Instituto Universitario de
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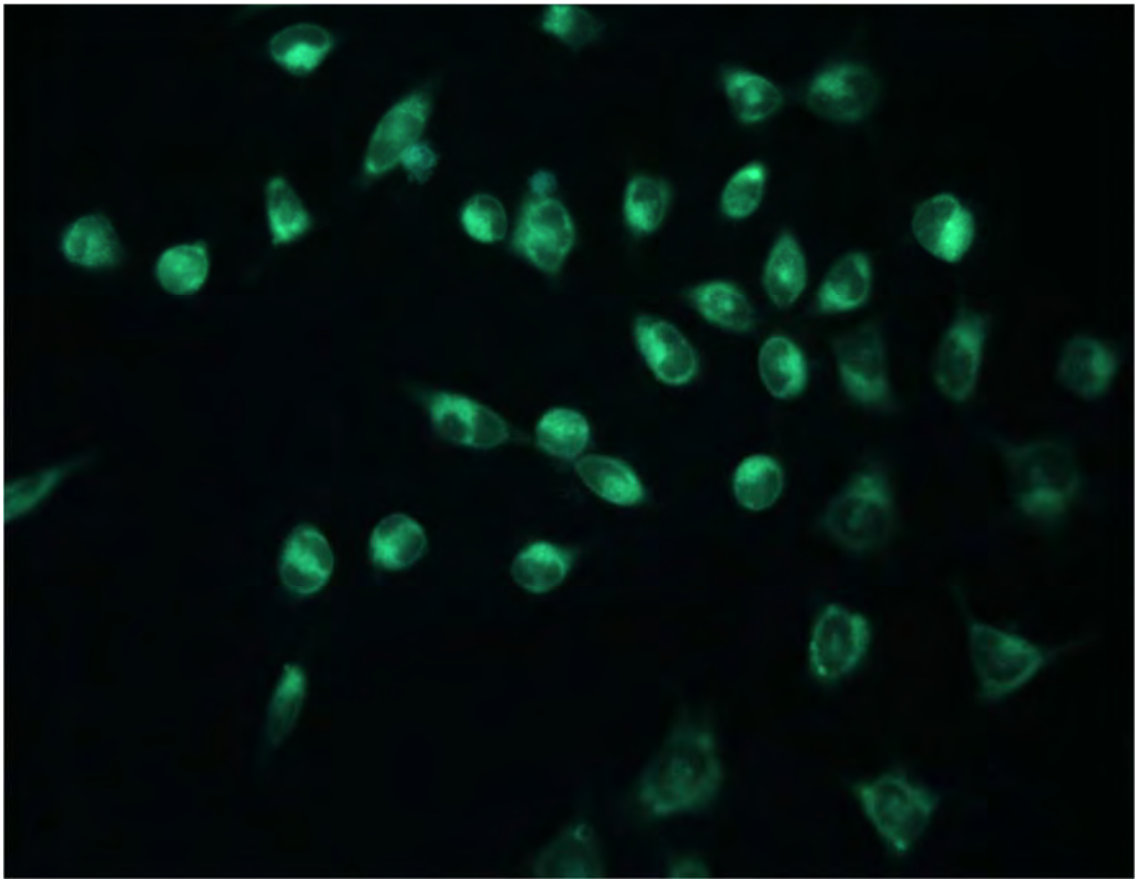
Universidad de La Laguna

EARLY PHARMACOLOGICAL PROFILING OF SMALL-MOLECULES: IDENTIFICATION OF SLC6A14 INHIBITORS



Trabajo de Fin de Máster

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La presente memoria de investigación ha sido realizada por Giada Carniglia 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. El trabajo forma parte de la línea de investigación *Diseño, descubrimiento y evaluación de fármacos anticancerígenos.*

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ABSTRACT

Our research focuses on recognising the efficacy and effectiveness of small molecules that inhibit the membrane transporter SLC6A14, which is expressed in most organs of the human body and overexpressed when cells become cancerous. Previous studies had highlighted the use of SLC6A14 as an anti-tumour therapeutic target. Using several low-cost methods of detection, we sought to study and prove the existence of the membrane transporter protein in different cell lines while testing the efficacy and effectiveness of our small inhibitory molecules.

RESUMEN

Nuestra investigación se centra en el reconocimiento de la eficacia y efectividad de pequeñas moléculas que inhiben el transportador de membrana SLC6A14, expresado en la mayoría de los órganos del cuerpo humano y sobreexpresado cuando las células son tumorales. Estudios anteriores habían destacado el uso de SLC6A14 como diana terapéutica antitumoral. Utilizando diferentes métodos de bajo coste, hemos intentado estudiar y probar la existencia de la proteína transportadora de membrana en diferentes líneas celulares, al tiempo que probamos la eficacia de nuestras pequeñas moléculas inhibidoras.

1. INTRODUCTION

SLC6A14 is the 14th member of solute carrier family 6, a family of Na^+- and Cl^--dependent solute transport systems, many of which are involved in transmembrane movement of neurotransmitters [1]. The encoded protein transports both neutral and cationic amino acids, except glutamate and aspartate, with a coupled sodium/chlorine transport, regulating the distribution of amino acids within the cell (Figure 1) [2]. In previous studies, using RNA-Seq method, SLC6A14 has been found to be the most expressed transporter in lung, stomach, colon, small intestine, salivary gland, skin, bladder, and endometrium, among others [3].

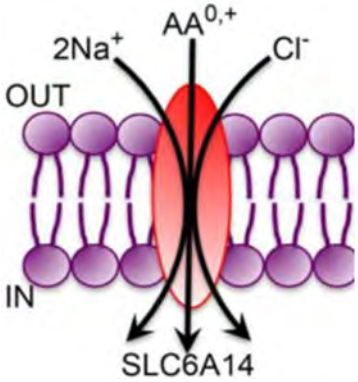


Figure 1. Transport model of SLC6A14. $AA^{0,+}$: neutral (net charge of zero on the molecule) and cationic (net positive charge on the molecule) amino acids. *Adapted from [2].*

Mutations in gene SLC6 have been associated with chromosome X and various diseases [4]. In fact, the overexpression and dysfunction of SLC6A14 appears in different diseases, even unrelated ones, like obesity [5] and cystic fibrosis [6], demonstrating the involvement of existing polymorphisms and other molecules interacting with SLC6A14 in cellular physiological metabolism. Tests have shown that amino acid demand is increased in several cancers. Nevertheless, SLC6A14 malfunctioning is correlated with several pathological states and it is upregulated in solid tumors. SLC6A14 overexpression is detected in diverse cancers [7] such as pancreatic, colorectal, breast and uterine cancer. The high expression of SLC6A14 is prognostic and unfavorable in various cancers, and in breast cancer it is expressed in estrogen receptor positive cells. The screening of several mammary epithelial cell lines showed that SLC6A14 is expressed robustly in some cancer cell lines, but not in all (Figure 2) [8]. In contrast, non-malignant cell lines do not express the transporter to the same extent.

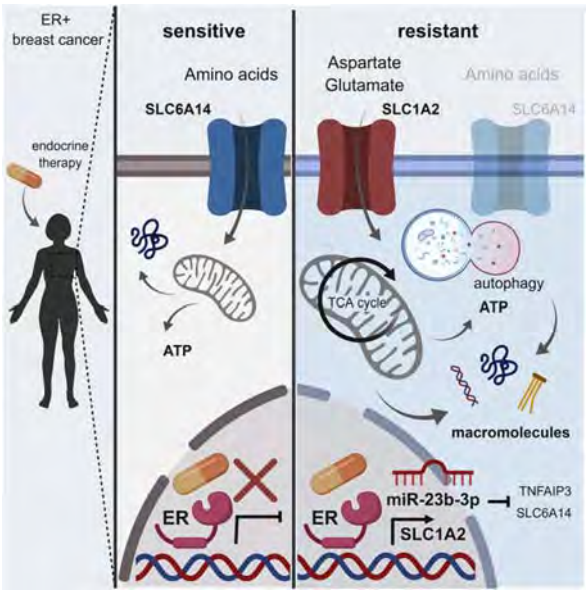


Figure 2. SLC1A2 and SLC6A14 are not concomitantly overexpressed or amplified in breast cancers and may be mutually exclusive. *Taken from [8]*.

Treatment of SLC6A14-positive tumor cells with α-methyltryptophan led to suppression of their colony-forming ability, whereas SLC6A14-negative cell lines were not affected. The blockade of SLC6A14 in these cells with α-methyltryptophan was associated with cell cycle arrest [9]. When compared to other transporters, SLC6A14 is recognized as a potential therapeutic target (Figure 3) [10] [11]. The known molecular mechanisms are still limited. Interestingly, the discovery of some substrates could have important impact in the design of new SLC6A14 inhibitors that could lead to tumor cell death [12]. Alternatively, its overexpression has been considered as a plausible drug delivery system [13].

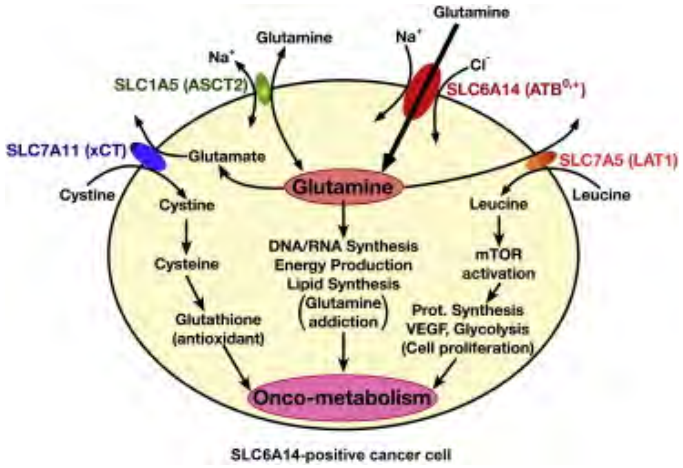


Figure 3. Relevance of SLC6A14 to other amino acid transporters and to tumor growth. *Taken from [11]*.

In our group, we are involved in the design and synthesis of tryptophan-based SLC6A14 inhibitors. However, the main limitation we face is the lack of a method to test in vitro SLC6A14 inhibition by small molecules. As a method to assess the early pharmacological profiling of these potential SLC6A14 inhibitors (iSLC6A14), in our group we have envisioned a fluorescence-based test. In particular, we are working on a cell-based assay where cells will be incubated with a fluorescent tagged substrate in the presence or absence of iSLC6A14 (Figure 4). With this test, we could determine if the uptake of amino acids by SLC6A14 is affected or not by the test compounds (iSLC6A14). Herein, we studied the scope and limitations of diverse topics related to the development of this phenotypic assay. In particular, we will focus on the characterization of the cell lines in relation to their expression levels of the transporter. In addition, we will study the scope and limitations of some fluorescent probe candidates prepared in our group.

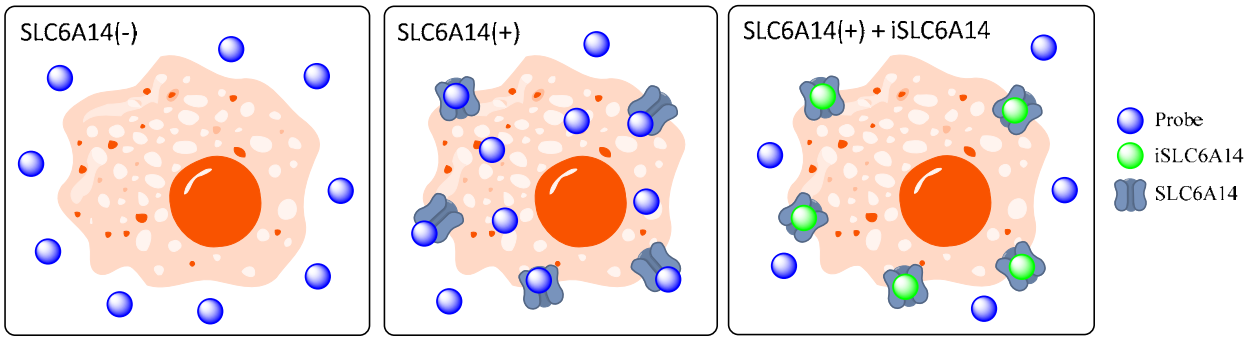


Figure 4. Fluorescence cell-based assay to identify iSLC6A14.

2. HYPOTHESIS

Some tumors overexpress the transmembrane transporter SLC6A14 to increase the uptake of amino acids for their metabolism. The inhibition of the uptake with small-molecule (SM) SLC6A14 inhibitors might provide new chemotherapeutics options for the treatment of cancer.

The biological effect of small-molecules (SMs) is reversible and usually rapid. Therefore, it allows the early characterization of phenotypic effects. SM effects produce *dose-response data*, where the level of phenotypic effects is dependent on the concentration of the SM. Biological assays are necessary to recognize and characterize the SM-protein interaction. In a phenotypic assay, compounds may interact with one or more targets or pathways not anticipated by a single mechanism-driven hypothesis. In essence, phenotypic approaches screen multiple mechanisms and targets simultaneously.

3. OBJECTIVES

At present, there is no method described in the literature to identify SM SLC6A14 inhibitors. The main objective of this work is to explore the scope and limitations of common experimental techniques when applied to the identification of SM SLC6A14 inhibitors. To achieve this objective, we will focus on:

1. The identification of the level of SLC6A14 expression in breast cancer cells.
2. The evaluation of the fluorescent probe candidates.

4. MATERIALS AND METHODS

4.1. Cell lines and cultures

For this study, we selected the human cervix cancer cell line HeLa and the human breast cancer cell lines HBL-100, MDA-MB-453, MCF-7, and T-47D. CEAMED S.A. kindly provided the cell line MDA-MB-453. Prof. G. J. Peters (VUmc, Amsterdam) kindly provided the other cell lines. Cell lines were grown in RPMI 1640 medium supplemented with fetal bovine serum (5% v/v), L-glutamine (2 mM) and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). Cell cultures were kept at 37 ºC in a humidified atmosphere of 5% CO_2 and maintained at low passage.

For all experiments, exponentially growing cells were trypsinized and resuspended in cell culture medium. Single cell suspensions were counted (Moxi Z, Orflo, IN, USA) and diluted to reach the appropriate cell densities for inoculation onto 60 mm cell culture dishes or multi-well plates.

4.2. Drugs and exposure

The fluorescent probes BLB0013, BLB0014, BLB0015 and BLB0016 (Figure 5) were used in this study and they were synthesized at our research group [14]. Samples were dissolved in DMSO at an initial concentration of 40 mM. When necessary, intermediate dilutions were prepared in fresh culture medium prior to inoculation onto plates.

Figure 5. Chemical structure of fluorescent probes based on Dns-Trp.

4.3. SLC6A14 expression

Western blot was used to determine the expression level of SLC6A14 in HBL-100, MCF-7, MDA-MB-453 and T-47D cell lines. To prepare protein extracts for western blot analysis, cells (50,000 cells) were seeded in 60 mm culture dishes and were grown until 80-90% confluence. Then, cells were washed twice with cold-PBS and lysed in ice-cold lysis buffer

freshly supplemented with protease and phosphatase inhibitors (20 mM Tris pH 8.0, 140 mM NaCl, 1% NP-40, 10% glycerol, 5 mM EDTA, 2 mM Na_3VO_4, 10 mM $Na_2P_2O_7$, 10 mM NaF, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 0.5 µg/mL pepstatin, and 1 mM PMSF). Lysates were incubated on ice for 10 min and then cell debris were spun down at 12,000 rpm for 10 min. After centrifugation, supernatants were transferred to new tubes and stored at −20ºC. Protein concentration was determined by the Bradford method [15].

Equal amounts of protein were resolved by SDS/PAGE and electro-transferred onto PVDF membranes. Membranes were blocked in Tris-buffered saline with Tween (TBST; 100 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 1% of BSA for 1 h and then incubated with the corresponding primary antibody for 2-16 h. After washing three times with TBST, membranes were incubated with HRP-conjugated secondary antibodies for 30 min, washed three times with TBST and bands were visualized by a luminol-based detection system with *p*-iodophenol enhancement.

4.4. Cell migration assay

In this assay, HeLa cells were seeded onto cell culture plates at densities of 50,000 cells/well. After 48 hours of incubation, a scratch was made in the monolayer culture of each well with the help of a sterile pipette tip. Then, the compounds were added to test wells and the plates incubated up to 30 h. Individual photographs (Axiovert 40 CFL, Carl Zeiss, Germany) of wells were taken at three different time points: 1) after making the scratch, 2) after 22 h of drug exposure, and 3) after 30 h of drug exposure. The gap width in each photograph was measured using image software (imageJ) and its plugin *Wound_healing_size_tool* [16]. The results allowed us to quantify cell migration using Equation 1, which compares the wounded area (A) at any time point (t) with respect to the initial value ($t = 0$).

$$Wound\ Closure\% = \left(\frac{A_{t=0} - A_{t=\Delta t}}{A_{t=0}}\right) x100\% \qquad \textbf{Equation 1}$$

4.5. Fluorescence microscopy

HeLa and MDA-MB-453 cells were seeded (50,000 cells/well) onto coverslips previously added to each well of a 6-well plate. On the next day, test compounds were added in concentrations of 40 µM (BLB0013 and BLB0014) and 100 µM (BLB0015 and BLB0016). After 24 h, cells were fixed with *p*-formaldehyde (PFA; 4% in PBS, 500 µL) for 20 minutes,

protected from light, and at room temperature. After fixation, 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.

5. RESULTS AND DISCUSSION

The amino acid transporter SLC6A14 is expressed in several cancer cells. Our first task was to search the literature to identify cell lines that overexpress the protein [SLC6A14 (+)], as well as cell lines that do not express the transporter [SLC6A14 (–)]. As a result, we found four candidate breast cancer cell lines that were also available in our cell line repository. Thus, we selected T-47D and MCF7 as representative of SLC6A14 (+) cells, and HBL-100 and MDA-MB-453 reported as SLC6A14 (–) [17]. In addition to SLC6A14, these breast cancer cell lines show different expression levels of other proteins (Table 1) that could help in the interpretation of the results.

Table 1. Expression status of selected proteins in breast cancer cell lines.

Cell line	SLC6A14	ER/PR	HER2	EGFR	β-Catenin
HBL-100	–	–	–	–	+
MCF7	+	+	–	–	+
MDA-MB-453	–	–	+	–	–
T-47D	+	+	–	–	+

5.1. SLC6A14 expression in breast cancer cells

Next, we checked the SLC6A14 expression levels in our cell lines by western blot. The western blot is the most widely used semiquantitative technique in most laboratories that need to check for the presence of a certain protein of interest, although it is a difficult technique to carry out. On top of that, we should recall that SLC6A14 is a membrane protein, which in general are more difficult to blot. An additional disadvantage we faced is related to the measurement of basal levels of expression without an overexpression control (i.e. cells transfected with the encoding DNA of interest).

We encountered several problems in carrying out the experimental setup. During the course of our investigations we had to remake several compounds used to produce the gel and carry out electrophoresis, which for some time did not allow us to obtain reproducible results. After several trials, we discovered recently that the elements causing the disturbances were β-mercaptoethanol and TEMED. Finally, we were able to characterize the expression of SLC6A14 in two of the cell lines included in this study (Figure 6). We found that T-47D cells express the amino acid transporter, whilst HBL-100 cells do not express the protein. This result

is consistent with the literature [17]. At present, the determination of the expression levels of SLC6A14 in the other cell lines remain incomplete.

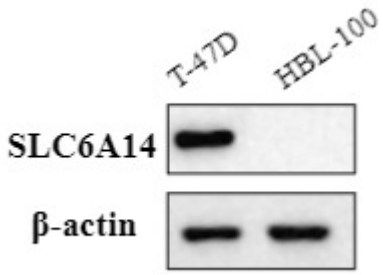


Figure 6. Expression of SLC6A14 in human breast cancer cells.

Noteworthy, Dejure *et al.* experienced similar difficulties when characterizing SLC6A14 expression by western blot [18]. They solved the situation testing by Sanger sequencing and by RT-qPCR. Unfortunately, those techniques are not available in our group.

5.2. Fluorescent probe candidate molecules

In our group, we have prepared a small set of tryptophan-based fluorescent compounds as potential candidates (Figure 5) for the so-called SLC6A14 inhibition assay [14]. These compounds are been characterized in our group using diverse biological assays and some preliminary data have been obtained (Table 2). In this work, we run complementary assays in order to determine the scope and limitations of said compounds as fluorescent probes for the SLC6A14 inhibition assay.

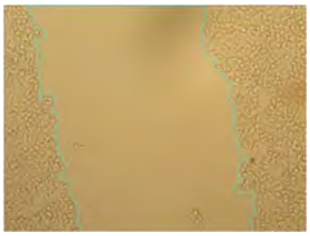
Table 2. Antiproliferative activity (GI_{50}, μM) against human cancer cell lines.

Cell Line (*origin*)	**BLB0013**	**BLB0014**	**BLB0015**	**BLB0016**
A549 (*lung*)	13 ± 0.8	11 ± 6.9	>100	>100
HBL-100 (*breast*)	15 ± 1.3	15 ± 6.8	>100	>100
HeLa (*cervix*)	2.5 ± 0.8	12 ± 4.8	>100	>100
MCF7 (*breast*)	7.1 ± 1.2	6.0 ± 0.31	>100	>100
MDA-MB-453 (*breast*)	3.9 ± 0.58	4.0 ± 0.03	>100	>100
SW1573 (*lung*)	11 ± 0.9	12 ± 6	>100	>100
T-47D (*breast*)	1.1 ± 0.34	15 ± 7.5	>100	>100
WiDr (*colon*)	11± 0.72	12 ± 5.4	>100	>100

5.3. Cell migration studies

As a model to study cell migration, we used the wound healing assay (or scratch assay). The basic steps involve creating a wound (scratch) in a monolayer culture, recording of the images at the beginning and at regular intervals during cell migration to close the wound, and the comparison of the images to quantify the migration rate of the cells. In this study, we selected HeLa cells since in our group they have shown the best cell line for this type of assay. From the four compounds selected for this study (Figure 5), BLB0013 and BLB0014 produced cell death (even at low micromolar concentration) and we were not able to study cell migration. In contrast, we treated HeLa cells with BLB0015 and BLB0016 (100 μM) and we were able to observe that the compounds did not interfere with cell migration (Table 3).

Table 3. Representative microscopy images of the wound healing assay for treated HeLa cells.

	Time of exposure (h)		
Compound (dose)	**0**	**22**	**30**
BLB0015 (100 μM)			
A (%)	100	40	36
BLB0016 (100 μM)			
A (%)	100	39	26

5.4. Cell uptake studies

To confirm whether the reduced cell viability was due to SLC6A14 inhibition, fluorescence microscopy was used to detect cellular uptake of the compounds. Fluorescent probe candidates can exert their inhibitory action on cell growth in three ways: (a) causing blockage of SLC6A14 transporter and inducing cellular starvation, (b) entering the cell by

passive diffusion or (c) being introduced by SLC6A14 transporter. In the last two cases, compounds exert their action within the cell and they represent unwanted effects.

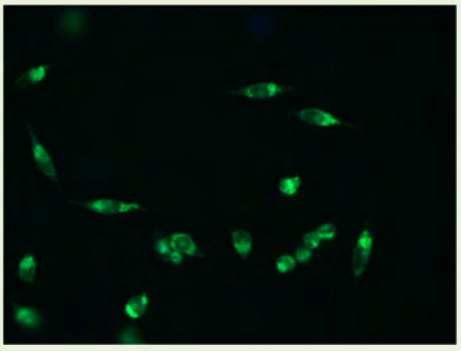
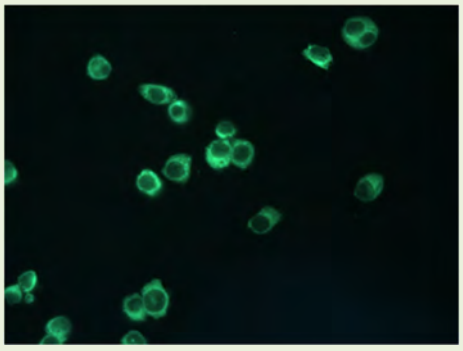
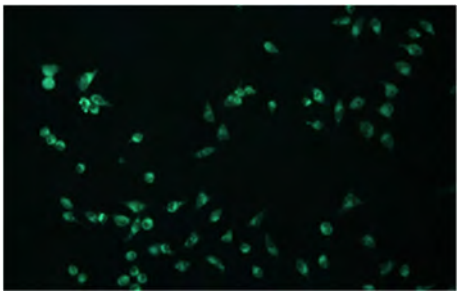
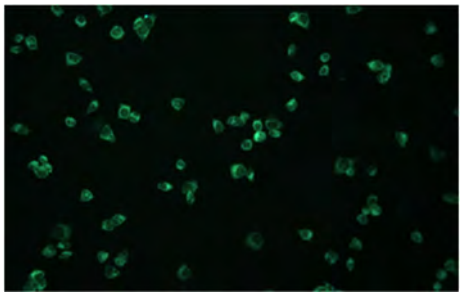
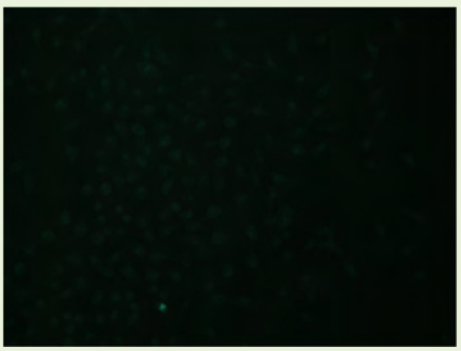
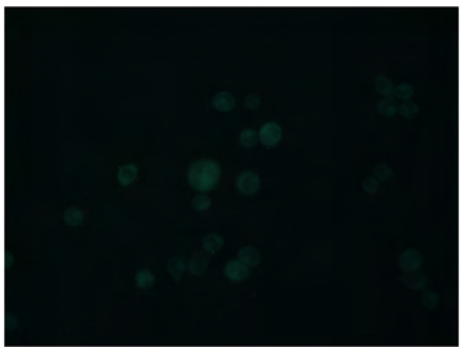
Compound	HeLa	MDA-MB-453
BLB0013		
BLB0014		
BLB0015		
BLB0016		

Figure 7. Fluorescence micrographs of HeLa and MDA-MB-453 cells exposed to compounds BLB0013, BLBL0014, BLB0015 and BLB0016.

HeLa [SLC6A14 (+)]and MDA-MB-453 [SLC6A14 (–)] cells were grown on coverslips by placing them inside each well of a 6-well plate before cell inoculation. This allowed easy staining of cells and visualizing the results. After incubation with the fluorescent probe candidates, we observed that BLB0013 and BLB0014 produced fluorescence inside both cell lines (Figure 7), which lead us to rule out SLC6A14 inhibition. We speculate that those compounds entered the cells by passive diffusion and induced antiproliferative effects by mechanisms other than SLC6A14 inhibition. In contrast, BLB0015 and BLB0016 did not fluoresce in any of the cell lines tested (Figure 7). This lack of uptake can be attributed to the carboxylate group, which provides the molecule with a net negative charge at physiological pH and thus prevents cellular uptake by passive diffusion. Overall, the fluorescent imaging data is consistent with the chemosensitivity testing results (Table 2) and show that the four compounds tested do not represent good candidates for a fluorescent assay to identify small molecule inhibitors of SLC6A14.

CONCLUSIONS

After a detailed literature search, we did not find enough information of SLC6A14 inhibition or even of methods to detect inhibition by small molecules of amino acid transporters. In view of the results, our group has envisioned the development of a fluorescent method to detect small molecule inhibitors of SLC6A14. From the results of this study, we can conclude that:

1. We determine the expression levels of SLCA14 in the human breast cancer cell lines T-47D and HBL-100, which were (+) and (–), respectively.
2. The fluorescent probe candidates BLB0013 and BLB0014 produced cell growth inhibition in cancer cells, whilst BLB0015 and BLB0016 were inactive.
3. BLB0013 and BLB0014 enter the cells by passive diffusion, whilst BLB0015 and BLB0016 do not enter cells.
4. The fluorescent probe candidates are not good candidates to detect small molecule inhibitors of SLC6A14.

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