UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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TRABALHO DE CONCLUSÃO DE CURSO

ISADORA SERRAGLIO FORTES

SYNTHESIS AND EVALUATION OF 2,3,4-SUBSTITUTED CHIRAL OXAZOLIDINES AGAINST PEDIATRIC CANCER CELLS

PORTO ALEGRE, DEZEMBRO DE 2018.

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Trabalho de Conclusão apresentado ao Curso de Farmácia da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do título de Farmacêutica.

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SYNTHESIS AND EVALUATION OF 2,3,4-SUBSTITUTED CHIRAL OXAZOLIDINES AGAINST PEDIATRIC CANCER CELLS

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ABSTRACT

It has previously been found a potent 2,3,4-oxazolidine series which was synthetized using the amino acid D-serine as the starting material. These compounds were assayed against cancer cell lines (HL60, JURKAT, LNCaP, MDA-MB-231, MCF-7, HCT-116) and their structure−activity relationship were investigated. The purpose of this work was to synthetize through seven steps, characterize and evaluate four 2,3,4-oxazolidines analogues against DAOY, SK-N-BE(2) and RD-ES pediatric cell lines. Compounds **5a** and **5b** designed by the extension of the structure of **1** were the most potent, being active against all cell lines ($IC_{50} \leq 7$ µM) and reduced ≥ 90% of cell viability at 25 µM for RD-ES and SK-N-BE(2). These derivatives were identified as novel anticancer agents including the novel direction towards pediatric cancer cells.

Keywords: pediatric cancer, anti-cancer; synthesis; oxazolidines; chiral

1. Introduction

Cancer is defined by the World Health Organization (WHO) as a group of diseases in which the principal characteristics are the growth of abnormal cells and the possibility of infiltration in adjacent tissues leading to metastasis [1]. Neoplasias consist in one of the most important cause of worldwide mortality, depending on the degree of economic development, life style and social factors [2]. In 2018, the International Agency for Research on Cancer (IARC) estimated 18.1 million new cancer cases and 9.6 million deaths [3]. The National Institute of Health (NIH), a part of the U.S. Department of Health and Human Services, estimated 10.590 new cases of morbidity in children with 0 to 14 years old and 1.180 probable death from this disease [4]. The most frequent causes in this age range are leukemia, lymphomas, brain and other central nervous system (CNS) tumors [4].

The cancer treatment is performed through chemotherapy, surgery, radiation therapy, immunotherapy, hormone therapy and steam cell transplant. The chemotherapy agents commonly used in pediatric cancer as medulloblastoma, neuroblastoma and Ewing Sarcoma are lomustine, vincristine, cisplatin, cyclophosphamide, doxorubicin, etoposide, carboplatin and ifosfamide [5,6,7]. However, factors such as low therapeutic index and severe side effects are still important challenges to be overcome.

During an *in vitro* screening to identify *hit* compounds, a series of molecules from a chemical library were tested at 50 μM in tumor cell lines and those that decrease antiproliferative activity in values greater than 50% were considered promising *hits* to identify IC_{50} values (the half maximal inhibitory concentration). Four 2,3,4-substituted chiral oxazolidines varying the substituent at the ring or stereochemistry showed cytotoxicity against Jurkat and HL60 cells [8].

In a previously structure-activity relationship showed that the presence of hydrophobic and electron withdrawing group NO_2 or $COOCH_3$ is important for the activity (Fig. 1). On the other hand, the presence of hydrophilic and electron withdrawing or hydrophobic and electron donor COOH e OMe, respectively, results in decreased activity. Besides, the stereochemistry *S* is essential for the activity and *para*-substituted compounds, generally, increased the selectivity index when compared *meta*-substituted compounds [9]. In this study, compound **1** (Fig. 1) showed activity against HL60 (human promyelocytic

leukemia cells), MDA-MB-231 (human adenocarcinoma mammary gland) and relevant selectivity index [9].

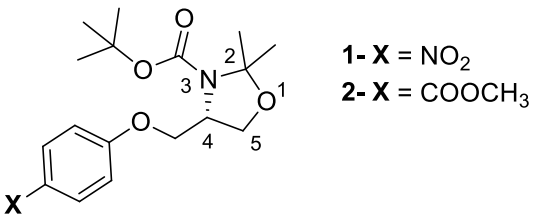


Fig. 1. Structure of 2,3,4-substituted oxazolidines

Introduction of an oxymethylene spacer or rigidification of the structure between benzene and oxazolidine ring influenced in activity of this class. In general, comparing compounds **3** and **4** with lead **1**, those were more active against LNCaP (prostate adenocarcinoma cells), HL60, JURKAT and MDA-MB-231 (breast carcinoma cells). Due to the difficulty of obtaining the alkenes and with similar IC_{50} values to **1** and **2**, the analogues using the oxymethylene spacer was chosen in the following studies (Fig 2) [10,11].

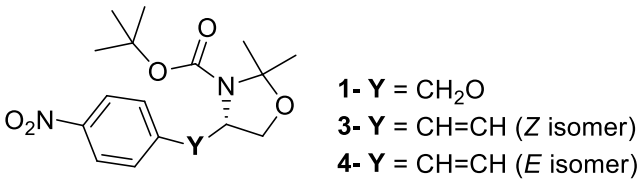


Fig. 2. Oxazolidines by modification of the spacer.

Among other replacements, it was observed that carbamate, oxazolidine ring and bulky group like *N*,*O*-terminal methyl group improve the cytotoxic activity. Finally, the extension of the structure with a second ring within an appropriate spacer in 4-position in first ring lead to low IC_{50} values (7-21 µM in HL60 and 5-22 µM in JURKAT). These values suggest that the insertion of a bulky group in that region of the molecule may increase the interaction with a hydrophobic pocket of a bioreceptor [11].

In a preliminary study for mechanism of action identification, it was observed about 90% and 80% of DNA fragmentation in HL60 cells after treatment with compounds **1** and **5a** at 50 µM, respectively. Besides, the compound **5a** showed about 50% DNA fragmentation on JURKAT cells [11]. Therefore, based on cytotoxic and DNA fragmentations results of compound **1** and analogue **5a**, the purpose of this work was to synthesize new derivatives as **5b** (**Fig. 3**), **6a** and **6b** (**Fig. 4**) in order to investigate relevant structure characteristics to activity of this class. Besides, this new series was evaluated against pediatric cancer cells – DAOY (medulloblastoma), SK-N-BE2

(neuroblastoma) and RD-ES (Ewing Sarcoma) – due to the clinical relevance of this type of disease.

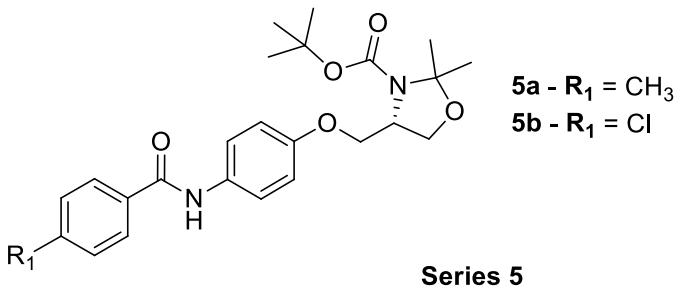


Fig. 3. Series 5 obtained by extension of the structure of lead **1**.

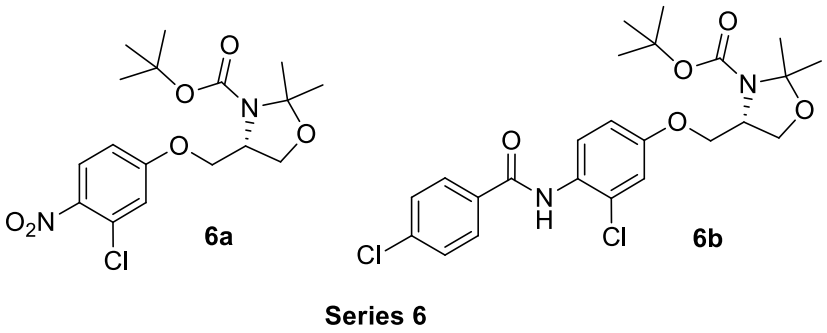


Fig. 4. Series 6 obtained by extension of the structure of **6a**.

2. Results and discussion

2.1 Chemistry

The **Fig. 5** demonstrates the strategy of synthesis to prepare final compounds **5a-b** and **6a-b** from commercially D-serine.

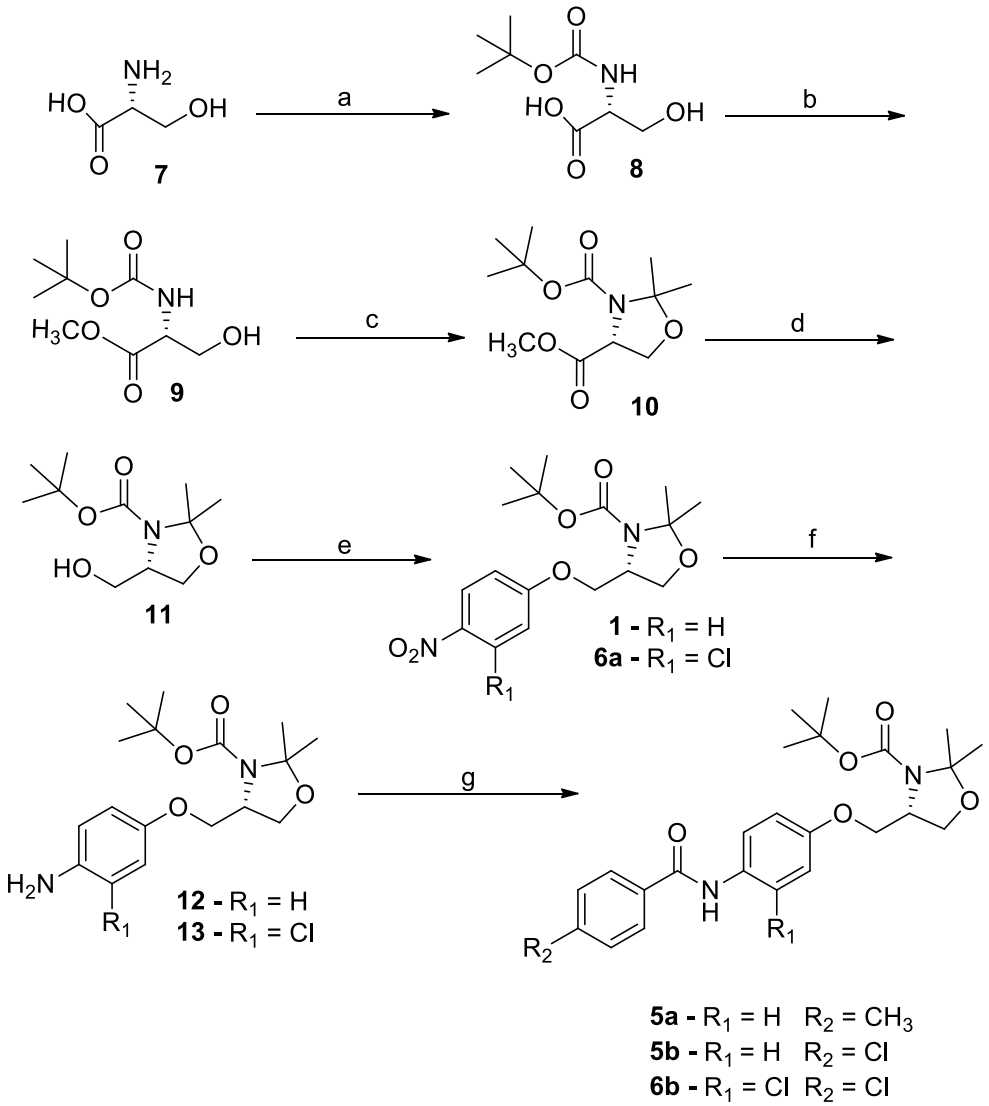


Fig. 5. Preparation of compounds **5a-b** and **6a-b**. Reagents and conditions: a) Boc_2O, NaOH, *t*-BuOH/H_2O (1:1), 0 ºC → r. t., 13 h; b) CH_3I, K_2CO_3, DMF, r. t., 1.5 h; c) DMP, $BF_3.OEt_2$, acetone, r. t., 2.5 h; d) $NaBH_4$, THF/MeOH (7:3), 0 ºC, 1 h; e) *p*-substituted phenol, PPh_3, DIAD, toluene, 80 ºC, 3 h; f) I) H_2, Pd-C, anhydrous THF, r. t. or $SnCl_2$, EtOH absolute, 70 ºC, 30 min. or hydrazine, Pd-C, isopropanol, r. t., 10 min.; g) Appropriate benzoic acid, EDC, DMAP, THF, r. t., 3 h.

First, the D-serine amino acid **7** was protected by treatment with di-*tert*-butyl dicarbonate in presence of NaOH in *t*-BuOH/water mixture to give compound **8** with 78% yield. After,

the acid group **8** was converted into methyl ester by treatment with methyl iodide and potassium carbonate in DMF to give **9** in 64% yield. With **9** in hand, we proceeded to the formation of oxazolidine ring by treatment with 2,2-dimethoxypropane in presence of $BF_3.Et_2O$ affording acetonide **10** (83% yield). This intermediate was reduced to alcohol **11** using $NaBH_4$ with 58% yield. Compounds **1** and **6a** were obtained using Mitsunobu conditions [12] with 70% and 75% yield, respectively. A mechanism propose of Mitsunobu reaction is illustrated in Fig. 6. First, triphenylphosphine reacts with DIAD (diisopropyl azodicarboxylate) by conjugated addition to generate a phosphonium intermediate. Next, the appropriate phenol was deprotonated by DIAD intermediate to give phenoxide ion and the phosphonium ion **I**. The nucleophilic attack by alcohol **11** upon phosphonium **I** affords intermediate **II** and finally, the nucleophilic attack by phenoxide ion upon intermediate **II** gives the desired product **1** or **6a** and triphenylphosphine oxide.

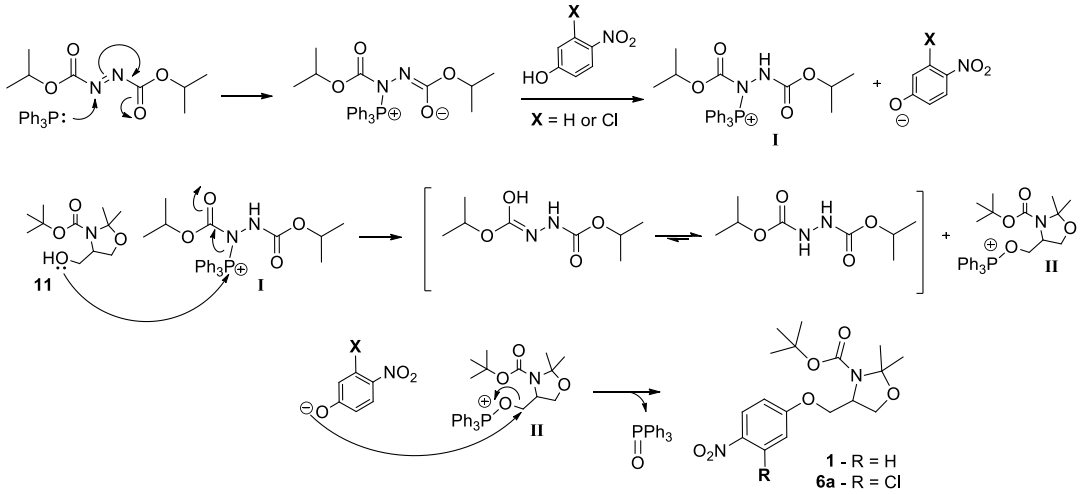


Fig. 6. Proposed mechanism for Mitsunobu reaction.

The intermediate **1** was reduced to **12** by catalytic hydrogenation in presence of H_2, Pd-C and anhydrous THF. The synthesis was performed by zinc oxidation in presence of sulfuric acid to give zinc sulfate and hydrogen gas, used to reduce nitro to amino group. After, compounds **5a** and **5b** were obtained by coupling with 4-methylbenzoic acid and 4-chlorobenzoic with 20% and 32% yield (two steps), respectively.

To synthetize **6a,** 3-chloro-4-nitrophenol **16** was prepared in a two steps process: firstly, a nitrosation reaction using 3-chlorophenol **14** and pyridine in mixture of sodium nitrite and sulfuric acid afforded **15** with 20% yield [13]. At last, the oxidation of nitroso group was carried out using hexacyanoferrate(III) in a solution of sodium hydroxide in water to give **16** with 70% yield (Fig. 7) [14].

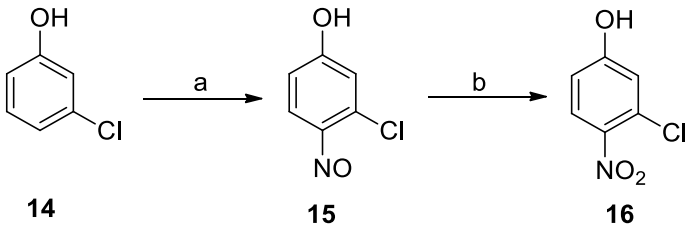


Fig. 7. Preparation of reagent **16**. Reagents and conditions: a) $NaNO_2$, H_2SO_4, ice, 0 °C → -10 ºC then 9**,** pyridine, 30 min; b) **15**, H_2O, NaOH, $K_3[Fe(CN)_6]$, r. t., 2 days.

Catalytic hydrogenation of **6a** led to the formation of a subproduct due to chloro reduction that possess virtually the same Rf. Better results were obtained using tin(II) chloride in ethanol [15] or hydrazine in the presence of Pd-C [16]. The compound **13** was obtained in both conditions with 27% and 17%, respectively. However, in the last approach the poor yield was due to degradation of oxazolidine ring.

2.2. Biological evaluation

2.2.1 Antiproliferative Activity

The antiproliferative activity of final compounds (**5a-b** and **6a-b**) was assessed on three human cancer cell lines, namely DAOY (medulloblastoma), SK-N-BE(2) (neuroblastoma) and RD-ES (Ewing's Sarcoma). These compounds showed good potency with IC_{50} values in some cases lower than 10 µM (Table 1), a value in which molecules could be considered a potential drug candidate.

The **6a** structure is similar to *hit* compound **1**, which demonstrated activity and selectivity against HL60 and Jurkat tumor cell lines. However, compound **6a** was inactive against SK-N-BE(2) and RD-ES at low concentrations (1-12.5 µM). It was observed that it began to inhibit cell proliferation at > 25 µM (Fig. 10).

Compounds **5a** and **5b** were synthetized to check if the introduction of a second ring enhanced the activity against these cell lines. These amides showed a similar activity for 3 cell lines, in which an increased in dose causes a greater cytotoxic effect. Comparing the activity of methylphenylamide derivative **5a** among the different cell lines, it was observed that this compound was a little bit more potent against the RD-ES cell (IC_{50} 4 µM) when compared to DAOY and SK-N-BE(2) cells (IC_{50} 5-7 µM), respectively (Fig. 8).

Compound **6b** with a second chloro group in the first ring at 3-position did not enhanced the activity, indicating that the introduction of an electron withdrawing in this region is unfavorable in this case (Fig. 11). This is a surprising result because in the previous study we have observed that with the derivatives bearing only one aromatic ring the introduction of an electron withdrawing group at *meta*-position have increased the potency. However, in this case as the compound has a higher molecular weight the hydrophobicity of chlorine group could enhance the log P and may reduce the cell uptake. Finally, remarkably, **5a** (Fig. 8) and **5b** (Fig. 9) compounds reduced ≥ 90% cell viability at 25 μM for RD-ES and SK-N-BE(2) reinforcing the potential of them to be used as novel anti-cancer agents.

Table 1
Antiproliferative activity of series **5a-b** and **6a-b** against cancer cells.

	IC_{50} (μM)[a]		
Compound	RD-ES	SK-N-BE(2)	DAOY
5a	4 (3 to 6)	7 (5 to 9)	5 (3 to 9)
5b	5 (2 to 9)	6 (3 to 11)	5 (4 to 6)
6a	>25	>25	>25
6b	11 (5 to 25)	7 (3 to 15)	13 (6 to 28)

[a]Half maximal inhibitory concentration (IC_{50}) represents the concentration of drug able to inhibit by 50% the *in vitro* growth.

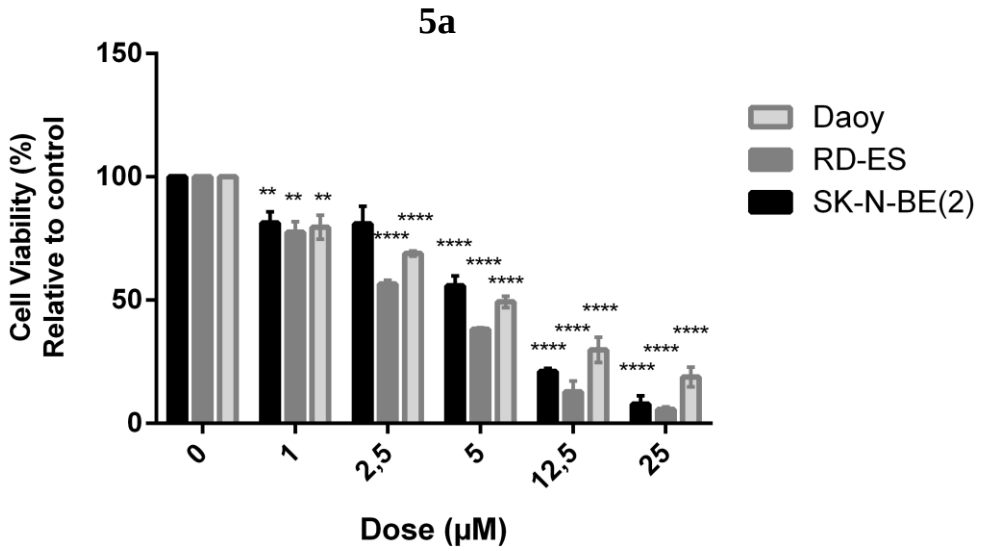


Fig. 8. Cell viability on the DAOY, RD-ES and SK-N-BE2 tumor cells lines after treatment with **5a** for 48 hours. Each data represents mean ± standard error of the mean (SEM) of at least three independent experiments. ** P < 0.01; **** P < 0.0001.

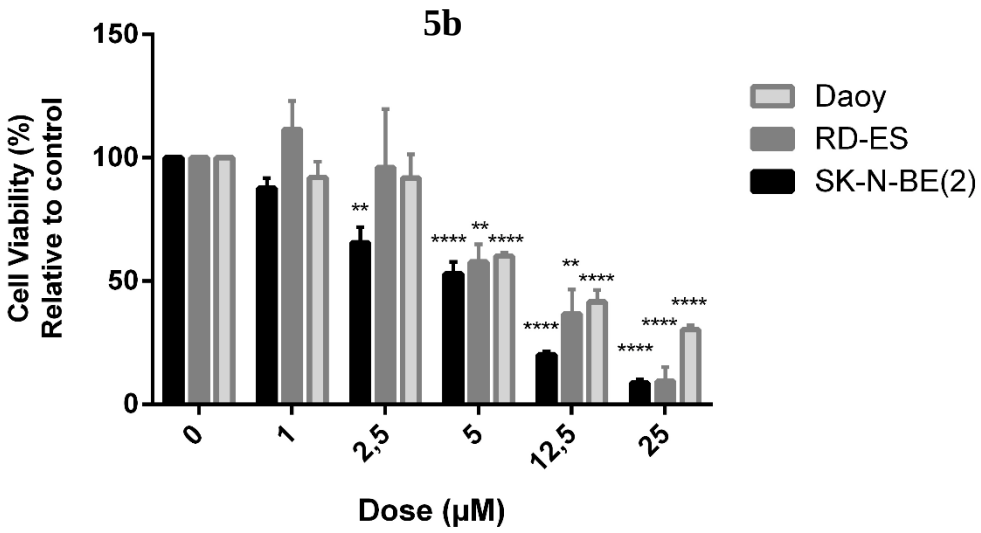


Fig. 9. Cell viability on the DAOY, RD-ES and SK-N-BE2 tumor cells lines after treatment with **5b** for 48 hours. Each data represents mean ± standard error of the mean (SEM) of at least three independent experiments. ** P < 0.01; **** P < 0.0001

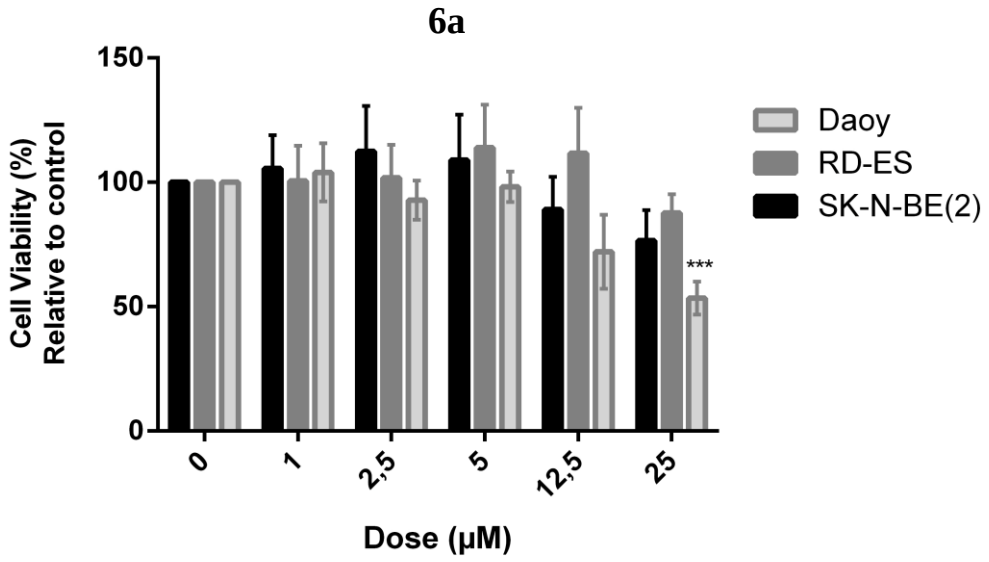


Fig. 10. Cell viability on the DAOY, RD-ES and SK-N-BE2 tumor cells lines after treatment with **6a** for 48 hours. Each data represents mean ± standard error of the mean (SEM) of at least three independent experiments. *** P < 0.001

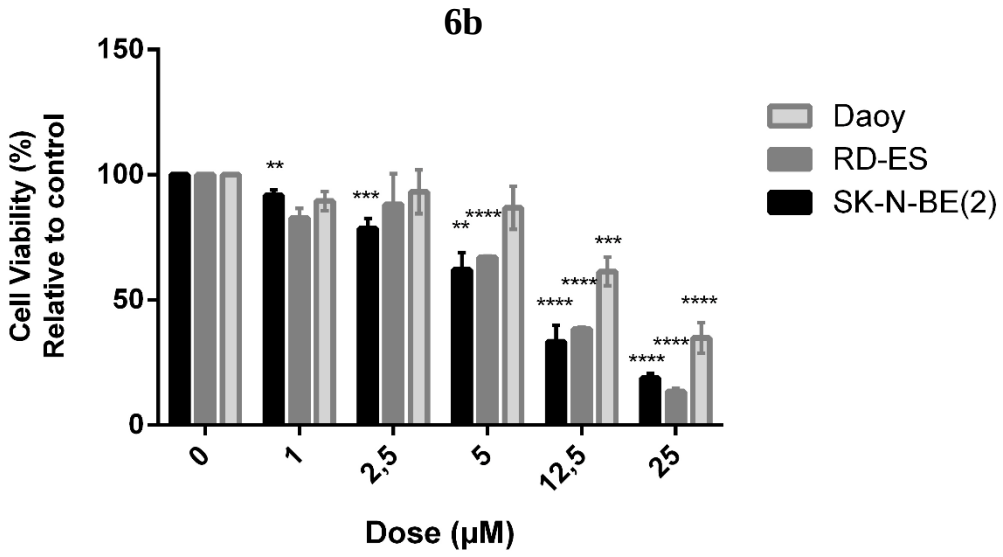


Fig. 11. Cell viability on the DAOY, RD-ES and SK-N-BE2 tumor cells lines after treatment with **6b** for 48 hours. Each data represents mean ± standard error of the mean (SEM) of at least three independent experiments. ** P < 0.01; *** P < 0.001; **** P < 0.0001

3. Conclusion

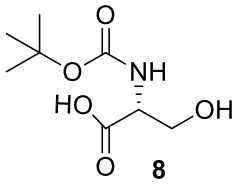
In this work, four final 2,3,4-substituted-oxazolidines analogues were synthesized by seven steps and cell viability on three cell lines was evaluated. The introduction of chloro group at hit compound **1** to give **6a** did not show active against SK-N-BE(2) and RD-ES at low concentrations, being active at > 25 µM. Compounds **5a** and **5b** designed by the extension of the structure with a second ring bearing a methyl or chloro group at 4-position, respectively, enhanced the activity for all cell lines ($IC_{50} \leq 7$ µM) and reduced about ≥ 90% of cell viability at 25 µM for RD-ES and SK-N-BE(2). Compound **6b** within chloro group in the first ring was less active than **5a** and **5b**. Thus, this work reinforces the potential of 2,3,4-substituted-oxazolidines to be considered as novel anticancer agents including the novel direction towards pediatric cancer cells.

4. Experimental section

4.1.1 General

^{1}H and ^{13}C NMR spectra were recorded with Bruker Ascend 400 MHz spectrometer in $CDCl_3$ or DMSO-d_6 solution. The proton and carbon chemical shifts (d) were referenced with tetramethylsilane (TMS) and the coupling constants (*J*) are given in hertz (Hz). THF and toluene were dried over Na/benzophenone and distilled prior to use. The solvents used in silica gel column chromatography and flash silica gel column chromatography were previously distilled. Column chromatography was performed on silica gel Fluka (Sigma-Aldrich) 0.035-0.070 mm. Flash column chromatography was performed on silica gel silica gel Fluka (Sigma-Aldrich) 0.040-0.063 mm.

4.1.2 Synthesis of *N*-[(1,1-dimethyletoxy)carbonyl]-D-serine 8



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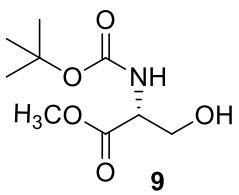
To a solution of D-serine **7** (1.0 g, 9.52 mmol) and NaOH (0.42 g, 10.5 mmol) in a mixture of H_2O/*t*-BuOH 1:1 (44 mL) di-*tert*-butyl dicarbonate (2.29 g, 10.48 mmol) was added at 0 ºC. After stirring for 1 h, the mixture was heated to room temperature. The resulting suspension was concentrated under reduced pressure to 1/3 of the initial volume and resuspended in water (22 mL). The aqueous layer was washed with hexane (3 x 20 mL) and carefully acidified with HCl 0.5 M (15 mL) to pH 2. The aqueous layer was extracted with EtOAc (4 x 30 mL), the organic layers were combined and washed with water (30 mL), dried over Na_2SO_4, filtered and concentrated to give **8** as a transparent oil (1.52 g, 78% yield). The product was used in the next step without previous purification. $C_8H_{15}NO_5$; 205 g/mol. The NMR spectroscopy data are identical to previously reported [9].

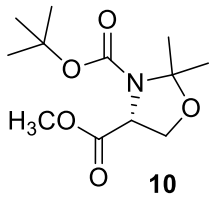
4.1.3 Synthesis of *N*-[(1,1-dimethyletoxy)carbonyl]-D-serine methyl ester 9



9

To a suspension of **8** (0.76 g, 3.71 mmol) and potassium carbonate (0.56 g, 4.07 mmol) in anhydrous DMF (4 mL), iodomethane (0.48 mL, 7.78 mmol) was added. The mixture was stirred at room temperature for 1.5 h then washed with water (10 mL) and EtOAc (4 x 10 mL). The organic layers were extracted with saturated solution of NaCl (2 x 20 mL), dried over Na_2SO_4, filtered and concentrated. Compound **9** was isolated as a transparent oil (0.52 g, 64% yield), then was used in the next step without previous purification. $C_9H_{17}NO_5$; 219 g/mol. The NMR spectroscopy data are identical to previously reported [9].

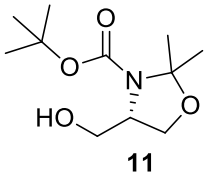
4.1.4 Synthesis of 1,1-dimethylethyl 4-methyl (*R*)- 2,2-dimethyl-3,4-oxazolidine-dicarboxylate 10



10

To a solution of **9** (0.5 g, 2.28 mmol) and 2,2-dimethoxypropane (4.2 mL, 34.2 mmol) in acetone (5.83 mL), $BF_3.Et_2O$ (0.112 mL, 0.915 mmol) was added. The mixture was stirred at room temperature for 2.5 h, then triethylamine was added (0.057 mL). The result solution was concentrated under reduced pressure and resuspended in saturated solution of $NaHCO_3$ (10 mL). The aqueous layer was extracted with diethyl ether (4 x 10 mL). The organic layers were combined, washed with water (10 mL), dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (hexane/EtOAc 9:1) and isolated as a transparent oil (0.49 g, 83% yield). $C_{12}H_{21}NO_5$; 259 g/mol. The NMR spectroscopy data are identical to previously reported. [9].

4.1.5 Synthesis of 1,1-Dimethylethyl (S)-4-(hydroxymethyl)-2,2-dimethyl-3-oxazolidinecarboxylate 11

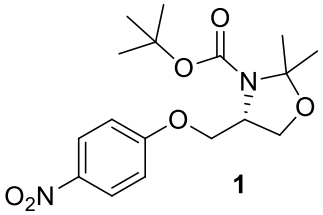


11

To a mixture of **10** (0.44 g, 1.7 mmol) in anhydrous THF (3 mL), $NaBH_4$ (0.64 g, 17 mmol) was added. The solution was stirred at 0 ºC for 25 min., then MeOH (1.2 mL) was added dropwise. After gas release, the mixture was stirred under reflux for 40 min. and

the solvent was evaporated. The residue was resuspended in EtOAc (20 mL), washed with water (2 x 10 mL), dried over Na_2SO_4, filtered and concentrated to give a transparent oil (0.23 g, 58% yield). The product was used in the next step without previous purification. $C_{11}H_{21}NO_4$; 231 g/mol. The NMR spectroscopy data are identical to previously reported [9].

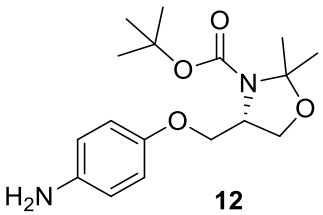
4.1.6 Synthesis of 1,1-Dimethylethyl (*S*)-2,2-Dimethyl-4-[(4-nitrophenoxy)methyl]-3-oxazolidinecarboxylate 1



1

To a mixture of **11** (0.4 g, 1.73 mmol), 4-nitrophenol (0.36 g, 2.60 mmol) and PPh_3 (0.73 g, 2.77 mmol) in anhydrous toluene (20 mL), DIAD (0.8 mL, 3.96 mmol) was added. The resulting solution was stirred at 80 ºC for 3 h, then EtOAc (80 mL) and 0,5 M NaOH (80 mL) was added. The organic layer was washed with water (2 x 80 mL), dried over Na_2SO_4, filtered, and the solvent was removed using a rotavapor. The crude product was purified by silica gel column chromatography (hexane/EtOAc 9:1) to afford a yellow oil (0.43 g, 71% yield). $C_{17}H_{24}N_2O_6$; 352 g/mol. The NMR spectroscopy data are identical to previously reported [9].

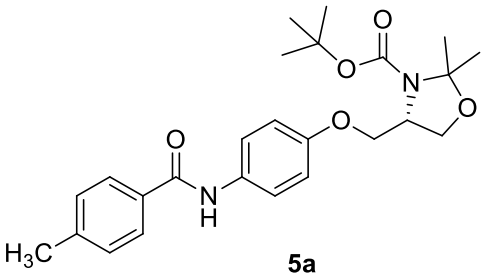
4.1.7 Synthesis of 1,1-Dimethylethyl (*S*)-2,2-Dimethyl-4-[(4-aminophenoxy)methyl]-3-oxazolidinecarboxylate 12



12

The synthesis of amine **12** was performed at room temperature in two flasks. Firstly, to a solution of **1** (0.05 g) in anhydrous THF (1mL) was added Pd-C 10% w/w (0,01 g, 0.094 mmol). Simultaneously, in other flask metallic zinc (1 g, 15.29 mmol), 0.1 M $CuSO_4$ (0.26 mL) and 3 M H_2SO_4 (5 mL) was added dropwise. The both systems were connected by a cannula. The solution of **12** was filtered, washed with THF (3 mL) and the resulting solution was used without further purification in the next step. $C_{17}H_{26}N_2O_4$; 322 g/mol.

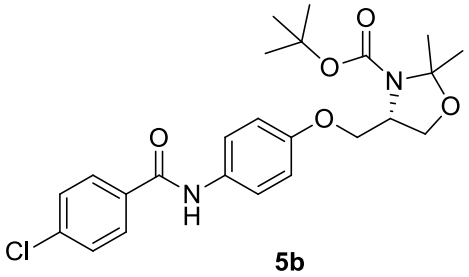
4.1.8 Synthesis of 1,1-Dimethylethyl (*S*)-2,2-dimethyl-4-[[4-(4-methylbenzoylamino)phenoxy]methyl]-3-oxazolidinecarboxylate 5a



5a

To a stirred suspension of 4-methylbenzoic acid (0.04 g, 0.249 mmol) in THF (3 mL) were added EDC (0.05g, 0.311 mmol) and DMAP (2.1 mg, 0.017 mmol) at room temperature. After 10 min., the solution of amine **12** was added and the mixture was stirred for 3 h, then EtOAc (20 mL) was added. The organic layer was washed with 0.5 M HCl (10 mL), followed by solution of 10% (w/v) $NaHCO_3$ (10 mL), water (2 x 10 mL), dried over Na_2SO_4, filtered and concentrated. The product was isolated as a white solid by flash silica gel column chromatography (hexane/EtOAc 8:2) (13 mg, 20% yield). $C_{25}H_{32}N_2O_5$; 440 g/mol. The NMR spectroscopy data are identical to previously reported [11].

4.1.9 Synthesis of 1,1-Dimethylethyl (*S*)-2,2-dimethyl-4-[[4-(4-chlorobenzoylamino)phenoxy]methyl]-3-oxazolidinecarboxylate 5b

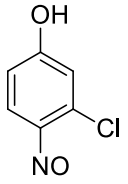


5b

To a stirred suspension of 4-chlorobenzoic acid (0.04 g, 0.249 mmol) in THF (3 mL) were added EDC (0.05 g, 0.311 mmol) and DMAP (2.1 mg, 0.017 mmol) at room temperature. After 10 min., the amine solution of **12** was added and the mixture was stirred for 3 h, then EtOAc (20 mL) was added. The organic phase was washed with 0.5 M HCl (10 mL), followed by 10% (w/v) $NaHCO_3$ (10 mL), water (2 x 10 mL), dried over Na_2SO_4, filtered and concentrated. The product was isolated as a white solid by flash silica gel column chromatography (hexane/EtOAc 8:2) (21 mg, 32% yield). $C_{24}H_{29}ClN_2O_5$; 460.95 g/mol.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.81 (d, 2H, J = 8.1 Hz), 7.53 (m, 2H,), 7.45 (d, 2H, J = 7.6 Hz), 6.95 (d, 2H, J = 7.6 Hz), 4.29-3.83 (m, 5H), 1.65-1.51 (s, 15H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) major rotamer: 164.63, 155.72, 152.38, 138.03, 133.39, 131.49, 128.48, 122.26, 115.02, 93.60, 80.64, 66.37, 65.21, 56.09, 28.43, 27.55, 24.32.

4.2.0 Synthesis of 3-chloro-4-nitrosophenol 15

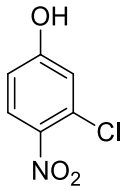


15

To a solution of sodium nitrite (3.28 g, 0.047 mol) in H_2SO_4 (32.8 mL, 0.614 mol) at 0 °C, ice (15.27 g, 0.85 mol) was added without stirring. After dissolution, the mixture was cooled to -10 ºC and a solution of 3-chlorophenol **14** (2.5 mL, 0.024 mol) in pyridine (17 mL, 0.211 mol) was slowly added under stirring for 30 min. The reaction was stopped after 5 min., then poured in water (320 mL). The crude product was purified by crystallization as follows. Firstly, MeOH (23 mL) was added under reflux at 60 ºC, then after complete solubilization, water (23 mL) was added. Next, charcoal was added and the hot mixture was filtered. The filtrate was allowed to stand until product precipitation was complete and finally it was filtered off to give a brown solid (0.71 g, 20% yield). $C_6H_4ClNO_2$; 157.55 g/mol.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.59 (s broad, 1H), 6.81 (s, 1H), 6.50 (dd, 1H, J = 10 Hz, J = 1.6 Hz).

4.2.1 Synthesis of 3-chloro-4-nitrophenol 16



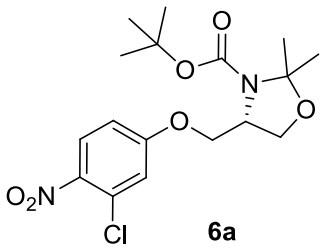
16

To a stirred solution of **15** (0.39 g, 2.50 mmol) and water (79 mL), NaOH (3.93 g, 0.098 mol) was added. After complete solubilization, potassium hexacyanoferrate(III) (3.93 g, 0.012 mol) was added. The mixture was stirred for 2 days at room temperature, then AcOH (11 mL) and EtOAc (3 x 75 mL) were added. The combined organic layers were

washed with water (125 mL), dried over Na_2SO_4, filtered and concentrated. The product was obtained as a brown solid (0.293 g, 68% yield). $C_6H_4ClNO_3$; 173.55 g/mol.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 11.27 (s broad, 1H, OH), 8.03 (d, 1H, J = 6 Hz), 7.02 (d, 1H, J = 4 Hz), 6.90 (dd, 1H, J = 6, 4 Hz).

4.2.2 Synthesis of 1,1-Dimethylethyl (S)-2,2-Dimethyl-4-[(3-chloro-4-nitrophenoxy)methyl]-3-oxazolidinecarboxylate 6a

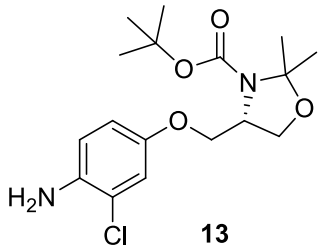


6a

The synthesis of **6a** was performed at 80 ºC for 3 h from compound **11** (0.23 g, 0.98 mmol) using **16** (0.26 g, 1.47 mmol), PPh_3 (0.41 g, 1.57 mmol) in anhydrous toluene (10 mL), then DIAD (0,45 mL, 2.26 mmol) was added. The mixture was extracted with EtOAc (40 mL) and 0.5 M NaOH (40 mL). The organic layer was washed with water (2 x 40 mL), dried over Na_2SO_4 and concentrated. The product **6a** was purified by silica gel column chromatography (hexane/EtOAc 9:1) and isolated as a yellow oil (0.24 g, 64% yield). $C_{17}H_{23}ClN_2O_6$; 386 g/mol.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.01 (d, 1H, J = 8 Hz), 7.12-6.92 (m, 2H), 4.30-3.94 (m, 5H), 1.67-1.52 (m, 15H).

4.2.3 Synthesis by 1,1-Dimethylethyl (S)-2,2-Dimethyl-4-[(3-chloro-4-aminophenoxy)methyl]-3-oxazolidinecarboxylate 13



13

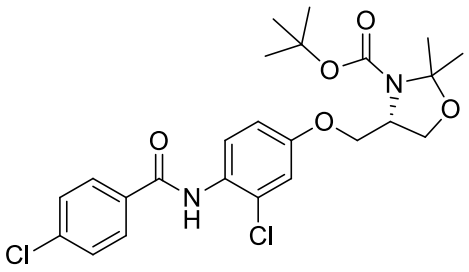
Method A

A mixture of **6a** (0.08 g, 0.21 mmol), Pd-C 10% (w/w) (0.01 g), hydrazine solution (0.147 mL) in isopropanol (1 mL) was stirred at 82 ºC for 10 min. The resulting solution was concentrated under vacuum and the product was purified by preparative TLC (thin-layer chromatography) (hexane/EtOAc 8:2) and isolated as a brown oil (0.013 g, 17% yield) which was used in the next step immediately. $C_{17}H_{25}ClN_2O_4$; 356.84 g/mol.

Method B

A mixture of **6a** (0.12 g, 0.31 mmol), tin(II) chloride (0.295 g, 1.55 mol), in ethanol absolute (2 mL) was stirred at 70 ºC for 30 min. The resulting solution was poured in ice (5 mL) and added $NaHCO_3$ (10 mL) until basic pH, then EtOAc (3 x 15 mL) was added, dried over Na_2SO_4 and concentrated. The product was purified by silica gel column chromatography (hexane/EtOAc 9:1) and isolated as brown oil (0.03 g, 27% yield) which was used in the next step immediately. $C_{17}H_{25}ClN_2O_4$; 356.84 g/mol.

4.2.4 Synthesis of 1,1-Dimethylethyl (*S*)-2,2-dimethyl-4-[[4-(4-chlorobenzoylamino)-3-chlorophenoxy]methyl]-3-oxazolidinecarboxylate 6b



6b

To a stirred suspension of 4-chlorobenzoic acid (10 mg, 0.062 mmol) in THF (2 mL) EDC (0.014 g, 0.090 mmol) and DMAP (1 mg, 0.006 mmol) were added at room temperature. After 10 min., the amine **13** (10 mg, 0.028 mmol) was added and the mixture was stirred at 80 ºC overnight. After, the same initially amount of 4-chlorobenzoic, EDC and DMAP was added and the reaction was stirred at 100 ºC for 3 h. The mixture was extracted with EtOAc (3 x 10 mL), water (1 x 10 mL), dried over Na_2SO_4, filtered and concentrated. The product was isolated by preparative TLC (hexane/EtOAc 95:5) then silica gel column chromatography (EtOAc:MeOH 1:1) as a brown oil (5 mg, 35% yield). $C_{24}H_{28}Cl_2N_2O_5$; 495 g/mol.

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.36 (d, 2H, J = 6.6 Hz), 8.18 (s, 1H, NH), 7.87 (d, 2H, J = 6.6 Hz), 7.51 (d, 1H, J = 8.4 Hz), 7.06-6.94 (m, 2H), 4.3-3.7 (m, 7H), 1.65-1.53 (m, 15H).

4.3 Cell lines

The RD-ES, SK-N-BE(2) and DAOY culture were prepared using the protocol previously described by Dr. Rafael Roesler's group (Universidade Federal do Rio Grande do Sul – Brazil). RD-ES (ATCC® HTB-166™) was cultivated in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B, enriched with 15% of fetal bovine serum (FBS). SK-N-BE(2) (ATCC® CRL-2271™) was mantained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B, enriched with F12 (1:1) and 10% FBS. Daoy (ATCC® HTB-186™) was cultivated in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B, enriched with 10% FBS.

4.3.1 Analysis of Cell Viability

DAOY, RD-ES and SK-N-BE(2) were cultivated in 96 wells plate at 4,000, 8,000 and 10,000 cells/well, respectively, in a final volume of 0.34 cm^2/well. The plates were pre-incubated in a 5% CO_2/95% air-humidified atmosphere at 37°C for 24 hours. All compounds were dissolved in dimethyl sulfoxide (DMSO), prior to dilution. The plates were treated with **5a-b** and **6a-b** and incubated in a 5% CO_2/95% air-humidified atmosphere at 37°C for 48 hours. A control with vehicle (DMSO) and other with cells were prepared to evaluate cytotoxicity and possible interferences of the cell culture. Cell viability count was estimated by hemocytometer with trypan blue and calculated in relation to control (vehicle without treatment). All assays were done in triplicates. The IC_{50} values were obtained using GraphPad Prism 6.

4.3.2 Statistical analysis

All results were expressed in graphics as the mean ± standard error of the mean (SEM) of three independents experiments performed, at least, in triplicate. Cell viability in the different dosages was compared in relation to control using Student's *t*-test. Statistical significance was determined as $P < 0.05$. The IC_{50} values were obtained by nonlinear regression using GraphPad Prism 6.

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Supplementary material

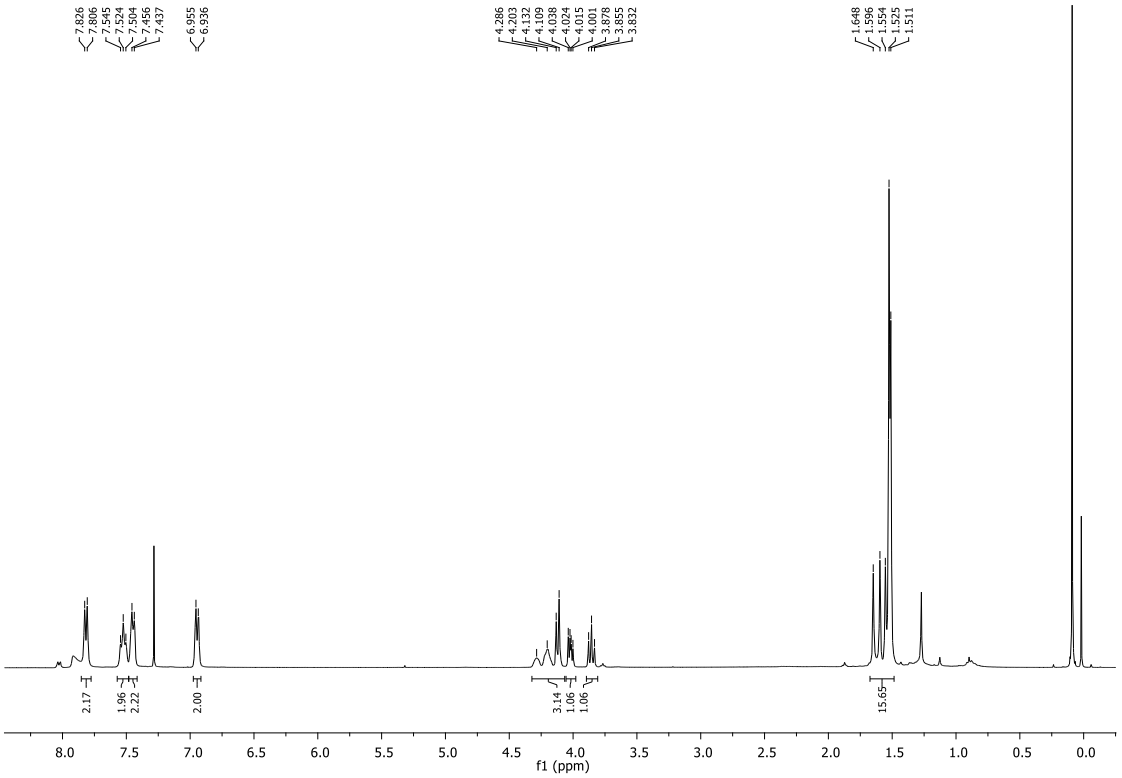

Fig 1. ^{1}H NMR spectrum in $CDCl_3$ of **5b**

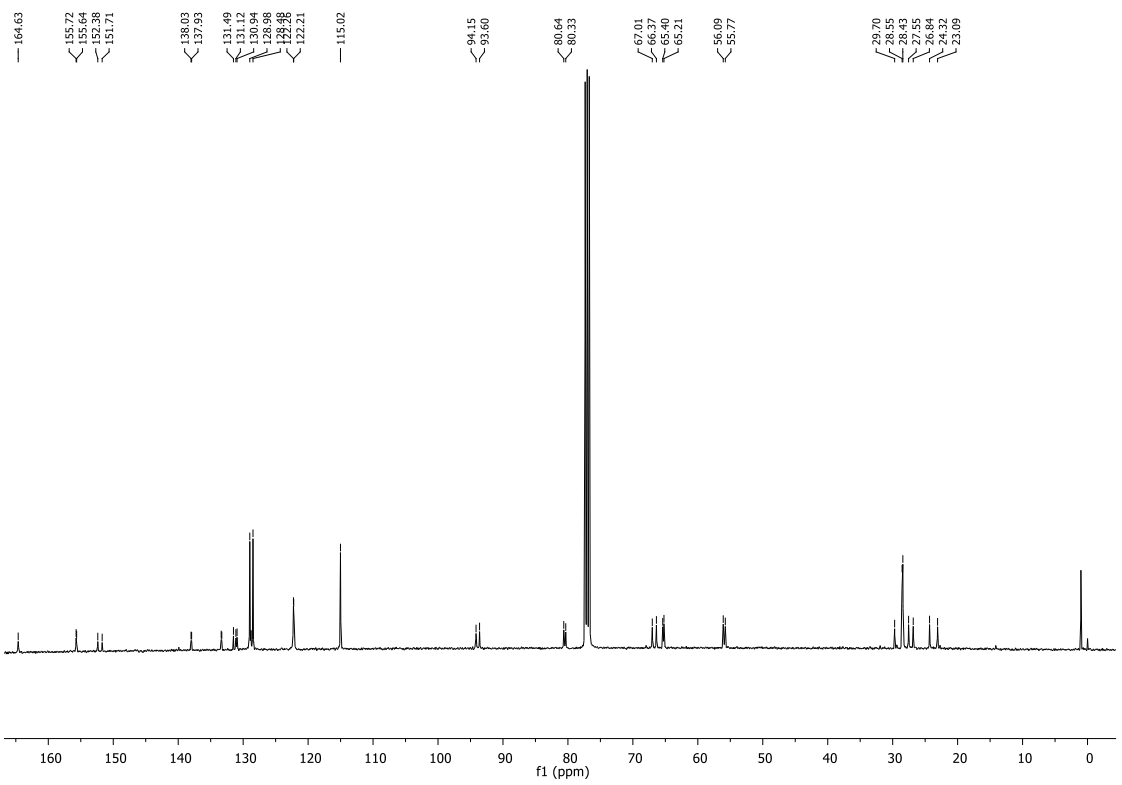

Fig 2. ^{13}C NMR spectrum in $CDCl_3$ of **5b**

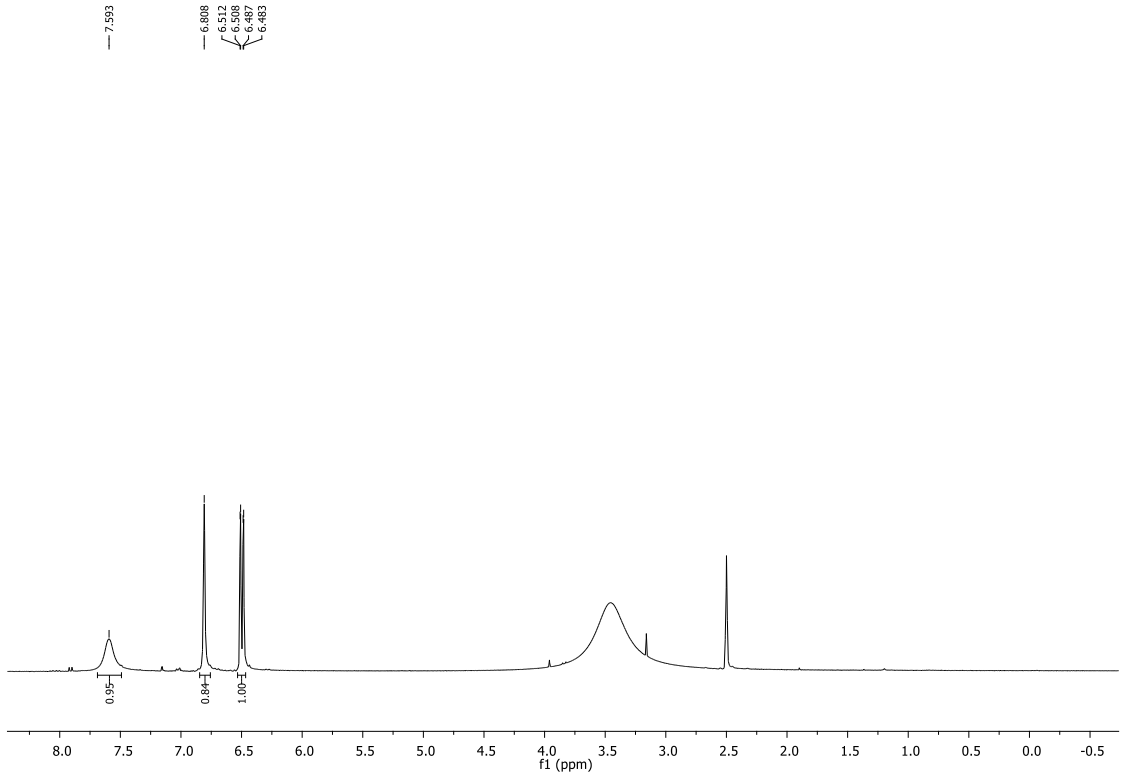

Fig 3. ^{1}H NMR spectrum in DMSO-d_6 of **15**

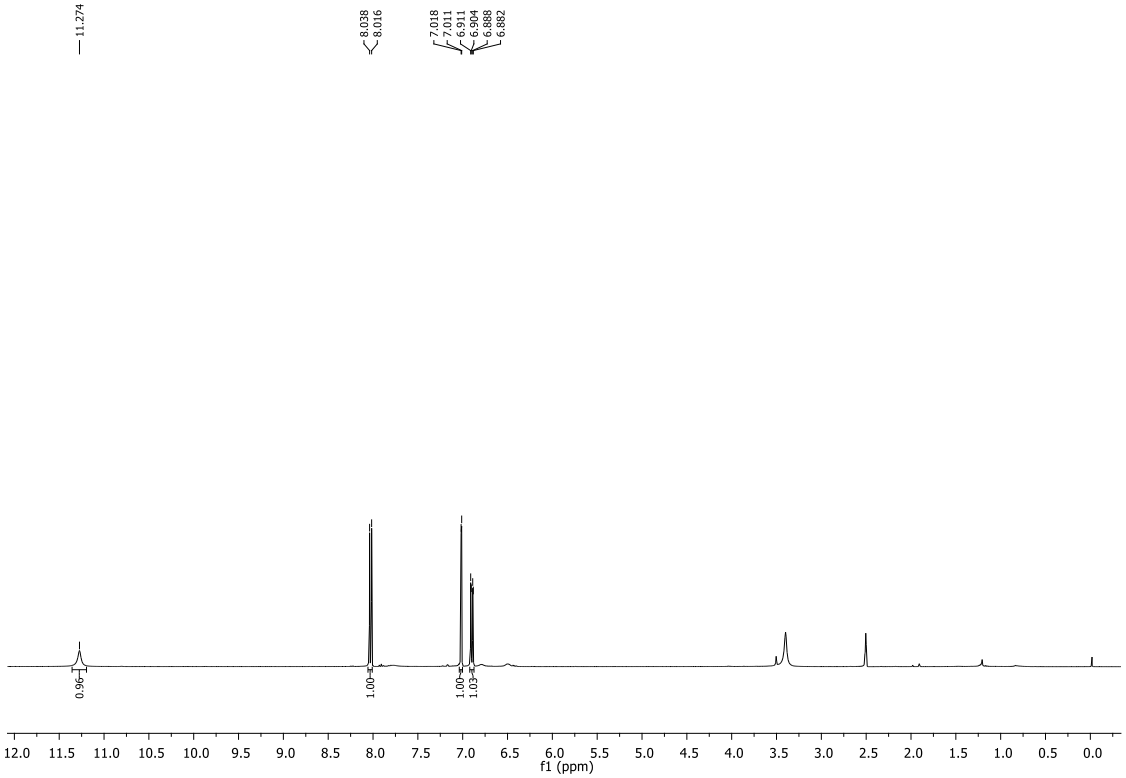

Fig 4. ^{1}H NMR spectrum in DMSO-d_6 of **16**

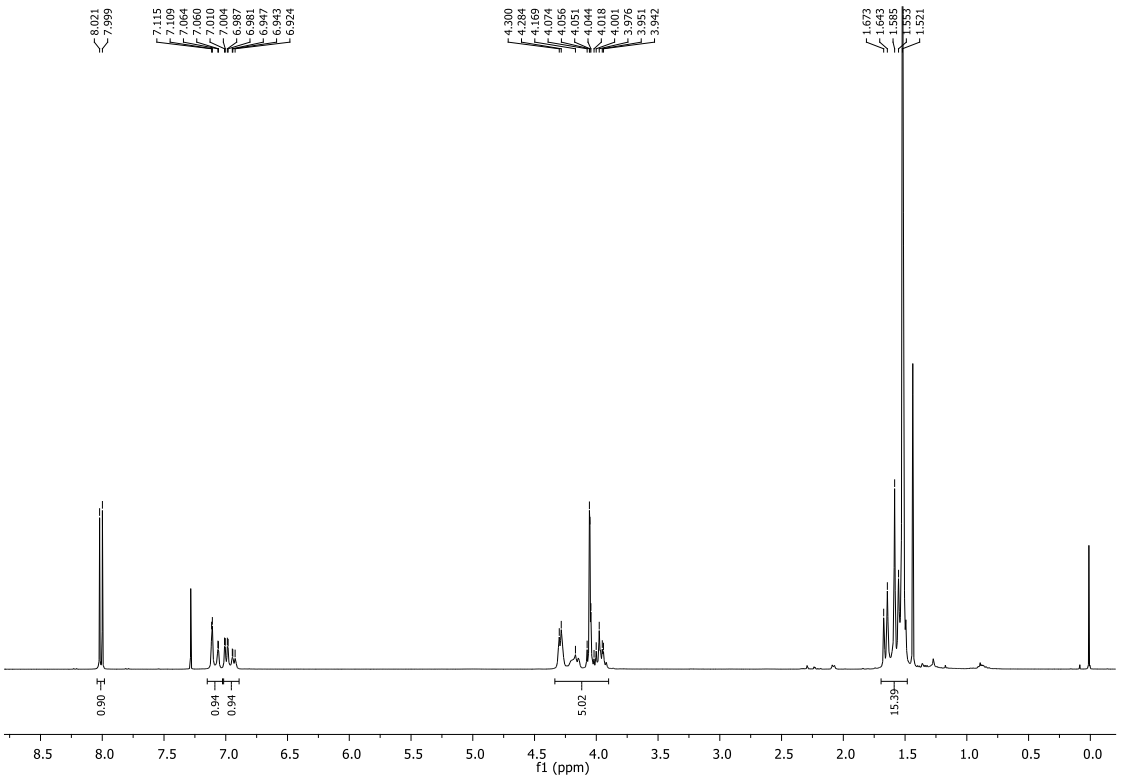

Fig 5. ^{1}H NMR spectrum in $CDCl_3$ of **6a**

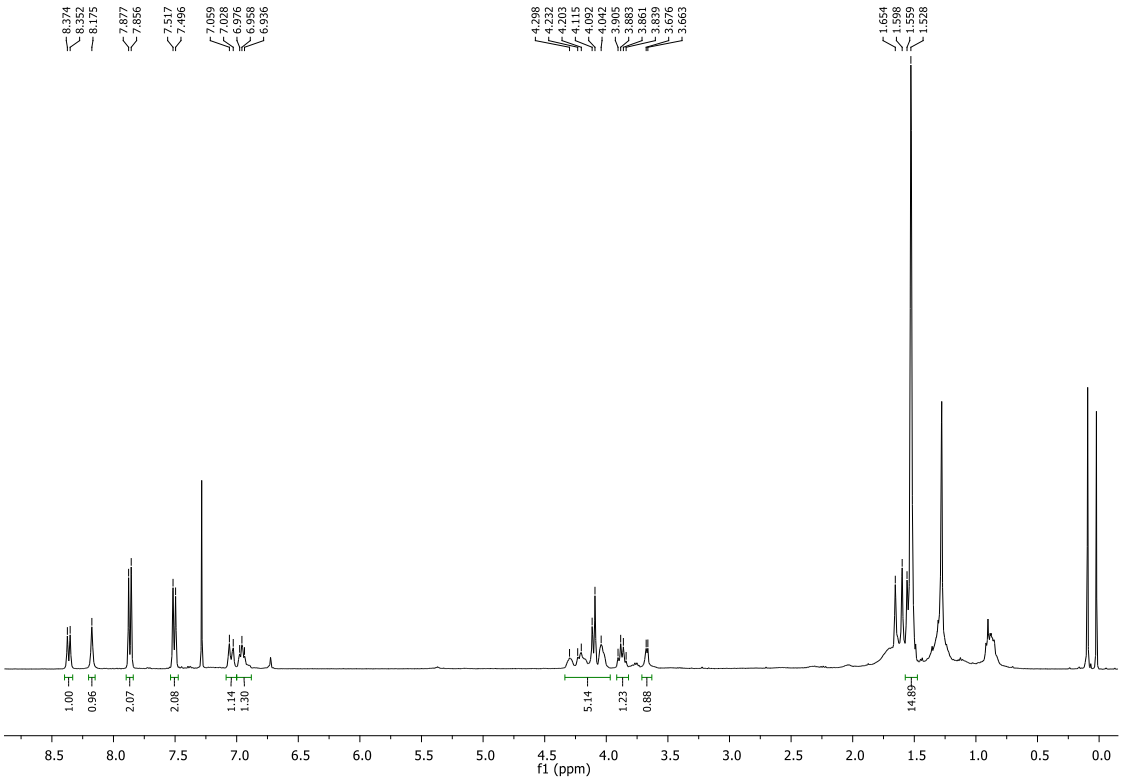

Fig 6. ^{1}H NMR spectrum in $CDCl_3$ of **6b**

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AUTHOR INFORMATION PACK

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