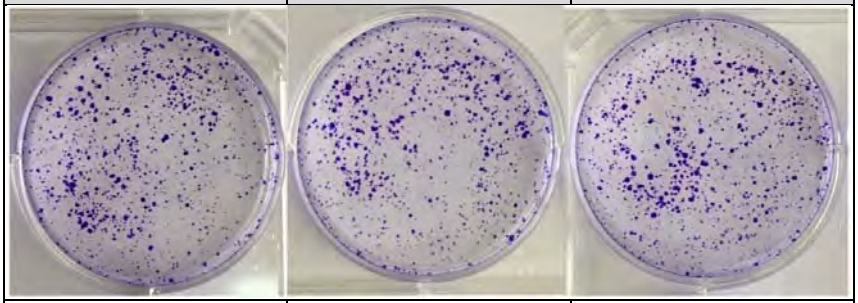
RESULTS & DISCUSSION

In spite of the fact that SLC6A14 transporter is well defined in many articles, there is scarce investigation in this field. No scientific articles that developed other possible candidates to inhibit SLC6A14 were found, with the exception of α-MT. The effects of α-MT on the disruption of cell growth was studied and proved in colorectal cancer, but the results showed that its activity needs concentrations in the millimolar range. Besides, there is a lack of experimental methods to analyze SLC6A14 inhibition. In this work, we tested the biological activity of a set of fluorescent probes, which could be potential candidates to analyze SLC6A14 inhibition by small molecules.

Evaluation of cell growth inhibition

The biological activity of fluorescent probe candidates was measured using the clonogenic assay. MCF7 cells showed different sensitivity to the compounds tested. The administration of BLB0013 and BLB0014 decreased cell viability and clonogenicity producing cell death, while BLB0015 and BLB0016 had no detectable cytotoxicity (Figure 3). Therefore, BLB0013 and BLB0014 are potentially active against MCF7 cancer cells. Nonetheless, BLB0015 and BLB0016 did not produce relevant changes in cell survival, which indicates their inactivity as possible inhibitors of cell growth.

A)

C	**BLB0013**	**BLB0014**
		
100	45.3±5.8	26.4±2.8
C	**BLB0015**	**BLB0016**
		
100	97.4±2.0	96.7±2.2

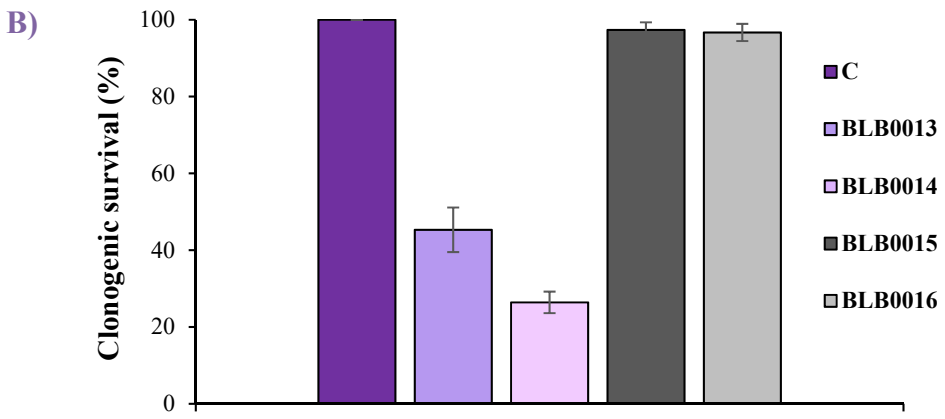


Figure 3. Representative clonogenic assay in MCF7 cell line. **A)** Images of stained wells without treatment (C, control) and after being treated with compounds (in %). **B)** Clonogenic survival of cells exposed to compounds compared to control.

Additionally, MCF7/PGP clonogenic assay (Figure 4) could not be measured due to low cell density. Colony formation assays require at least 50 cells [19], which were not present in our MCF7/PGP assay of the fluorescent probes.

However, by comparison, BLB0015 and BLB0016 did not show noticeable antiproliferative activity against all tested cells. Little difference in BLB0013 and BLB0014 sensitivity was detected between MCF7 and MCF7/PGP. Results indicate that BLB0013 in concentration of 1.5 µM has lower potential in MCF7/PGP than in MCF7. On the contrary, BLB0014 in concentration of 15 µM induces growth inhibition in both cell lines. Among them, BLB0014 shows greater cytotoxic potential than BLB0013.

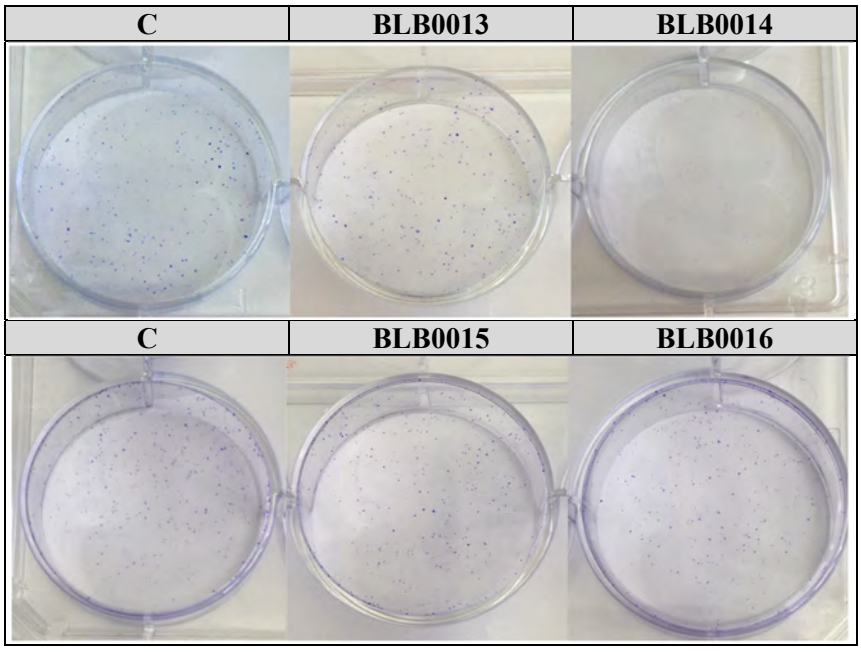


Figure 4. Representative clonogenic assay in MCF7/PGP cell line.

Chemosensitivity testing

Using paclitaxel as positive control (known substrate of P-gp), the concentration at which 50% of cell growth is inhibited (GI_{50}) was tested for different compounds. For better comparison of the data, the resistance factor (Rf) for a given compound was defined as the ratio of GI_{50} values against the P-gp overexpressing and the wild-type cell lines, respectively. On the one hand, in the absence of verapamil and in co-treatment with it, similar Rf was recorded for all the compounds tested (comparing them with the positive control, paclitaxel) (Table 1). This result indicates that none of the compounds are substrates of P-gp.

On the other hand, GI_{50} for BLB0015 and BLB0016 is greater than 100 µM in both cell lines, whilst BLB0013 and BLB0014 is below that value. National Cancer Institute (NCI) establishes 100 µM as the limit concentration to consider a compound as active, therefore, we can consider BLB0015 and BLB0016 as inactive and BLB0013 and BLB0014 as potentially active compounds.

Table 1. Antiproliferative activity (GI_{50}) of compounds in MCF7 and MCF7/PGP cell lines. Gi_{50} and SD in µM. Rf = $GI_{50\,(MCF7/PGP)}$ / $GI_{50\,(MCF7)}$

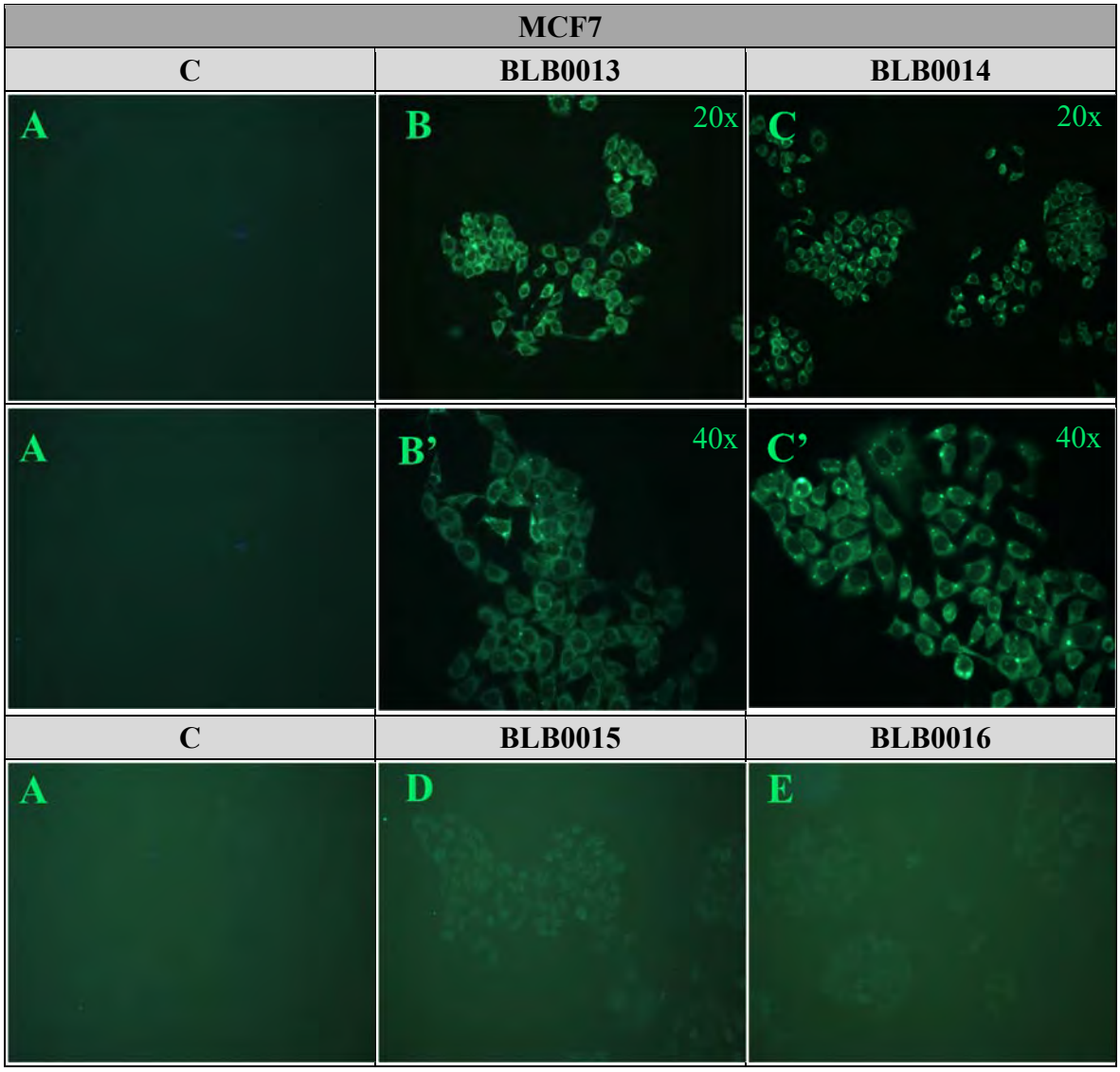
	Without Verapamil				
	MCF7		**MCF7/PGP**		
Compounds	GI_{50}	SD	GI_{50}	SD	Rf
Paclitaxel	7.2E-5	4.0E-5	0.13	0.011	**1810**
BLB0013	7.1	1.2	12	1.0	**1.7**
BLB0014	6.0	0.31	17	1.4	**2.8**
BLB0015	>100		>100		–
BLB0016	>100		>100		–

	With Verapamil				
	MCF7		**MCF7/PGP**		
Compounds	GI_{50}	SD	GI_{50}	SD	Rf
Paclitaxel	1.9E-3	4.1E-4	3.2E-3	1.2E-3	**1.7**
BLB0013	2.5	0.15	6.4	0.47	**2.6**
BLB0014	1.9	0.34	13	0.21	**6.8**
BLB0015	>100		>100		–
BLB0016	>100		>100		–

Cellular uptake of the compounds

To confirm whether the reduced cell viability was due to SLC6A14 inhibition, fluorescence microscopy was used to detect cellular uptake of the compounds. Compounds bound to fluorescent probe can exert their inhibitory action on cell growth in three ways: (1) causing blockage of SLC6A14 transporter and inducing cellular starvation, (2) entering the cell by passive diffusion or (3) being introduced by SLC6A14 transporter. In the last two cases, compounds exert their action within the cell.

BLB0013 and BLB0014 produced fluorescence inside cells, which lead us to rule out SLC6A14 inhibition. BLB0015 and BLB0016 did not fluoresce in any of both cell lines tested (Figure 5). This can be attributed to the carboxylate group, which prevents cellular uptake by passive diffusion. Overall, the fluorescent imaging data is consistent with the clonogenic assay and the chemosensitivity testing results.



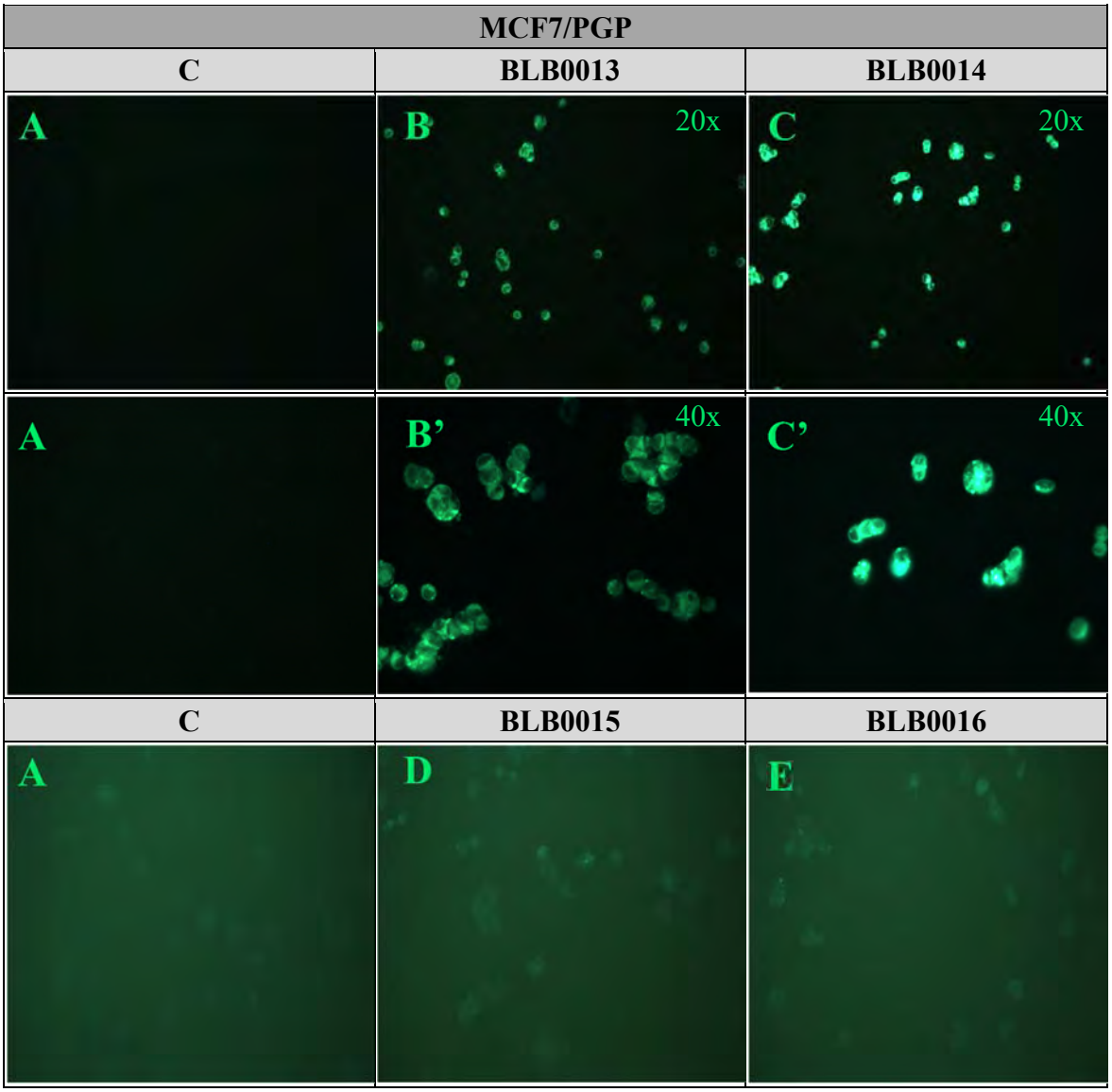


Figure 5. Fluorescence micrographs of MCF7 and MCF7/PGP cells treated with different concentrations of compounds. (A) Control, (B) BLB0013, (C) BLBL0014, (D) BLB0015 and (E) BLB0016.

We can speculate that those compounds that were taken up by the cell (BLB0013 and BLB0014) induced antiproliferative effects by mechanisms other than SLC6A14 inhibition. Additionally, probes BLB0015 and BLB0016 did not induce growth inhibition. In summary, the four compounds tested do not represent good candidates for a fluorescent assay to identify small molecule inhibitors of SLC6A14.

CONCLUSIONS

As we observed in the literature search, there is not enough information of SLC6A14 inhibition or even of methods to detect amino acid transporters inhibition by small molecules. In view of the results, our group has envisioned the development of a fluorescent method to detect small molecule inhibitors of SLC6A14. In this study, we tested the biological activity against breast cancer cells of four fluorescent probe candidates. From the results, we can conclude that:

1. The fluorescent probes BLB0013 and BLB0014 produce cell growth inhibition in MCF7 cells, whilst BLB0015 and BLB0016 are inactive.
2. The probes BLB0013 and BLB0014 are not substrates of P-gp, so the absence of activity of BLB0015 and BLB0016 due to P-gp extrusion can be discarded.
3. BLB0013 and BLB0014 enter the cells by passive diffusion, whilst BLB0015 and BLB0016 do not enter cells.
4. The fluorescent probes are not good candidates to detect small molecule inhibitors of SLC6A14.

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