Instituto Universitario de
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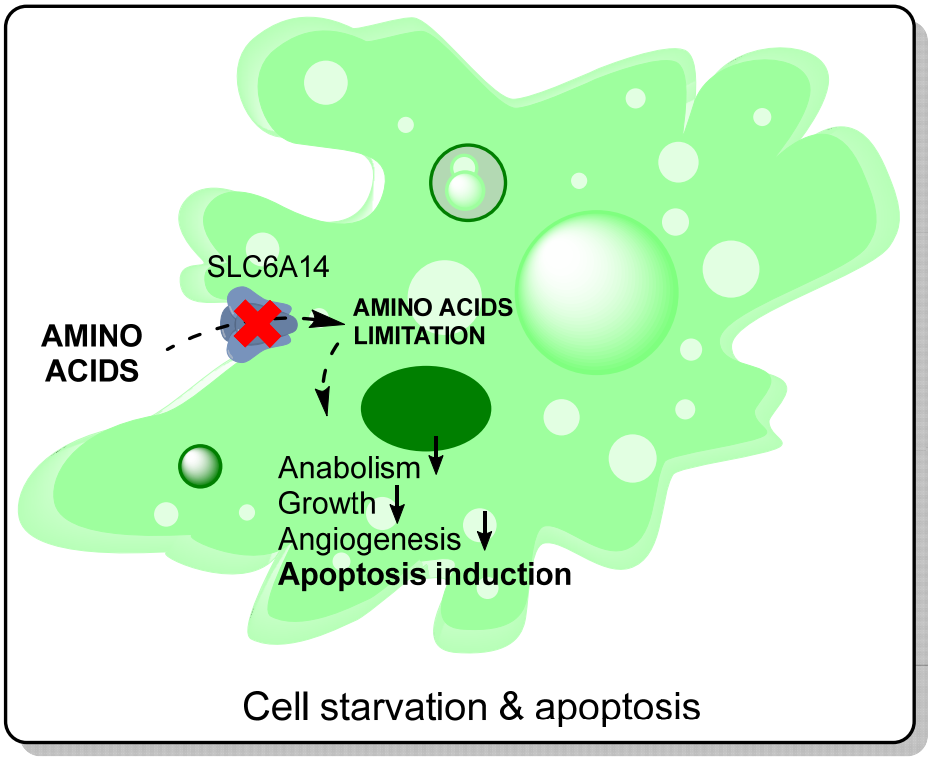
Universidad de La Laguna

DISCOVERY OF SELECTIVE INHIBITORS OF SLC6A14



Trabajo de Fin de Grado

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La presente memoria de investigación ha sido realizada por el alumno Juan Benamer Díaz Pérez durante el curso académico 2019-2020 en las instalaciones del Instituto Universitario de Bio-Orgánica "Antonio González" (IUBO-AG) 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.*

La Laguna, a 5 de julio de 2020.

Fdo. José M. Padrón

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ABSTRACT

Amino acids play a key role in human biology. Currently they are object of study because they are involved in various proliferative diseases, including cancer. The amino acid transporter SLC6A14 has been identified overexpressed in diverse tumors. SLC6A14 represents a druggable target for cancer treatment. However, there is only one reported inhibitor of the protein, α-methyltryptophan, which has very low potency. Thus, inhibitors of SLC6A14 are in need.

In this study we planned the preparation of a small library of tryptophan-based dipeptides (TBDs). The compounds were designed based on the previous results of the group. In addition to TBDs, we prepared a fluorescent derivative of tryptophan. We chose dansyl as fluorescent group. A fluorescent probe was synthesized, characterized and tested in vitro against human solid tumor cells. The results indicate that the fluorescent compound is not suitable for SLC6A14 inhibition assays.

RESUMEN

Los aminoácidos juegan un papel clave en la biología humana. Actualmente son objeto de estudio porque están involucrados en diversas enfermedades proliferativas, incluido el cáncer. El transportador de aminoácidos SLC6A14 se ha identificado sobreexpresado en diversos tumores. SLC6A14 se presenta como un objetivo farmacológico para el tratamiento del cáncer. Sin embargo, solo hay un inhibidor reportado de la proteína, el α-metiltriptófano, que tiene una potencia muy baja. Por lo tanto, se necesitan inhibidores de SLC6A14.

En este estudio planteamos la preparación de una pequeña biblioteca de dipéptidos a base de triptófano. Los compuestos fueron diseñados en base a los resultados previos del grupo. Además de los dipéptidos, preparamos un derivado fluorescente de triptófano. Elegimos dansilo como grupo fluorescente. Se sintetizó una sonda fluorescente, se caracterizó y se probó in vitro contra células tumorales humanas. Los resultados indican que el compuesto fluorescente no es adecuado para los ensayos de inhibición de SLC6A14.

INTRODUCTION

Amino acids play a fundamental role in cell development. Many diseases depend on the metabolism of amino acids to perpetuate their survival, and a clear example is cancer. The function of amino acids is not only to be part of the protein structure, but they also act as secondary metabolites and bioactive molecules [1]. The mechanisms involved in the regulation of cancer progression by amino acids are barely known. Therefore, amino acid transporters represent relevant pharmacological targets. Some cancer cells depend on this transporter for cell proliferation, however, we could block this channel with either small molecules or monoclonal antibodies in order to stop cancer cell growth. Inhibition of this transporter would decrease the synthesis of amino acids, lipids and nucleotides, would slow down the action of the mTOR protein, blocking angiogenesis and produce cell death by starvation (Figure 1).

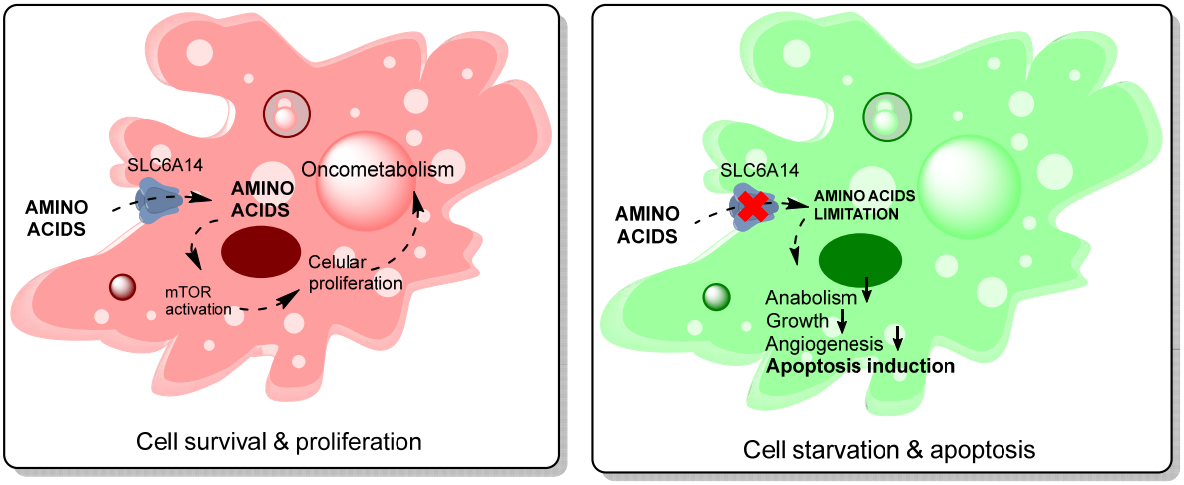


Figure 1. Role of SLC6A14 in cell proliferation and consequences of its inhibition.

Amino acids cannot cross cell membranes, which is why cells express a multitude of transporters in their plasma membranes to favor their passage to the cytosol [2]. Among the known amino acid transporters, SLC6A14 (solute carrier family 6 member 14) is the only amino acid transporter that has broad substrate specificity [3]. SLC6A14, also known as $ATB^{0,+}$, can transport inside cells 18 of the 20 proteinogenic amino acids (glutamic and aspartic acid are not transported). 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].

The treatment of colon cancer cells with α-methyltryptophan (α-MT), an inhibitor of SLC6A14 (iSLC6A14), induces amino acid deprivation, decreases mTOR activity, increases autophagy, promote apoptosis, and suppresses cell proliferation and invasion [6]. Furthermore, α-MT does not affect healthy cells. At this point, the design and synthesis of small molecule inhibitors of the amino acid transporters comes into play. Therefore, the inhibition of amino acid uptake in cancer cells represents a plausible strategy to discover new drugs with antiproliferative activity and potential application in cancer treatment.

In our group, we have discovered two families of potential iSLCA14 (Figure 2). The first family corresponds to glutamic acid-based dipeptides (GABDs). GABDs were able to inhibit all cancer solid tumors tested although tryptophan dipeptides showed no detectable activity against HBL-100 cells, which do not express the transporter SLC6A14 [7]. The second family includes naphthol-derived Betti bases (NDBBs). From a small library of NDBBs, fourteen compounds were less active in HBL-100 breast cancer cells (SLC6A14 negative) than towards the breast cancer cell line T-47D (SLC6A14 positive) [8]. NDBBs behave as tryptophan mimetics, blocking SLC6A14 and induced antiproliferative effects.

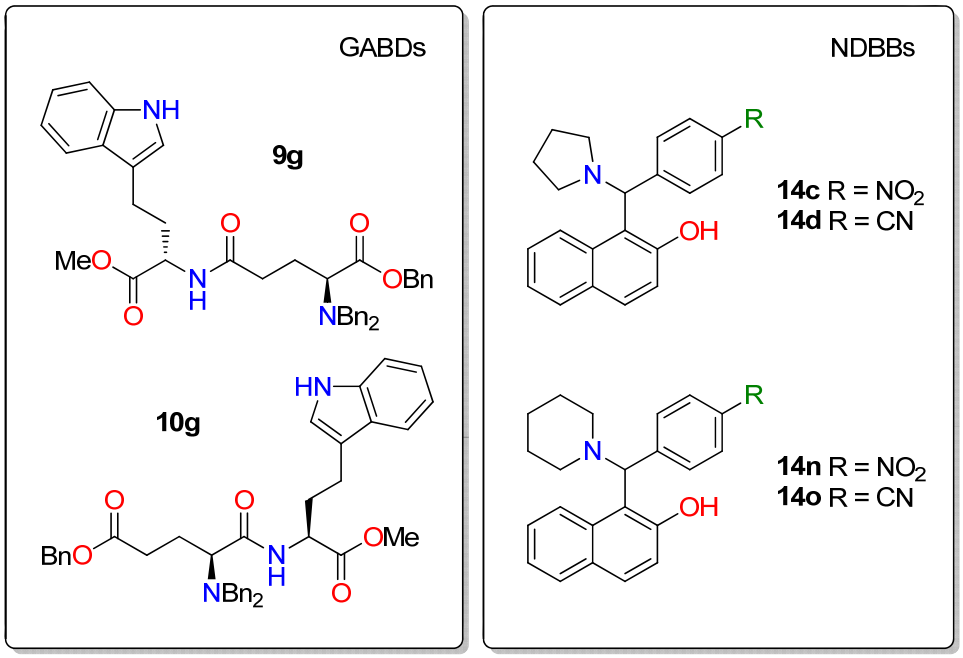


Figure 2. Chemical structure of potential SLC6A14 inhibitors. Left: GABDs. Right: NDBBs.

In this work, we have focused on the synthesis of tryptophan derivatives, which, as we have already seen, has a potentially inhibitory action against the SLC6A14 transporter. This would give us a clear view of the search for new therapeutic targets against cancer and would help us to demonstrate that the use of tryptophan derivatives may be candidates as new antitumor drugs.

HYPOTHESIS

The growth inhibition effects of α-MT have been demonstrated in tumor cells that overexpress the transporter SLC6A14. Therefore, α-MT represents a drug lead structure to design new potential iSLC6A14 that could induce cell death by starvation (Figure 3).

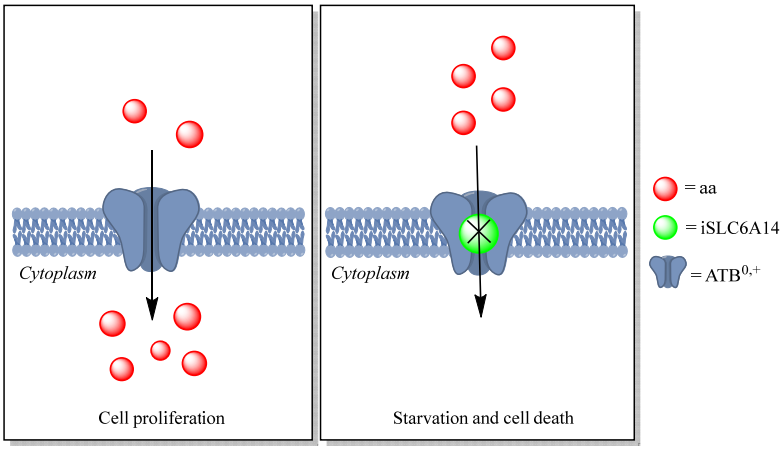


Figure 3. Schematic representation of the effect of SLC6A14 inhibition on amino acid cellular uptake.

OBJECTIVES

The general objective of this work is to obtain iSLC6A14 with enhanced pharmacological profile. To achieve this main 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 4).

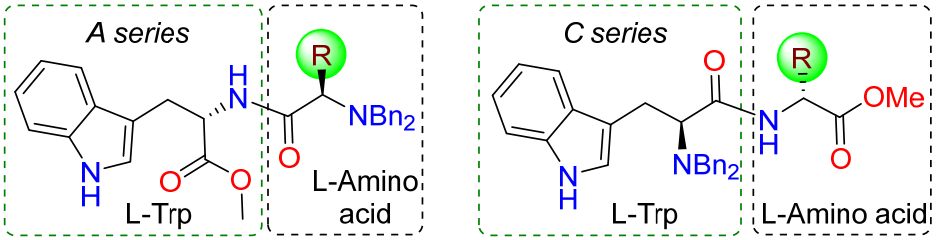


Figure 4. Chemical structure of the proposed tryptophan based-dipeptides.

2. To prepare a fluorescent probe to develop a SLC6A14 inhibition assay (Figure 5).

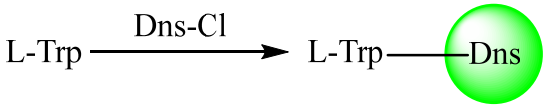


Figure 5. Chemical structure of the proposed fluorescent probe. *Dns = Dansyl.*

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 petroleum ether (PE) as eluent. Compounds were visualized by use of UV light, ninhydrin (0.2% in ethanol) and phosphomolybdic acid (2.5% in ethanol).

Characterization of compounds

Optical rotations were measured in $CHCl_3$ with a polarimeter at the sodium line. $^1H/^{13}C$ NMR spectra of the samples as $CDCl_3$ solutions were recorded at 400/100 MHz or at 500/125 MHz or 600/150 MHz, respectively, at 298 K. Chemical shifts (δ) are quoted in ppm and referenced to internal TMS (δ = 0 ppm) for ^1H NMR and $CDCl_3$ (δ = 77.0 ppm) for ^{13}C NMR; coupling constants (J) are quoted in hertz (Hz).

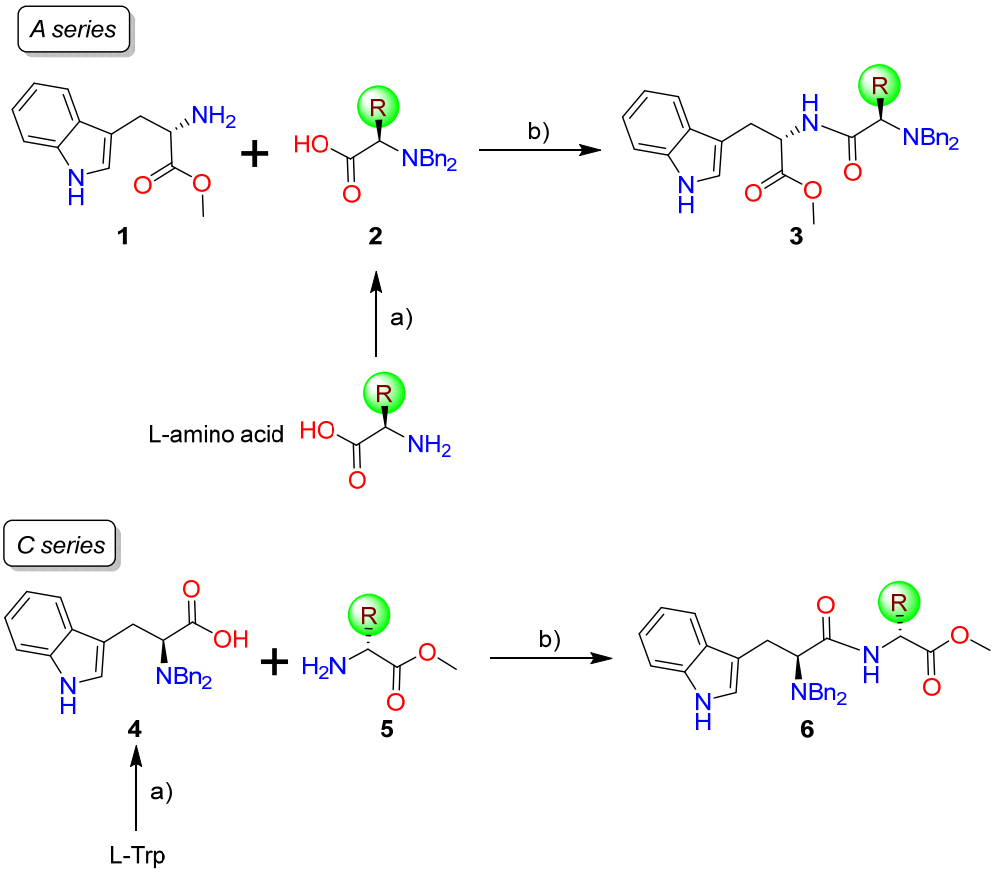
Experimental procedures

*Methyl ((5-(dimethylamino)naphthalen-1-yl)sulfonyl)-L-tryptophanate (**7**).* To a solution of L-tryptophan methyl ester hydrochloride (1 g, 3.9 mmol) in dry dichloromethane (39 mL) at room temperature, triethylamine (1.7 mL, 8.6 mmol) and dansyl chloride (1.15 g, 4.3 mmol) were added sequentially. The reaction was stirred overnight, after which time the solvent was evaporated. The residue was purified by flash chromatography on silica gel to give **7** as a yellow oil (963 mg, 55% yield). $[\alpha]_D^{20}$ = –3.46º (*c* 0.101, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ 2.86 (s, 6H), 3.17 (dd, J = 16.4, 6.6 Hz, 1H), 3.42 (dd, J = 16.4, 7.0 Hz, 1H), 3.66 (s, 3H), 3.81 (dd, J = 6.8, 6.8 Hz, 1H), 7.01 (m, 2H), 7.19 (m, 2H), 7.33 (d, J = 7.5 Hz, 1H), 7.55 (m, 3H), 8.24 (dd, J = 0.8, 7.2 Hz, 1H), 8.33 (d, J = 8.8 Hz, 1H), 8.39 (brs, 1H), 8.53 (d, J = 8.4 Hz, 1H), 10.79 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.5 (s), 151.9 (s), 143.3 (s), 136.5 (s), 133.0 (s), 128.3 (d), 127.9 (d), 127.4 (s), 126.3 (d), 124.9 (d), 123.7 (d), 123.2 (d), 121.7 (d), 119.9 (s), 119.8 (d), 118.8 (d), 115.2 (d), 111.1 (d), 109.7 (s), 59.5 (d), 51.9 (q), 45.4 (q), 29.6 (t). HRMS (EI): calcd for $C_{24}H_{25}N_3O_4S$ $(M+Na)^+$: 474.1463, found: 474.1465.

RESULTS & DISCUSSION

For the synthesis of TBDs as potential iSLC6A14, different approaches are possible. First, we need considering whether the tryptophan scaffold would form the peptide bond to the other amino acids through the amino (*A series*) or the carboxyl group (*C series*).

In the preliminary studies, the reported iSLC6A14 **9g** and **10g** (Figure 2) are linked through the amino group of tryptophan. Thus, the synthesis of TBDs from the *A series* would use as starting material L-tryptophan methyl ester (**1**), which will be reacted with diverse *N*,*N*-dibenzyl amino acids (**2**) (Scheme 1). These intermediates will be prepared by the benzylation of the corresponding free amino acid [9]. Compounds **1** and **2** can be coupled to provide dipeptides **3** using 2-(1H-benzotriazole-1yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as coupling agent, DMF as solvent, and *N*,*N*-diisopropylethyl amine as base [10].



Scheme 1. Synthesis of proposed TBDs from the *A series*. Reagents and conditions: a) BnBr, KOH, K_2CO_3, H_2O, Δ; b) TBTU, DIPEA, DMF, rt, overnight.

For the synthesis of TBDs from the *C series* we will use a similar strategy. Thus, *N*,*N*-dibenzyl tryptophan (**4**) will be prepared by the benzylation of the free amino acid [9]. The

commercially available amino acid methyl ester (**5**) will be coupled to **4** in order to obtain the dipeptide **6**.

It has been reported that SLC6A14 can transport D-tryptophan inside cells [4]. Therefore, it seems plausible to apply the proposed methodology to obtain TBDs using D-tryptophan as starting material. Furthermore, the library of compounds can be enlarged if we consider using the corresponding D-amino acids (enantiomers) of **2** and **5**. We speculate that this library will aid in the discovery of more selective iSLC6A14.

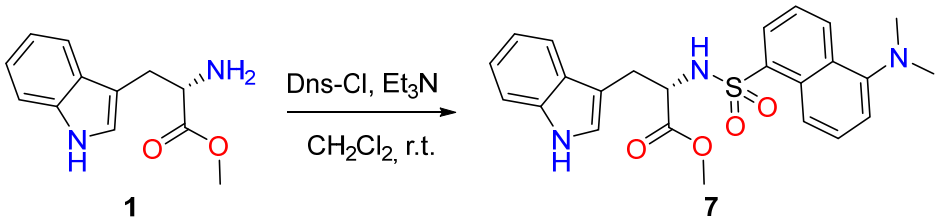
Other possible modifications of the core structure of the TBDs relate to the alkyl substituent on the ester group or the benzyl groups on the amino group. However, these modifications will require more elaborated synthesis and have not been considered in this preliminary study.

Notice to the reader: At this point of the work, we suffered COVID-19 lockdown and we were unable to continue with the proposed TBD synthesis.

In order to test the inhibition of amino acid uptake by iSLC6A14, our group is interested in the development of an assay, since there are no commercial kits available. We pursue a fluorescent assay that could determine if the uptake of amino acids by SLC6A14 is affected by the TBDs. Our group is involved in the development of a cell-based assay where cells will be incubated with a fluorescent tagged substrate in the presence or absence of inhibitors. Fluorescence is a technique widely used in research and a must for many researches, and its use is continually increasing. Among the myriad of uses, fluorescent probes are employed to detect protein location and activation, identify protein complex formation and conformational changes and monitor biological processes in vivo. Furthermore, profiling drug candidates across dynamic in vitro and in vivo live imaging assays can provide unique insights into therapeutic mode-of-action and robust quantification of transient responses. We reported earlier the synthesis and evaluation of a dansyl (Dns) probe of the tubulin depolymerizing agent DTA0100 [11]. Similarly, our strategy seeks bonding a fluorescent probe to a molecule that has some kind of affinity for SLC6A14. In first instance, we considered using L-tryptophan as substrate and dansyl as the fluorescent group (Figure 5).

Our first attempt was the reaction of L-tryptophan with dansyl chloride (DnsCl) in water. NaOH (2 equivalents) was added to dissolve the amino acid and to neutralize the resulting HCl. The reaction was set at a temperature of 60ºC for 6 h. However, we were not able to observe progression of the reaction. Under this reaction conditions DnsCl remained as a solid in suspension. Longer reaction times produced the slow degradation of DnsCl without the formation of the expected fluorescent derivative, indicating that DnsCl is sensitive to strong basic media. Next, we tried the reaction changing the solvent. In this occasion, we used as solvent a 1:1 mixture of acetone:$NaHCO_3$ solution (0.1 M, pH = 8.3). Under this reaction conditions the reactants were not completely soluble and the reaction did not take place.

To overcome the solubility problems, we decided to change the starting material. Thus, we reacted L-tryptophan methyl ester (**1**) with DnsCl in dichloromethane (Scheme 2). As base we used triethylamine. The mechanism of the reaction involves the displacement of the chloride group of DnsCl by the amino group of **1**. The fluorescent compound **7** was obtained in 55% yield. Noteworthy, the reaction did not proceed to completion and conditions were not optimized.



Scheme 2. Synthesis of fluorescent probe **7**.

Compound **7** 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 all cell lines. Thus, compound **7** cannot be used as fluorescent probe for the SLC6A14 inhibition assay. Interestingly, compound 7 is more active in T-47D cells (SLC6A14 positive) than in HBL-100 cells (SLC6A14 negative). Thus, the results point out three relevant consequences: a) **7** enters cells independently of SLC6A14 transport, presumably by passive diffusion, b) affects more those cell lines overexpressing SLC6A14, and c) SLC6A14 is not the only cellular target.

Table 1. Antiproliferative activity (GI_{50}) of compound **7** against solid tumor cell lines.[a]

	Cell line					
	A549	HBL-100	HeLa	SW1573	T-47D	WiDr
7	13±0.9	16±1.5	<1.0	11±0.8	1.3±0.3	11±0.7

[a] Values represent the mean of three independent experiments (µM ± standard deviation).

CONCLUSIONS

1. The synthesis of tryptophan derivatives can lead to the discovery of new candidate drugs for the treatment of cancer. Studies show that both tryptophan and its derivatives have a potentially inhibitory activity on the SLC6A14 transporter.

2. We proposed the preparation of tryptophan-based dipeptides as potential inhibitors of the amino acid transporter SLC6A14.

3. We have synthesized a fluorescent probe based on tryptophan and dansyl chloride. The compound was characterized and tested against human solid tumor cell lines. The fluorescent derivative showed antiproliferative activity against all cell lines tested.

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