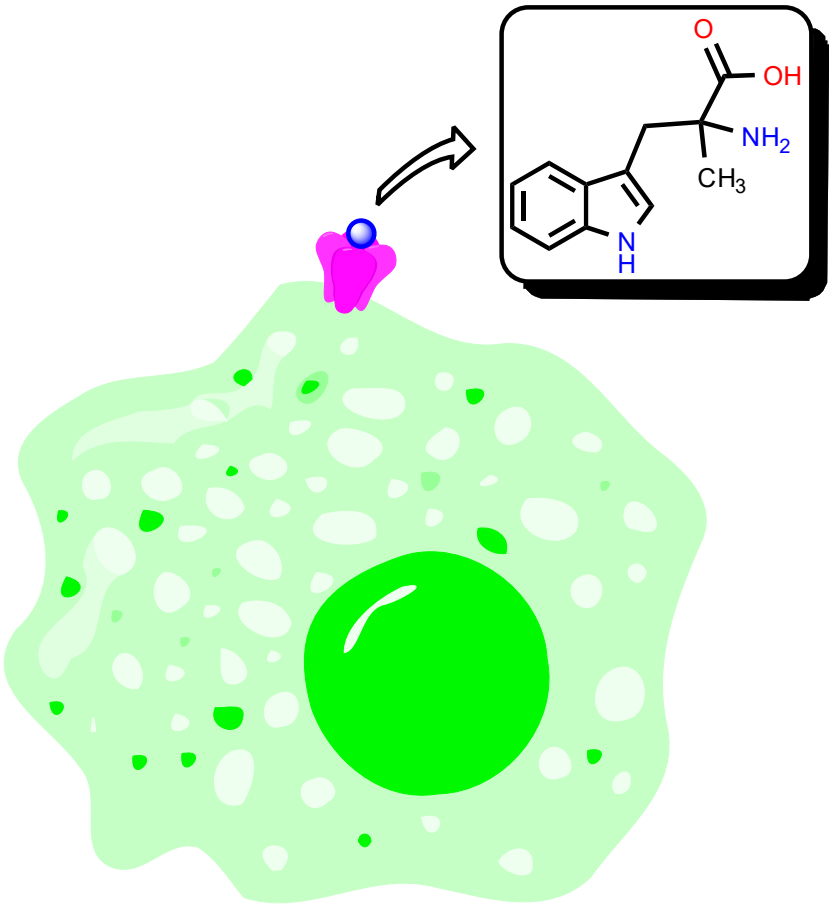
Instituto Universitario de
Bio-Orgánica Antonio González
Universidad de La Laguna

SELECTIVE INHIBITORS OF SLC6A14



Trabajo de Fin de Máster

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La presente memoria de investigación ha sido realizada por Juan Benamer Díaz Pérez 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

The overexpression of the amino acid transporter SLC6A14 has been identified in cancer cells. It is believed that the inhibition of this transporter could be an alternative as an effective treatment against cancer. The only known inhibitor of this transporter is α-methyl-tryptophan. Unfortunately, its use as a drug is unfeasible because millimolar quantities are needed to obtain a pharmacological effect. The design of inhibitory molecules of this transporter, and the design of a fluorescent probe for an inhibition assay are the pillars of this work.

RESUMEN

Se ha identificado una sobreexpresión del transportador de aminoácidos SLC6A14 en células cancerígenas. Se cree que la inhibición de este transportador podría ser una alternativa como tratamiento efectivo contra el cáncer. El único inhibidor conocido de este transportador el α-metil-triptófano. Desafortunadamente su uso como fármaco es inviable debido a que se necesitan cantidades milimolares para obtener un efecto farmacológico. El diseño de moléculas inhibidoras del transportador SLC6A14, y el diseño de una sonda fluorescente para un ensayo de inhibición son los objetivos de esta memoria de investigación.

INTRODUCTION

Cancer refers to a multitude of diseases where abnormal cell overgrowth occurs with really dangerous results for the subject who suffers from it. Many diseases, where cancer is also included, depend on the metabolism of amino acids to cause this pathological overgrowth in the patient [1]. It should be noted that amino acids cannot cross the membrane by passive diffusion, which is why our cells express specific transporters to help internalize these amino acids [2]. These transporters represent a very important pharmacological target, since the inhibition of amino acid metabolism within these tumor cells can provoke tumor cell death and slow down the progress of the disease. Inhibition of this type of amino acid transporters can cause death by starvation since the synthesis of amino acids, lipids and nucleotides would be affected, causing a decrease in the activity of the mTOR protein and causing cell death (Figure 1).

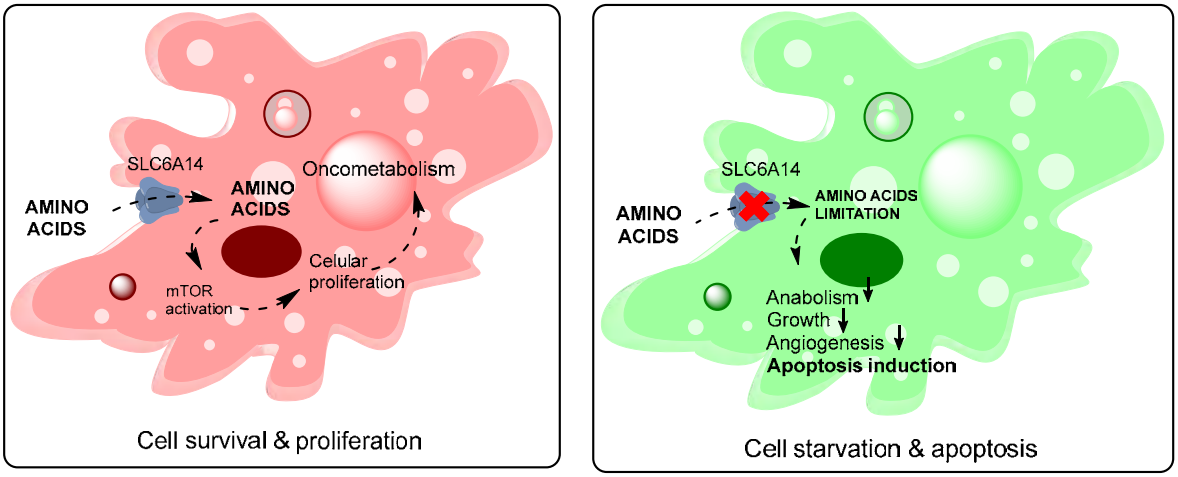


Figure 1. Role of SLC6A14 in cell proliferation and consequences of its inhibition.

Within this type of specific amino acid transporters there is a transporter called SLC6A14 (member 14 of the family of solute transporters 6) which is the only one amino acid transporter that has broad substrate specificity [3]. Furthermore, overexpression of this transporter has been seen in tumor cells versus healthy cells. Thus, the selective blocking of the SLC6A14 transporter may be a good therapeutic strategy as a cancer treatment. SLC6A14, also known as $ATB^{0,+}$, can transport 18 of the 20 amino acids into cells, excluding glutamic acid and aspartic acid from this list. In addition to natural amino acids, SLC6A14 can transport D-amino acids such as D-serine, D-alanine, D-methionine, D-leucine and D-tryptophan [4]. SLC6A14 is a Na^+/Cl^- coupled amino acid transporter whose expression is up-regulated in various types of cancer, such as colon, pancreas and breast [5].

Currently, the only known inhibitor of SLC6A14 (iSLCA14) transporter is α-methyl-tryptophan (α-MT). The mechanism of action of α-MT is based on the blockade of this transporter, causing it to induce amino acid deprivation, decrease mTOR activity, increase autophagy, promote apoptosis, and suppress cell proliferation and invasion [6].

Unfortunately, the use of α-MT is not feasible because the minimum concentration necessary to produce a pharmacological effect in cell cultures is 1.5 mM, which makes it impossible for a galenic formulation. This is where the organic synthesis of new small inhibitory molecules of amino acid transporters comes into play.

In our group, we have discovered two groups of potential iSLCA14 (Figure 2). The first group includes glutamic acid-based dipeptides (GABDs). GABDs had antiproliferative activity in all solid tumors tested. However, the tryptophan dipeptides did not show activity against the HBL-100 cell line, which does not overexpress the transporter [7]. The second group corresponds to the naphthol derivatives of Betti bases (NDBBs). Of the small library of molecules of NBDDs, fourteen compounds were less active in HBL-100 breast cancer cells than in T-47D breast cancer cells [8]. HBL-100 cells are SLC6A14(–) since they do not express the transporter. In contrast, T-47D cells are SLC6A14(+) because they overexpress the amino acid transporter. NBDDs behave like tryptophan mimetic structures inducing antiproliferative activity. However, the lack of a method to test SLC6A14 inhibition did not allow to confirm the aforementioned GABDs and NBDDs as iSLC6A14.

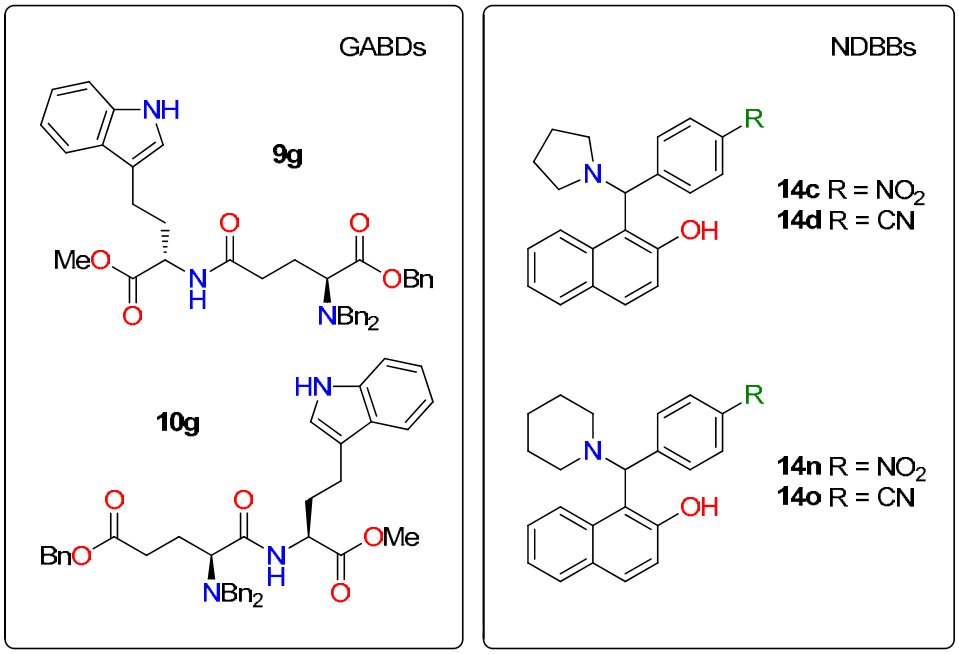


Figure 2. Chemical structure of candidate iSLC6A14. Left: GABDs. Right: NDBBs.

Our interest in this work is focused on the search for new possible antitumor drugs, and some possible candidates may be tryptophan-based dipeptides. As we have already mentioned, GABDs have a potential inhibitory activity against the SLC6A14 transporter, which would demonstrate that these dipeptides based on tryptophan can be used as antitumor drugs. In addition to the design and synthesis of iSLC6A14, we are involved in the development of a method to assess these iSLC6A14 candidates. In our group, we have envisioned a fluorescence-based test (Figure 3), which requires the development of a fluorescent tagged substrate. The synthesis and evaluation of some probe candidates will be presented.

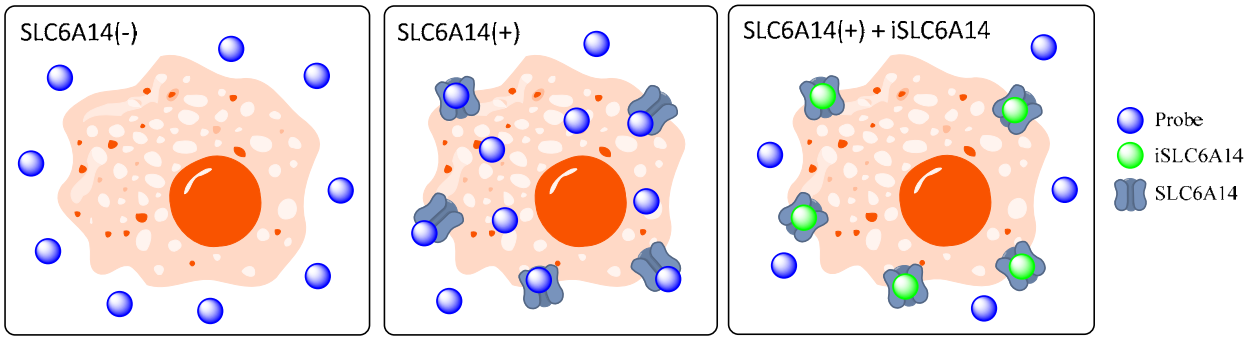


Figure 3. Fluorescence cell-based assay to identify iSLC6A14.

HYPOTHESIS

α-MT has a potentially inhibitory activity against the SLC6A4 transporter. Therefore, α -MT represents a possible lead for the synthesis of selective SLC6A14 inhibitors with better pharmacological profiles (Figure 4).

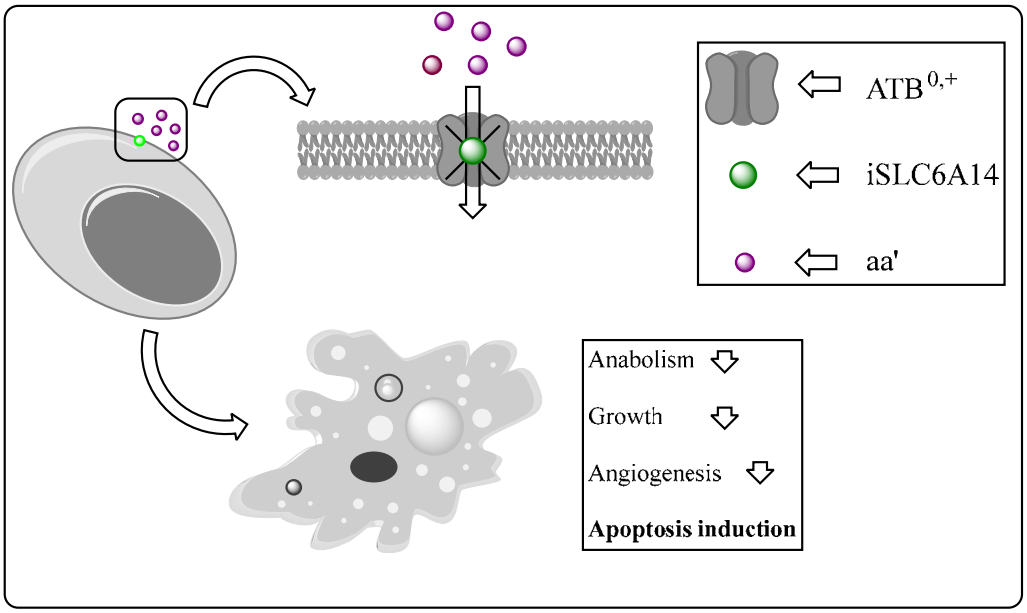


Figure 4. Schematic representation of the effect of SLC6A14 inhibition on amino acid cellular uptake.

OBJECTIVES

The general objective of this work is to obtain an inhibitor of the SLC6A14 transporter with an improved pharmacological profile. To achieve this general objective, we propose the following specific objectives:

1. To design and to synthesize a small library of tryptophan-based dipeptides (TBDs) as potential iSLC6A14 (Figure 5).

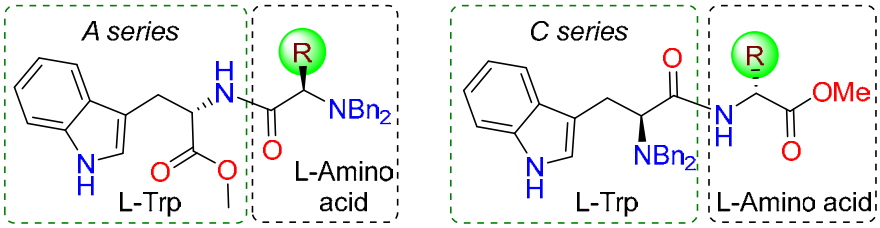


Figure 5. Chemical structure of the proposed tryptophan based-dipeptides.

2. To prepare a tryptophan-based fluorescent probe to develop a SLC6A14 inhibition assay (Figure 6).

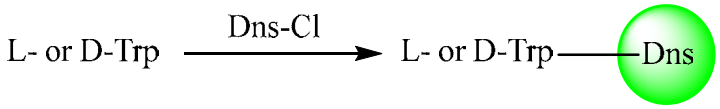


Figure 6. Chemical structure of the proposed fluorescent probe candidate.

MATERIALS AND METHODS

Chemicals and reagents

All the solvents and reagents were used as received from the commercial sources. Reactions were monitored using thin-layer chromatography (TLC) on aluminum packed percolated Silica Gel 60 F254 plates. Flash column chromatography was carried out with silica gel 60 (particle size less than 0.020 mm) by using appropriate mixtures of ethyl acetate and *n*-hexane as eluent. Compounds were visualized by use of UV light, ninhydrin (0.2% in ethanol) and phosphomolybdic acid (2.5% in ethanol).

Experimental procedures

(S)-methyl 2-(5-(dimethylamino)naphthalene-2-sulfonamido)-3-(1H-indol-3-yl)propanoate ***(13)***. To a solution of L-tryptophan methyl ester hydrochloride (1.0 g, 3.9 mmol) in dry dichloromethane (19.5 mL) at room temperature, triethylamine (1.7 mL, 11.7 mmol) and dansyl chloride (1.9 g, 7.02 mmol) were added sequentially. The reaction was stirred for 48 hours, after which time the solvent was evaporated. The residue was purified by flash chromatography on silica gel to give as a yellow oil (1.4 g, 82% yield) as a light green solid.

*((5-(dimethylamino)naphthalen-1-yl)sulfonyl)-L-tryptophan(****14****).* To a solution of **13** (508 mg, 1.13 mmol) in dioxane (4.5 mL), NaOH (49 mg, 1.24 mmol) was added. The reaction was stirred until completion, as shown on TLC. Then, 5% HCl (2 mL) was added and the mixture was extracted with AcOEt (3 x 20 mL). The organic phase was dried with $MgSO_4$, filtered, concentrated and the crude was purified by chromatographic column to give **14** (454 mg, 92% yield) as a yellow solid.

5-(Dimethylamino)-N-(3-hydroxypropyl)naphthalene-1-sulfonamide ***(15)***. To a solution of 5-aminopentan-1-ol and Et_3N in anhydrous CH_2Cl_2 under argon atmosphere at 0 ºC was added dansyl chloride. The reaction was stirred overnight and then it was quenched with H_2O (90 mL) and extracted with CH_2Cl_2. The organic phase was dried with $MgSO_4$, filtered, concentrated and the crude was purified by chromatographic column to give **15** (2.26 g, 88% yield) as a yellow solid.

5-((5-(dimethylamino)naphthalene)-1-sulfonamido) pentyl (tert-butoxycarbonyl) tryptophanate ***(16)***. To a solution of 2-((*tert*-butoxycarbonyl) amino)-3-(1*H*-inden-3-yl)

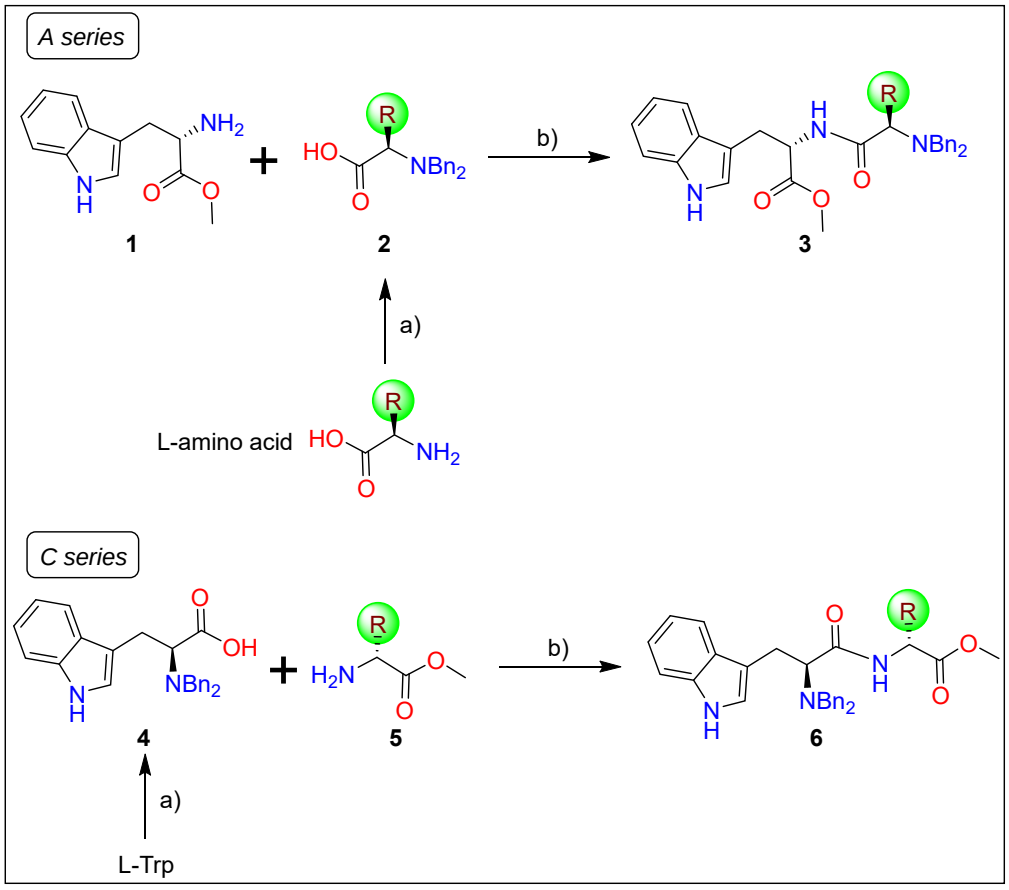
propanoic acid (1.0 g, 3.27 mmol) and **15** (1.1 g, 3.27 mmol) in anhydrous CH_2Cl_2 (33 mL) under argon atmosphere was added *N*,*N*–dicyclohexylcarbodiimide (743 mg, 3.60 mmol) and DMAP (4 mg, 0.033 mmol). The reaction was stirred overnight and when the reaction was over, the solvent was concentrated and the crude was purified by flash chromatography on silica gel to give **16** (1.47 g, 72% yield) as a yellow–green oil.

RESULTS & DISCUSSION

Tryptophan-based dipeptides

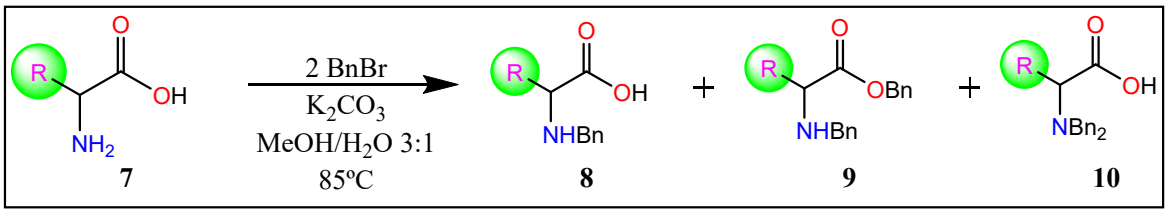
For the synthesis of TBDs as inhibitors of the SLC6A14 transporter, different approaches are possible (Scheme 1). It must be considered that we can form dipeptides based on tryptophan by forming the peptide bond at the amino end (*A series*) as well as at the carboxylic end (*C series*).

In previous studies, the reported iSLC6A14 were linked at the amino terminus of tryptophan. Then, for the synthesis of TBDs, we can use tryptophan methyl ester as starting material, which we will make it react with different *N*,*N*-dibenzylated amino acids. The coupling between the amino end of tryptophan and the carboxylic end of the different amino acids can be performed using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as the coupling agent, DMF as solvent and *N*,*N*-diisopropylethylamine as base.



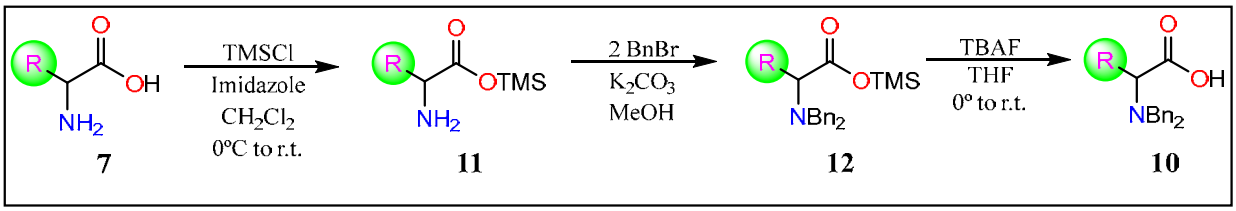
Scheme 1. Proposed synthesis of TBDs. Reagents and conditions: a) i) TMSCl, imidazole, CH_2Cl_2, 0º to r.t., ii) BnBr, K_2CO_3, MeOH, Δ, iii) TBAF, THF, 0ºC to r.t. b) TBTU, DIPEA, DMF, r.t., overnight.

The *N*,*N*-dibenzylation of free amino acids (**7**), despite being an apparently easy process, turn to a nightmare and has not been accomplished until present. Several unsuccessful attempts were tried. Our group reported the tribenzylation of glutamic acid using stoichiometric amounts of the benzylating agent [9]. Thus, we studied the benzylation of amino acids (having the free carboxylic end) under the same conditions, i.e. a 3:1 methanol:water mixture and K_2CO_3 as base (Scheme 2). Instead, we used just 2 equivalents of benzyl bromide in order to obtain the *N*,*N*-dibenzylation of the amino group and preventing the excess of benzyl bromide from reacting with the carboxylic acid. Unfortunately, these reaction conditions caused the formation of unwanted by-products. In the best scenario, we got the *N*-benzylated compound **8** and the *N*,*O*-dibenzylated derivative **9**. The desired *N*,*N*-dibenzylated of *N*, was obtained in a very low yield to be considered viable for the preparation of TBDs. Despite the different conditions tried, we were not able to get **10**.



Scheme 2. Benzylation of free amino acids.

To solve the problem of the nucleophilicity of the hydroxyl group of the carboxylic acid, we have considered an alternative approach for the synthesis of the *N*,*N*-dibenzylated amino acids (Scheme 3). We propose to start from the amino acid and carry out a protection at the acidic terminus with trimethylsilane chloride to give **11**. Then, it should follow the dibenzylation on the nitrogen to obtain **12**. Finally, the deprotection of the ester will give *N*,*N*-dibenzylated amino acid **10**. This alternative pathway has not been tried in the laboratory since we have directed our efforts to the preparation of the fluorescent probe candidate.



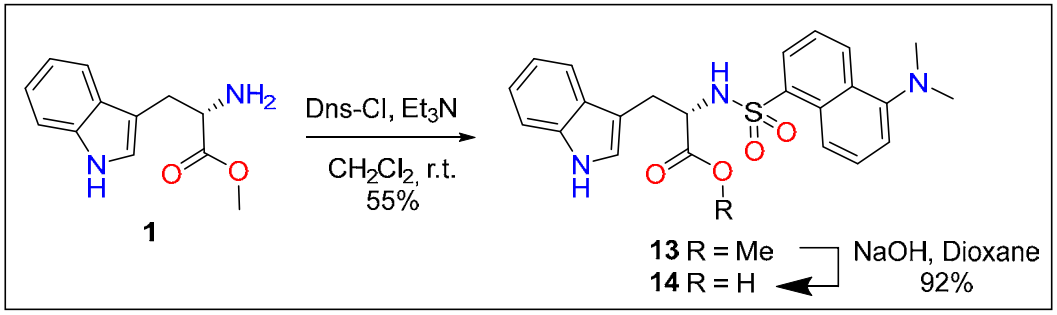
Scheme 3. Proposed alternative synthesis for *N*,*N*-dibenzylated amino acids.

Tryptophan-based fluorescent probes

In order to identify iSLC6A14, our group is involved in the development of an inhibition assay, since currently there are no commercial kits or non-radioactive methods reported in the literature to study this biological effect. We have envisioned a fluorescence assay to determine if the absorption of amino acids is influenced by TBDs (Figure 3). Fluorescence assays have been used throughout the history of science to determine a multitude of biological processes in vivo, to identify formation as the conformation of protein complexes, or to identify their location, among other things.

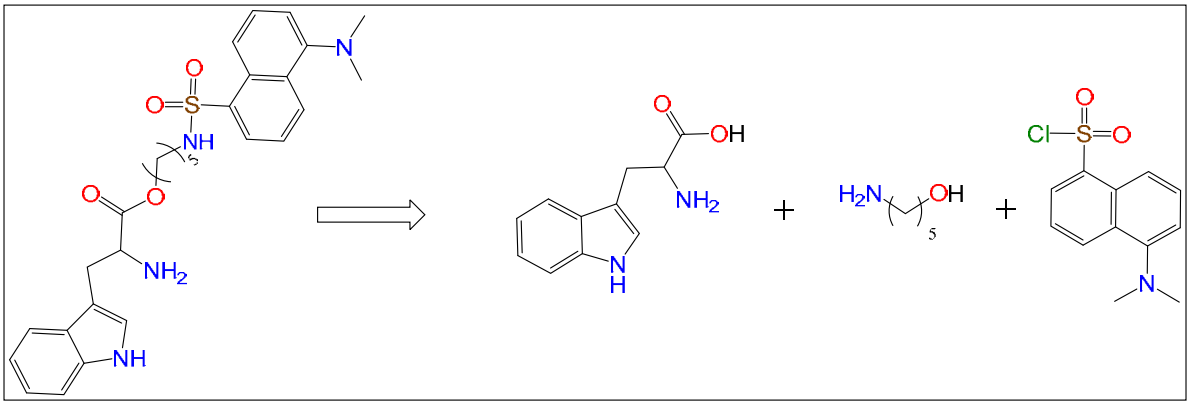
In essence, our approach needs the development of a fluorescent probe that will be internalized exclusively by the cells that express the transporter. Previously, our group reported the synthesis and evaluation of a dansyl (Dns) probe of the tubulin depolymerizing agent DTA0100 [10]. Analogously, we consider as initial approach binding the fluorescent tag Dns to a molecule that behaves as a transported substrate and has a certain affinity for SLC6A14. For this purpose, we selected the amino acid tryptophan.

In a previous work, we reported the synthesis of *N*-Dns-tryptophan methyl ester (**13**) (Scheme 4) [11]. Compound **13** was tested against a panel of six human solid tumor cell lines to check for its antiproliferative activity. The results (Table 1) show that the compound is able to induce antiproliferative effects in SLC6A14(+) and SLC6A14(–) cell lines. Thus, compound **13** is not a valid probe for the SLC6A14 inhibition assay.



Scheme 4. Synthesis of *N*-Dns-tryptophan methyl ester (**13**).

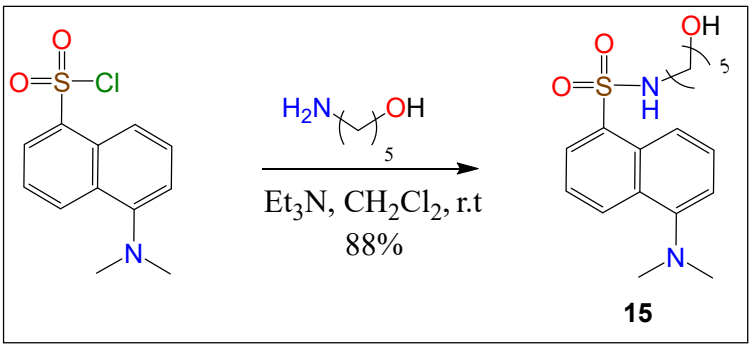
In this study, we continued the preparation of Dns derivatives of tryptophan to be studied as fluorescent probe candidates. Thus, we submitted **13** to saponification under standard conditions to afford acid **14** in 92% yield (Scheme 4). We considered the synthesis of additional tryptophan derivatives bearing the Dns group on the carboxylic acid. This time, the insertion of the Dns group has to be done using a linker (Scheme 5) since the direct coupling to the acid group of tryptophan is not possible.



Scheme 5. Retrosynthetic analysis.

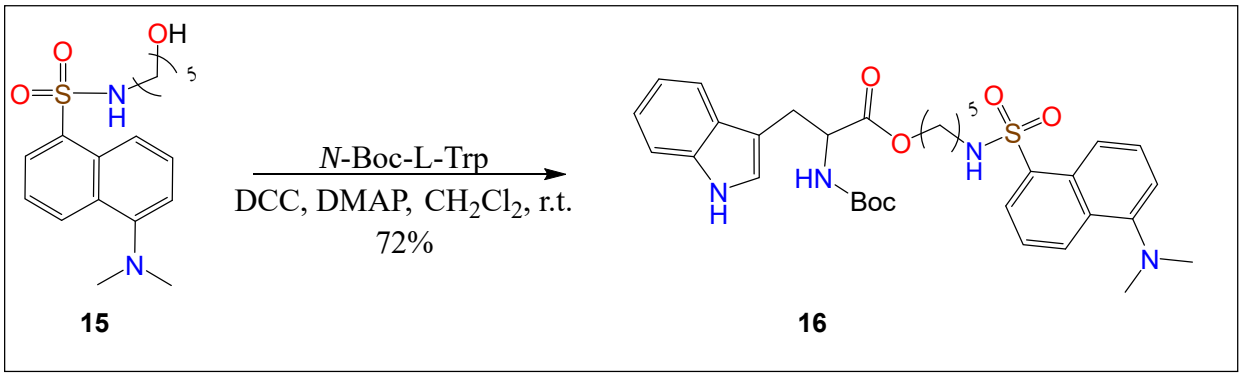
Our synthetic strategy is based on the attachment of Dns to a linker. For this role, we selected 5-amino-1-pentanol. The Dns group will bind the amino group through a sulfonamide bond and tryptophan will link the alcohol trough an ester bond.

The first step was the coupling of 5-amino-1-pentanol to DnsCl using 2 equivalents of triethylamine as base and reaction catalyst, and dichloromethane as reaction solvent (Scheme 6). The reaction was successful with a yield of 88% to afford intermediate **15**. The mechanism of the reaction involves the displacement of the chloride group of Dns-Cl by the amino group of 5-amine-1-pentanol.



Scheme 6. Synthesis of intermediate **15**.

The second step was the coupling of the alcohol to the substrate. We used commercially available *N*-Boc-L-Trp as reagent to link to the intermediate **15**. The coupling was carried out by a Steglich esterification reaction using DCC as the coupling agent and DMAP as the catalyst in dichloromethane as the solvent (Scheme 7). Compound **16** was obtained in 72% yield.



Scheme 6. Synthesis of intermediate **16**.

Finally, we attempted to remove the Boc group under standard conditions, i.e. trifluoroacetic acid (10 equiv.) in dichloromethane. Although the reaction proceeded smoothly, during the workup the compound decomposed. Therefore, the preparation of this fluorescent probe candidate remains to be completed.

Biological evaluation

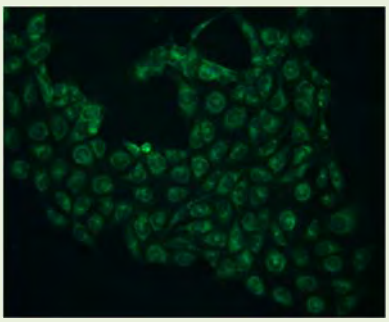
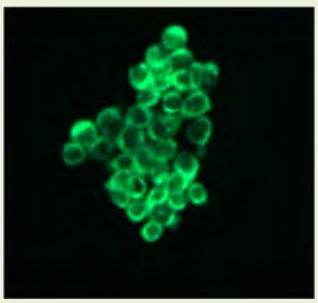
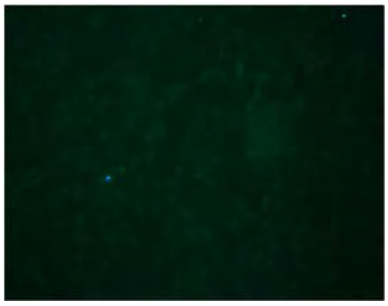
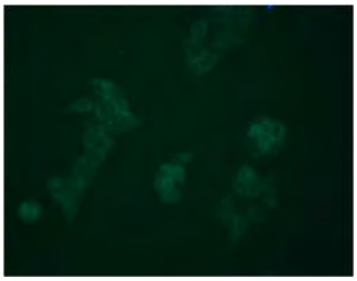
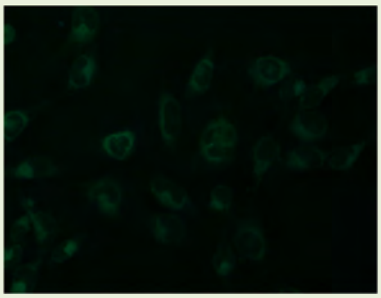
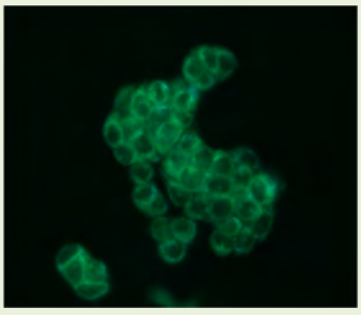
In our group, the antiproliferative activity of the fluorescent probes was studied against SLC6A14(+) and SLC6A14(–) cell lines [12] [13]. The results are shown in Table 1. Besides the tryptophan analogs **13**, **14** and **16**, the non-tryptophan intermediate **15** was tested also for comparison purposes. From the growth inhibition data, we can infer some preliminary considerations. In general, we have observed in our screening program that HBL-100 is a cell line more sensitive to the compounds than T-47D, as observed for non-trytophan derivative **15**. However, compounds **13** and **16** are more active in T-47D cells (SLC6A14 +) than in HBL-100 cells (SLC6A14 –). We speculate that the difference on the expression level of SLC6A14 could explain this trend. In addition, compound **16** is less active than **13**, which shows that the fluorescent tag should be better placed on the carboxyl side. Compound **14** is inactive (GI_{50} > 100 µM, the maximum test concentration) in both cell lines. This result can be a consequence of the carboxylate group that at physiological pH is in ionized form, which does not allow the molecule to cross the cell membrane by passive diffusion.

Table 1. Antiproliferative activity (GI_{50}, µM) against human breast cancer cell lines.

Cell Line	SLC6A14	13	14	15	16
HBL-100	–	15 ± 1.3	>100	69 ± 16	40 ± 3.9
T-47D	+	1.1 ± 0.34	>100	86 ± 25	20 ± 2.3

In addition to the growth inhibition tests, the cellular uptake of the probe candidates was tested using fluorescence microscopy. The results are shown in Table 2. Compounds **13** and **16** internalize in both cell lines, whilst **14** does not enter cells. These results are consistent with the growth inhibition data from Table 1. One interesting observation that needs to be confirmed with quantitative methods, is the apparent larger uptake of **13** and **16** in the SLC6A14 (+) cell line.

Table 2. Fluorescence micrographs of HBL-100 and T-47D cells exposed to probes **13**, **14**, and **16**.

Compound	HBL-100	T-47D
13		
14		
16		

Overall, the results point out three relevant consequences: a) **13** and **16** enter cells independently of SLC6A14 transport, presumably by passive diffusion, b) **13** and **16** affect more those cell lines overexpressing SLC6A14, and c) SLC6A14 is not the only cellular target.

CONCLUSIONS

In the literature, there is not enough information of iSLC6A14 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) tryptophan-based dipeptides as potential iSLC6A14 and b) a fluorescent method to detect small molecule iSLC6A14. In this study, we explored these two subjects. From the results, we can conclude that:

1. The synthesis of tryptophan-based dipeptides could not be accomplished, being the main problem the direct synthesis of dibenzyl amino acids from amino acids.

2. We prepared a small set of dansylated tryptophan derivatives using general synthetic methods.

3. We found that the position of the dansyl group in the tryptophan scaffold modulates the biological response.

4. The fluorescent compounds did not show complete selectivity towards the SLC6A14 (+) cell line.

REFERENCES

[1] Wu G. (2013). Functional amino acids in nutrition and health. Amino Acids, 45, 407–411.

[2] Zhang, L., Sui, C., Yang, W., & Luo, Q. (2020). Amino acid transporters: Emerging roles in drug delivery for tumor-targeting therapy. Asian Journal of Pharmaceutical Sciences, 15, 192–206.

[3] Sikder, M., Yang, S., Ganapathy, V., & Bhutia, Y. D. (2017). The Na^+/Cl^--coupled, broad-specific, amino acid transporter SLC6A14 ($ATB^{0,+}$): Emerging roles in multiple diseases and therapeutic potential for treatment and diagnosis. The AAPS Journal, 20, 12.

[4] Hatanaka, T., Huang, W., Nakanishi, T., Bridges, C. C., Smith, S. B., Prasad, P. D., Ganapathy, M. E., & Ganapathy, V. (2002). Transport of D-serine via the amino acid transporter $ATB(^{0,+})$ expressed in the colon. Biochemical and Biophysical Research communications, 291(2), 291–295.

[5] Palazzolo, L., Paravicini, C., Laurenzi, T., Adobati, S., Saporiti, S., Guerrini, U., Gianazza, E., Indiveri, C., Anderson, C., Thwaites, D. T., & Eberini, I. (2019). SLC6A14, a pivotal actor on cancer stage: When function meets structure. SLAS Discovery, 24(9), 928–938.

[6] Sikder, M., Sivaprakasam, S., Brown, T. P., Thangaraju, M., Bhutia, Y. D., & Ganapathy, V. (2020). SLC6A14, a Na^+/Cl^--coupled amino acid transporter, functions as a tumor promoter in colon and is a target for Wnt signaling. The Biochemical Journal, 477, 1409–1425.

[7] Silveira-Dorta, G., Martín, V. S., & Padrón, J. M. (2015). Synthesis and antiproliferative activity of glutamic acid-based dipeptides. Amino Acids, 47, 1527–1532.

[8] Puerta, A., Galán, A. R., Abdilla, R., Demanuele, K., Fernandes, M. X., Bosica, G., & Padrón, J. M. (2019). Naphthol-derived Betti bases as potential SLC6A14 blockers. Journal of Molecular Clinical Medicine, 2, 35–40.

[9] Padrón, J. M., Silveira-Dorta, G., & Martín, V. S. (2014). Direct synthesis of polybenzylated glutamic acid monoesters: Disambiguation of *N*,*N*-dibenzylglutamic acid α- and γ-benzyl esters. Synlett, 25(15), 2166–2170.

[10] Podolski-Renić, A., Banković, J., Dinić, J., Ríos-Luci, C., Fernandes, M. X., Ortega, N., Kovačević-Grujičić, N., Martín, V. S., Padrón, J. M., & Pešić, M. (2017). DTA0100, dual topoisomerase II and microtubule inhibitor, evades paclitaxel resistance in P-glycoprotein overexpressing cancer cells. European Journal of Pharmaceutical Sciences, 105, 159–168.

[11] Pérez Díaz, J. B. (2020). Discovery of selective inhibitors of SLC6A14. Undergraduate Thesis. Universidad de La Laguna.

[12] Carniglia, G. (2021). Early pharmacological profiling of small-molecules: identification of SLC6A14 inhibitors. Master Thesis. Universidad de La Laguna.

[13] Fumero Cruz, P. (2021). Early pharmacological profiling of SLC6A14 inhibitors. Undergraduate Thesis. Universidad de La Laguna.