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Article

Cytotoxic Activity of Synthetic Chiral Amino Acid Derivatives

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Cancer is a chronic degenerative disease considered one of the most important causes of death worldwide. In this context, a series of dual-protected amino acid derivatives was synthesized and evaluated as potential novel anticancer agents. The 40 derivatives were prepared in up to three reaction steps. The cytotoxic activities were screened *in vitro* against a panel of tumor and non-tumor cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Among the synthesized derivatives, three of them showed promising activity against cancer cells with half-maximal inhibitory concentration (IC_{50}) ranging between 1.7-6.1 μM. The most promising derivative, bearing both a lipophilic *N*-alkyl diamine moiety and a protected amino acid scaffold showed a selectivity index of 3.4 towards tumor cells. The *N*-alkyl diamine moiety seems to play a crucial role in the enhancement of the anticancer activity. On the other hand, the incorporation of an amino acid scaffold resulted in increase in the selectivity towards cancer cell lines.

Keywords: amino acid, carbamate, diamine, anticancer activity

Introduction

Nowadays cancer clinically appears as one of the most important diseases worldwide, with growing incidence and mortality rates. In 2018, only in the United States, more than 1.7 million new cancer cases and more than 0.6 million cancer deaths are expected to occur.[1] Clinically, lung, breast, colorectal and prostate appears among the most diagnosed cancer types, being responsible for 42.7% of new cases.[2] Besides, since chemotherapy drugs presents only moderate selectivity towards growing abnormal cells, the death of healthy cells frequently occurs, resulting in toxicity and several side-effects for the patient during cancer treatment.[3,4]

In this context, the development of new anticancer agents, especially those with new mechanisms of action, fewer side effects and lower toxicity in the clinic appears as a great challenge in medicinal chemistry.[5] In addition, another great clinical challenge is the advent of new anticancer drugs capable of selectively acting in different types of cancer cells, making the treatment more general and simpler. Finally, most cancer drugs consists of very complex chemical structures, such as paclitaxel and vinblastine, which demands several synthetic steps for their preparation, with a direct impact in cancer treatment cost.[6] Thus, the discovery of new candidates based on small molecules, bearing simple and accessible precursors is essential for increasing the accessibility of cancer treatment.

Due to the great potential of carbamate in medicinal chemistry, we envisioned that it is a promising building-block for the design of new anticancer candidates.[7-10] For example, many important clinically employed anticancer drugs, with different mechanisms of action, bear this scaffold, such as the nucleoside analogue capecitabine, the topoisomerase I inhibitor irinotecan and the taxanes, such as paclitaxel, docetaxel and carbazitaxel.

Moreover, amino acids also appears as interesting moieties in organic synthesis and it use has several advantages, such as the low cost of natural-occurring amino acids, the easy access, the presence of one or more defined stereocenters and the possibility of functionalization in both the amine or carboxyl moiety, greatly enhancing the molecule complexity in only one or a few reaction steps.[11-15] Furthermore, the increased amino acid uptake has been described for some types of cancer, making it a promising building block for enhancing the selectivity towards cancer

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cells.[16] Finally, some anticancer drugs, such as Melphalan and Eflornithine, presents a substituted amino acid scaffold.

Over the last years our research group has prepared several complex amino acid derivatives, some of them with promising biological activity.[17,18] Thus, we envisioned that the preparation of new derivatives by the coupling of a carbamate and an amino acid moiety would result in new derivatives with potential applications in cancer treatment. Besides, the modification of the amino acids employed, associated with carboxyl group functionalization by different amines, would allow the access to several biologically promising stable amide derivatives. In this framework, we describe herein the preparation of thirty-eight dual-protected amino acid derivatives and two analogues, as well as their cytotoxic profile against cancerous and non-cancerous cells.

Experimental

General remarks

All chemicals were used as purchased, without further purification. Solvents were dried following standard procedures and all reaction mixtures were carried out in flame-dried glassware. Analytical thin layer chromatography (TLC) was performed on TLC plates and visualized employing a ninhydrin (2,2-dihydroxyindane-1,3-dione) indicator. Yields refers to chromatographically purified and spectroscopically pure derivatives. The chemical shifts are reported in ppm relative to the solvent residual peak (chloroform 7.28 ppm). The ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz and ^{13}C NMR at 125 MHz. Chemical shifts are reported in ppm using the following peak pattern abbreviations: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; pent, pentet; m, multiplet. Infrared (IR) spectra were recorded on a PerkinElmer 1720 FTIR spectrometer in the region of 4000-600 cm^{-1} as an average of 120 scans. High resolution mass spectra were recorded in the positive ion mode using a time-of-flight (TOF) mass spectrometer equipped with an electron spray ionization (ESI) source. Melting points and optical rotations were recorded, respectively, on a melting point apparatus and a polarimeter.

General procedure for the synthesis of *N*-alkyl diamines

The two *N*-alkyl diamine derivatives were synthesized employing literature protocols.[5] Initially, 40 mmol of the diamine was added in methanol (50 mL) and then alkyl bromide or chloride (8 mmol) was slowly added to the reaction mixture. The reaction was kept at reflux for 48 h. Then, the solvent was rotary evaporated and a liquid-liquid extraction employing water/dichloromethane performed. The organic layer was dried and the solid recrystallized in hot hexane. After filtration, the desired mono-alkyl diamine derivatives were isolated as white solids. In an attempt to compare the amino acid moiety influence in the anticancer activity, the half-maximal inhibitory concentration (IC_{50}) of these intermediates were also assayed.

General procedure for the preparation of amino acid derivatives

The amino acid derivatives were prepared employing a methodology developed by our research group.[19] Initially, the coupling between the *tert*-butyloxycarbonyl (Boc) protecting group and amino acids (L-alanine, D,L-alanine, D-alanine, L-valine, L-leucine, L-isoleucine or L-phenylalanine, 1.0 mmol) was done by adding to a solution of 1,4-dioxane/water (40:60 v/v) the amino acid and 50 mg of Na_2CO_3. The mixture was stirred until complete dissolution and then Boc_2O (1.5 mmol) was added. The reaction was kept at magnetic stirring for 18 h and then purified employing literature protocols.[20]

For the amide bond formation, the previously prepared Boc amino acids (1.0 mmol) were transferred to a flame-dried glassware vial with dichloromethane (5.0 mL) and then EDC (3-dimethylaminopropyl)-*N*-ethylcarbodiimide) hydrochloride (1.1 mmol) was added. The reaction stirred at 0 °C for 30 min, and then washed twice with distilled water. The organic layer was immediately transferred to a vial with the amine nucleophile (2.0 mmol) and the (±)-camphorsulfonic acid organocatalyst (0.1 mmol, 10 mol%). After 24 h the dichloromethane was removed and the desired compounds purified by recrystallization, liquid-liquid extraction or column chromatography.

Boc deprotection of two of the previously prepared products was carried out following literature protocols,[21] in which the derivatives (1 mmol) and trifluoroacetic acid (22 mmol) were added in dichloromethane (5 mL) and kept under magnetic stirring for 3 h, affording the salts **33** and **34** after solvent removal.

Characterization data

tert-Butyl (*S*)-(1-(butylamino)-1-oxopropan-2-yl)carbamate (**1**)

The product was prepared according to the general procedure and obtained after recrystallization in ethanol/water as a yellow solid (302 mg, 69%); mp 72.1-72.0 °C; $[\alpha]_D^{20}$ –10 (*c* 1.0, CH_2Cl_2); IR (KBr) ν / cm^{-1} 3316, 3262, 2964, 2925, 2865, 1686, 1653, 1520, 1447, 1242, 1163,

1023; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.30 (br, 1H), 5.09 (br, 1H), 4.11 (br, 1H), 3.23 (q, 2H, *J* 6.1 Hz), 1.51-1.41 (m, 11H), 1.37-1.28 (m, 5H), 0.90 (t, 3H, *J* 7.3 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.7, 155.7, 80.2, 50.2, 39.3, 31.7, 28.4, 20.1, 18.5, 13.8; HRMS (ESI-TOF) *m/z*, calcd. for $C_{12}H_{24}N_2NaO_3^+$ [M + Na]$^+$: 267.1685, found: 267.1702.

tert-Butyl (*R*)-(1-(diethylamino)-1-oxopropan-2-yl) carbamate (**35**)

The product was prepared according to the general procedure and obtained after liquid-liquid extraction in dichloromethane/water as a yellow oil (358 mg, 73%); $[\alpha]_D^{20}$ –10° (*c* 1.0, CH_2Cl_2); IR (KBr) ν / cm^{-1} 3430, 3295, 2973, 2928, 2876, 1704, 1634, 1453, 1362, 1247, 1169, 1054; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.44 (d, 1H, *J* 7.5 Hz), 4.48 (pent, 1H, *J* 7.0 Hz), 3.42-3.38 (m, 1H), 3.28-3.26 (m, 2H), 3.23-3.16 (m, 1H), 1.35 (s, 9H), 1.21 (d, 3H, *J* 6.8 Hz), 1.14 (t, 3H, *J* 7.1 Hz), 1.03 (t, 3H, *J* 7.1 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.1, 155.1, 79.3, 46.1, 41.7, 40.3, 28.3, 19.6, 14.5, 12.8; HRMS (ESI-TOF) *m/z*, calcd. for $C_{12}H_{24}N_2NaO_3^+$ [M + Na]$^+$: 267.1668, found: 267.1685.

tert-Butyl (*R*)-(1-morpholino-1-oxopropan-2-yl)carbamate (**36**)

The product was prepared according to the general procedure and obtained after liquid-liquid extraction in dichloromethane/water as a yellow oil (421 mg, 73%); $[\alpha]_D^{20}$ –14° (*c* 1.0, CH_2Cl_2); IR (KBr) ν / cm^{-1} 3427, 3308, 2977, 2924, 2850, 1698, 1639, 1440, 1234, 1162, 1109, 1016; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.50 (d, 1H, *J* 6.9 Hz), 4.57 (pent, 1H, *J* 7.2 Hz), 3.69-3.65 (m, 6H), 3.59-3.49 (m, 2H), 1.42 (s, 9H), 1.28 (d, 3H, *J* 6.9 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.5, 155.2, 79.8, 67.1, 66.9, 52.1, 46.0, 42.5, 28.5, 19.4; HRMS (ESI-TOF) *m/z*, calcd. for $C_{12}H_{22}N_2NaO_4^+$ [M + Na]$^+$: 281.1477, found: 281.1490.

tert-Butyl (*R*)-(1-((2-methoxyphenyl)amino)-1-oxopropan-2-yl)carbamate (**37**)

The product was prepared according to the general procedure and obtained after recrystallization with ethanol/water as a white solid (236 mg, 74%); mp 129.1-130.0 °C; $[\alpha]_D^{20}$ –12° (*c* 1.0, CH_2Cl_2); IR (KBr) ν / cm^{-1} 3328, 3268, 2977, 2924, 1673, 1658, 1540, 1519, 1460, 1254, 1155, 1022, 744; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.43 (br, 1H), 8.35 (dd, 1H, *J* 8.0 Hz, *J* 1.1 Hz), 7.05 (t, 1H, *J* 7.3 Hz), 6.95 (t, 1H, *J* 7.7 Hz), 6.87 (d, 1H, *J* 7.8 Hz), 5.06 (br, 1H), 4.34 (br, 1H), 3.87 (s, 3H), 1.46-1.44 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8, 155.6, 148.2, 127.5, 124.0, 121.2, 119.9, 110.1, 80.4, 55.8, 51.2, 28.5, 18.5; HRMS (ESI-TOF) *m/z*, calcd. for $C_{15}H_{22}N_2NaO_4^+$ [M + Na]$^+$: 317.1477, found: 317.1502.

tert-Butyl (*R*)-(1-((4-bromophenyl)amino)-1-oxopropan-2-yl) carbamate (**38**)

The product was prepared according to the general procedure and obtained after recrystallization with ethanol/water as a white solid (265 mg, 78%); mp 156.6-157.2 °C; $[\alpha]_D^{20}$ –10° (*c* 1.0, CH_2Cl_2); IR (KBr) ν / cm^{-1} 3394, 3261, 2977, 1678, 1506, 1367, 1248, 1162, 817; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.73 (br, 1H), 7.37-7.35 (m, 4H), 5.11 (d, 1H, *J* 6.4 Hz), 4.33 (br, 1H), 1.45 (s, 9H), 1.42 (d, 3H, *J* 7.1 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.2, 156.4, 137.1, 132.0, 121.4, 116.9, 81.0, 50.9, 28.4, 17.5; HRMS (ESI-TOF) *m/z*, calcd. for $C_{14}H_{19}BrN_2NaO_3^+$ [M + Na]$^+$: 365.0477, found: 365.0474.

Cell culture

Murine metastatic melanoma (B16F10), murine fibroblast (NIH3T3), and human hepatocellular carcinoma (HepG2) cells were kindly provided by Dr Anésia Aparecida dos Santos (Departamento de Biologia Geral, Universidade Federal de Viçosa, Minas Gerais, Brazil). Human breast adenocarcinoma (MDA-MB-231) cell was kindly provided by Dr Sandra Martha Gomes Dias (Centro Nacional de Pesquisa em Energia e Materiais, Laboratório Nacional de Biociências, Campinas, São Paulo, Brazil). Human cervix adenocarcinoma (HeLa) and human embryonic kidney (HEK293) cells were kindly provided by Dr Jörg Kobarg (Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil). Cell lines were grown in RPMI-1640 (Roswell Park Memorial Institute, Sigma, Darmstadt, Germany) or DMEM (Dulbecco's Modified Eagle Medium, Gibco, Dublin, Ireland) medium supplemented with 10% (v/v) fetal bovine serum (FBS) (LGC Biotecnologia, São Paulo, Brazil), 100 g mL^{-1} streptomycin and 100 units *per* mL penicillin (Sigma, Darmstadt, Germany) at pH 7.2 and 37 °C under 5% CO_2 atmosphere.

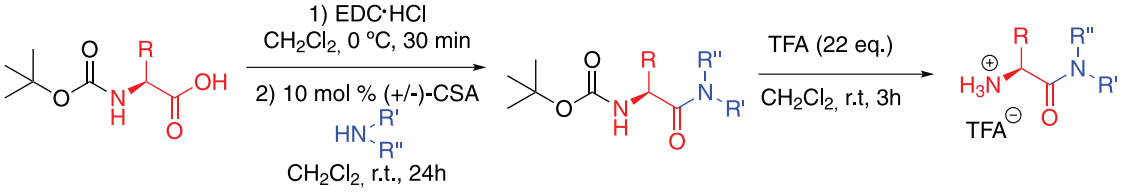
Cell viability assay

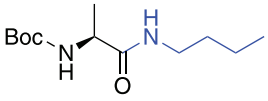
Each cell line were seeded in 96-well plates at the density of 10^4 cell *per* well containing 100 µL of complete medium and 100 µL of each compound solution at 10 µM or at different concentrations (0-200 µM) for IC_{50} determination. The compounds were diluted in RPMI medium with 10% FBS and 0.4% dimethylsulfoxide (DMSO) (v/v, Sigma, Darmstadt, Germany). After 48 h of culture, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) (5 mg mL^{-1}, Sigma, Darmstadt, Germany) was added to the wells. After 3 h at 37 °C, the MTT solution was removed and it was added 100 µL *per* well of DMSO to solubilize the formazan. Absorbance was measured at 540 nm in a microplate reader (SpectraMax M5, Molecular Devices). Analyses were performed using Microsoft Excel (Microsoft Office Software) and GraphPad Prism (GraphPad Software Inc.).[22] Each experimental procedure was performed in triplicate.

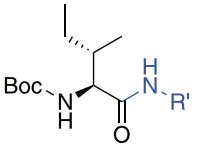
Results and Discussion

By employing the synthetic methodology developed by our research group,[23] 32 derivatives from L-amino acids and 2 from D,L-alanine were prepared in moderate to good yields. Furthermore, the previously prepared *N*-alkyl diamine **39** was successfully employed as nucleophile in the attainment of the derivative **8**. A detailed list of the synthesized compounds is described in Scheme 1.

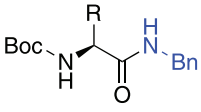




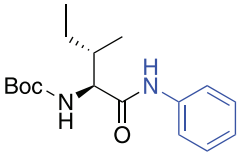
(**1**) (69%)



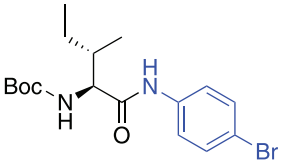
(**2**) R' = $(CH_2)_5CH_3$ (78%)
(**3**) R' = $(CH_2)_7CH_3$ (67%)
(**4**) R' = $(CH_2)_9CH_3$ (80%)
(**5**) R' = $(CH_2)_{11}CH_3$ (77%)
(**6**) R' = $(CH_2)_{13}CH_3$ (77%)
(**7**) R' = $(CH_2)_3NH_2$ (67%)
(**8**) R' = $(CH_2)_4NH(CH_2)_{11}CH_3$ (78%)



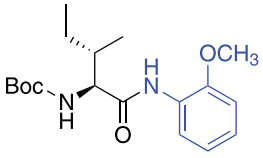
(**9**) R = (2S)-*s*Bu (70%)
(**10**) R = Me (72%)
(**11**) R = Bn (73%)
(**12**) R = *i*Bu (79%)
(**13**) R = *i*Pr (81%)



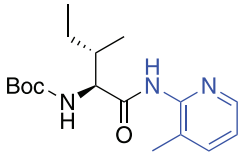
(**14**) (73%)



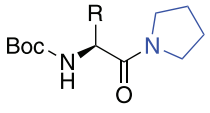
(**15**) (76%)



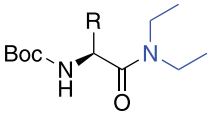
(**16**) (70%)



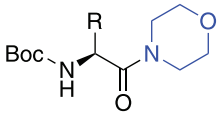
(**17**) (52%)



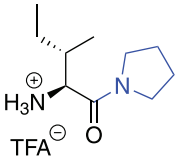
(**18**) R = (2S)-*s*Bu (73%)
(**19**) R = Me (64%)
(**20**) R = *i*Pr (68%)
(**21**) R = Bn (85%)



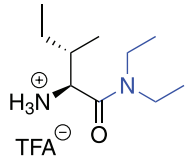
(**22**) R = (2S)-*s*Bu (72%)
(**23**) R = Me* (67%)
(**24**) R = *i*Pr (79%)
(**25**) R = *i*Bu (76%)
(**26**) R = Bn (72%)



(**27**) R = (2S)-*s*Bu (86%)
(**28**) R = Me (71%)
(**29**) R = Me* (65%)
(**30**) R = *i*Pr (75%)
(**31**) R = *i*Bu (73%)
(**32**) R = Bn (76%)



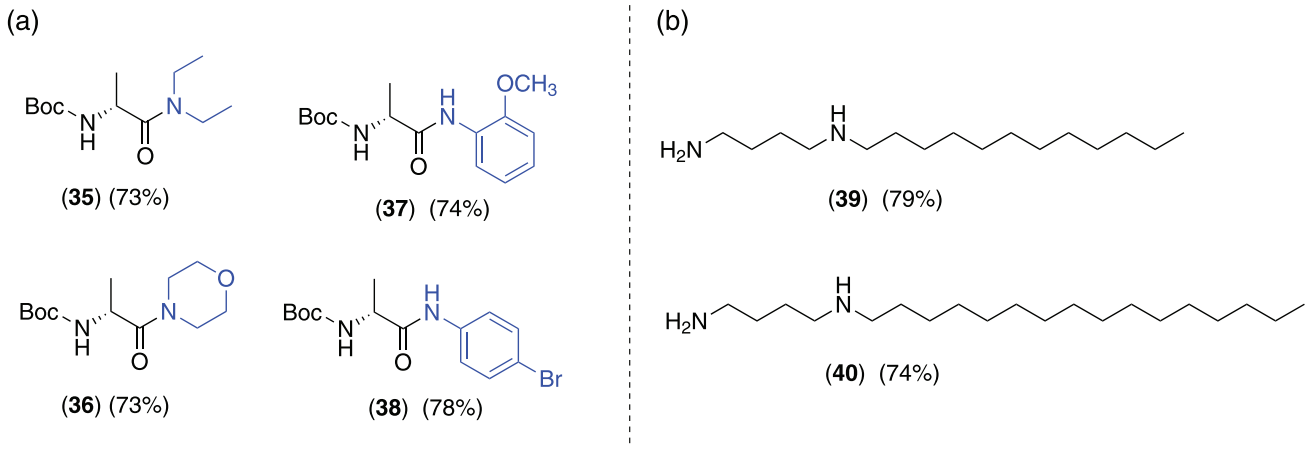
(**33**) (86%)



(**34**) (84%)

*Compounds **23** and **29**: racemic mixtures.

Scheme 1. Synthesized L-amino acid derivatives.



Scheme 2. (a) Synthesized D-amino acid derivatives; (b) synthesized *N*-alkyl diamines.

Besides, in an attempt to compare the influence of the product stereochemistry in the anticancer activity, four new derivatives were prepared employing the D-alanine as initial substrate (Scheme 2a). Finally, in addition to the *N*-alkyl diamine **39**, a more lipophilic derivative was prepared (**40**) aiming to evaluate the side-chain size in the biological activity (Scheme 2b). It is worth mentioning that in addition to other techniques for the characterization of the relative and absolute stereochemistry of the products, the derivative **37** had its X-ray crystallographic structure determined (Figure 1).

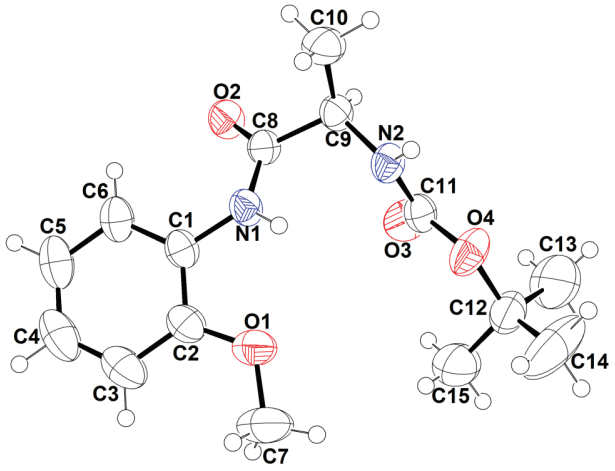


Figure 1. X-ray crystallographic structure of **37** (anisotropic displacement ellipsoids are drawn at the 50% probability level).

The cytotoxic effects of the synthesized amino acid derivatives **1-40** were firstly screened employing murine metastatic melanoma-B16F10, human cervix adenocarcinoma-HeLa, murine fibroblast-NIH3T3 (non-tumor cell line), and human embryonic kidney-HEK293 (non-tumor cell line), using the well-established (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay.[24] The results are shown in Table 1.

The most promising derivatives in the previous assay were then selected and had their half-maximal inhibitory concentration determined for the four cell lines previously employed and for two other tumor cells: MDA-MB-231, a breast adenocarcinoma cell, and HepG2, a hepatocellular carcinoma.

In general, three of the derivatives (**8**, **39** and **40**) showed promising cytotoxicity, mostly towards cancer cells. Interestingly, these most active compounds have in their structure an *N*-alkyl diamine moiety. Considering all forty compounds, compound **7** (in which propanediamine was employed as nucleophile instead of an *N*-alkyl diamine) showed no inhibition of tumor cells. On the other hand, a similar analogue with the incorporation of a lipophilic sidechain (compound **8**) was the only derivative to inhibit B16F10 tumor cell line in more than 90%. It is important to mention that metastatic B16F10 is known to be very resistant to anticancer agents,[25] which highlight the result obtained.

In the tested concentration, most of the compounds were almost inactive towards the tumor cells evaluated, with a growth percentage relative to control ranging between 70-100%. All derivatives prepared though the use of secondary amines nucleophiles (**18-32**) presented poor inhibition over B16F10 cells (above 70% of cell viability after 48 h of treatment) and most of them stimulated the growth of HeLa cells. Moreover, the use of Boc deprotected derivatives (**33-34**) as well as all four derivatives from D-alanine (**35-38**) failed to effectively inhibit the cancer cells.

Furthermore, by analyzing the data some interesting observations could be found. For example, compound **14** stimulated the cellular growth of tumor cells, while showing a small inhibition over non-tumor cells. Besides, a direct influence of the increase in the activity and the lipophilicity of the derivatives could not be traced. As when

Table 1. Effect of synthesized amino acid derivatives on cell viability. Cell viability was determined using the MTT assay. The percentage of inhibition was calculated considering the cells treated with the vehicle (DMSO), considered as 100% of cell viability

Compound[a] / 10 μM	Cell line / % of viable cell			
	B16F10[b]	HeLa[c]	NIH3T3[d]	HEK293[e]
1	74.9 ± 8.7	91.5 ± 1.3	69.0 ± 9.4	96.8 ± 2.3
2	80.8 ± 4.3	94.7 ± 12.7	53.7 ± 11.9	95.3 ± 1.3
3	66.2 ± 5.4	39.8 ± 0.6	66.5 ± 5.0	96.1 ± 4.1
4	63.0 ± 0.9	40.6 ± 1.0	78.1 ± 9.0	94.3 ± 6.4
5	72.3 ± 11.5	90.3 ± 10.9	70.2 ± 19.0	92.3 ± 5.7
6	74.8 ± 24.1	108.6 ± 3.1	75.5 ± 1.3	92.1 ± 5.1
7	98.1 ± 27.5	104.6 ± 1.4	66.0 ± 16.2	93.9 ± 4.4
8	9.4 ± 0.5	20.1 ± 0.7	34.5 ± 4.5	17.8 ± 5.3
9	84.2 ± 12.0	93.0 ± 1.8	94.5 ± 18.3	92.5 ± 5.8
10	75.2 ± 29.2	99.5 ± 5.2	58.8 ± 6.9	95.6 ± 5.3
11	77.2 ± 10.1	126.4 ± 4.9	77.5 ± 18.7	95.5 ± 5.6
12	83.1 ± 10.5	90.6 ± 20.4	68.2 ± 18.8	96.2 ± 2.7
13	75.6 ± 1.8	119.9 ± 2.2	62.2 ± 28.8	96.9 ± 2.4
14	106.8 ± 13.6	115.5 ± 5.5	74.0 ± 19.1	92.2 ± 4.9
15	92.4 ± 25.1	89.8 ± 9.4	41.8 ± 5.0	91.0 ± 2.8
16	96.2 ± 11.9	103.7 ± 7.0	46.4 ± 3.8	94.9 ± 7.0
17	70.4 ± 4.6	102.5 ± 15.6	57.5 ± 3.6	95.4 ± 5.2
18	75.9 ± 19.6	98.9 ± 8.7	53.1 ± 4.9	94.6 ± 4.1
19	71.7 ± 13.2	105.2 ± 4.0	62.5 ± 3.8	98.4 ± 0.4
20	84.3 ± 10.4	96.8 ± 1.6	69.2 ± 16.0	94.6 ± 3.5
21	71.5 ± 13.8	109.6 ± 5.2	79.4 ± 4.1	88.0 ± 2.4
22	84.2 ± 4.0	104.1 ± 9.0	87.7 ± 13.4	94.9 ± 3.4
23	82.4 ± 12.5	118.5 ± 4.7	65.7 ± 6.7	94.4 ± 3.6
24	82.4 ± 9.1	126.7 ± 3.0	74.3 ± 20.6	96.1 ± 3.7
25	81.9 ± 6.7	120.2 ± 3.6	87.6 ± 11.5	90.9 ± 5.7
26	76.3 ± 7.8	95.3 ± 4.7	64.1 ± 18.5	96.3 ± 4.0
27	81.3 ± 4.1	99.3 ± 7.7	69.3 ± 15.8	98.5 ± 0.7
28	76.2 ± 4.4	97.4 ± 2.2	73.6 ± 4.7	91.4 ± 2.0
29	92.5 ± 12.9	117.5 ± 1.4	83.3 ± 7.5	93.1 ± 5.8
30	74.6 ± 11.1	124.7 ± 3.1	75.9 ± 17.5	90.2 ± 3.6
31	73.3 ± 1.7	114.2 ± 8.5	91.3 ± 11.2	94.8 ± 6.9
32	74.6 ± 11.0	118.7 ± 15.6	80.4 ± 2.6	93.7 ± 5.7
33	73.0 ± 9.1	101.7 ± 4.1	98.6 ± 3.8	92.2 ± 5.5
34	79.2 ± 9.9	104.3 ± 1.1	62.2 ± 18.2	93.9 ± 4.8
35	80.6 ± 5.8	111.8 ± 9.2	54.3 ± 6.0	94.3 ± 3.8
36	79.5 ± 3.6	84.3 ± 15.7	86.2 ± 0.4	94.3 ± 4.9
37	88.0 ± 4.2	87.6 ± 3.3	75.1 ± 11.5	90.0 ± 0.8
38	88.3 ± 5.1	84.7 ± 4.9	96.1 ± 10.3	95.2 ± 5.1
39	10.3 ± 0.2	25.1 ± 1.1	36.1 ± 5.2	13.5 ± 0.7
40	12.0 ± 0.2	18.7 ± 1.0	26.9 ± 4.7	55.7 ± 19.8

[a]B16F10, HeLa, NIH3T3, and HEK293 cells were treated with 10 μM of each compound for 48 h. The values are expressed as the means ± standard deviation of triplicated experiments; [b]B16F10: murine metastatic melanoma; [c]HeLa: human cervix adenocarcinoma; [d]NIH3T3: murine fibroblast (non-tumor cell line); [e]HEK293: human embryonic kidney (non-tumor cell line).

analyzing the series of compounds **1-6**, the most active compounds were the mid-sized chain derivatives **3** and **4**, with considerable decrease in cellular growth inhibition by altering the carbon chain size.

In the following experiments, the three most promising compounds (**8**, **39** and **40**) had their half-maximal inhibitory concentration determined (Table 2) for six cell lines. The results indicated that these three derivatives showed anticancer activity with IC_{50} ranging from 1.9 to 6.1 μM. While in *N*-alkyl diamine **39** the IC_{50} for one of the tumor cell lines (MDA-MB-231) was higher than for the tested normal cells, the same profile was not observed for the longer chain derivative **40**. On the other hand, compound **40** in some cases presented similar half-maximal inhibitory concentrations for normal and tumor cells, such as for MDA-MB-231 (IC_{50} 3.1 μM) and NIH3T3 (IC_{50} 4.4 μM), indicating a poor selectivity.

The most promising result was detected for the derivative **8**, bearing both the *N*-alkyl diamine aliphatic sidechain and the amino acid moiety, with IC_{50} for all cancer cells near 2.0 μM and for normal cells above 6.0 μM. Most interesting, the comparison of compound **8** (the Boc-protected amino acid bearing a *N*-alkyl diamine moiety) and **39** (the *N*-alkyl diamine) showed considerable differences in the activity. While the incorporation of the amino acid scaffold reduced the IC_{50} for all tumor cells (especially for MDA-MB-231, with an almost three-fold decrease in IC_{50}), the inverse behavior was detected in normal cells, with a small increase in IC_{50} for HEK293 and a three-fold increase for NIH3T3. These three compounds probably inhibits cell growth by acting in the polyamine biosynthesis/metabolism pathway, as occur in other diamine-based in drugs (such as Eflornithine).[26] On the other hand, the enhanced selectivity of compound **8** might be involved with the increase in amino acid uptake in some cancer cells, by the overexpression of L-type amino acid transporter-1 (LAT-1).[9]

Finally, the selectivity index was calculated by diving the mean IC_{50} for normal cells by the mean IC_{50} for tumor strains and the results are described in Table 3. Once more, the compound **8** showed the best overall result, with a selectivity index of 3.4. Although the selectivity index and the activity can be further enhanced, we believe that derivative **8** is a promising building block for the development of new anticancer candidates. Thus, the preparation of new amino acid derivatives, bearing *N*-alkyl diamines of different sidechain lengths is under development by our research group and will be reported in the due course along with more in-depth studies regarding the mechanism of action in the intracellular context.

Conclusions

In summary, a series of 40 compounds (including thirty two L-amino acid, two D,L-amino acid and four D-amino acid derivatives, as well as, two protected amino acid salts and two *N*-alkyl diamines) were synthesized and evaluated towards their cytotoxic activity over four tumor and two non-tumor cells. Among the synthesized derivatives, three bearing an *N*-alkyl diamine sub-unity exhibited the best

Table 2. Half-maximal inhibitory concentration (IC_{50}) values for the treatments with compounds **8**, **39** and **40**

Compound	Cell line - IC_{50} / μM[a]					
	B16F10[b]	MDA-MB-231[c]	HepG2[d]	HeLa[e]	NIH3T3[f]	HEK293[g]
8	1.8 ± 2.1	2.3 ± 4.3	1.7 ± 3.0	1.7 ± 4.3	6.6 ± 5.2	6.0 ± 1.1
39	2.2 ± 1.4	6.1 ± 2.5	1.9 ± 2.7	2.0 ± 7.6	2.2 ± 3.8	5.8 ± 1.2
40	2.4 ± 2.4	3.1 ± 1.9	2.3 ± 2.8	2.1 ± 5.0	4.4 ± 5.0	9.7 ± 1.8

[a]Indicated cell lines were treated with increasing concentrations (0-200 μM) of each compound for 48 h. Cell viability was determined using the MTT assay. The values are expressed as the means ± standard deviation of triplicated experiments; [b]B16F10: murine metastatic melanoma; [c]MDA-MB-231: breast adenocarcinoma; [d]HepG2: hepatocellular carcinoma; [e]HeLa: cervix adenocarcinoma; [f]NIH3T3: murine fibroblast (non-tumor cell line); [g]HEK293: human embryonic kidney (non-tumor cell line).

Table 3. Selectivity index determination and mean half-maximal inhibitory concentration (IC_{50}) values for the treatments in normal and cancer cells

Compound	IC_{50} / μM		
	Non-tumor cell lines	Tumor cell lines	Selectivity index[a]
8	6.3 ± 3.2	1.9 ± 3.4	3.4
39	4.0 ± 2.5	3.1 ± 3.6	1.3
40	7.1 ± 3.4	2.5 ± 3.0	2.8

[a]Selective indexes were calculated by dividing the IC_{50} mean value for non-tumor cell lines by the IC_{50} mean values for tumor cells.

overall inhibitory activity, presenting IC_{50} values at the low micromolar range. Notably, compound **8** was the most potent derivative, presenting IC_{50} about 2.0 μM on tumor cells. Furthermore, the inclusion of an amino acid moiety in the *N*-alkyl diamine seems to play an important role in the selectivity towards cancer cells, increasing both the activity and the selectivity index. To the best of our knowledge, this consists the first time that the class of dual-protected amino acid derivatives hereby reported were assayed toward their anticancer activity. Due to the promising results found during this study, the preparation of new derivatives based on the structure of compound **8** is ongoing in our laboratory.

Supplementary Information

Crystallographic data (excluding structure factors) for the structures in this work were deposited in the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 1884757. Copies of the data can be obtained, free of charge, via www.ccdc.cam.ac.uk/conts/retrieving.html or from the Cambridge Crystallographic Data Centre, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033. E-mail: deposit@ccdc.cam.ac.uk.

Supplementary data (copies of ^{1}H NMR, ^{13}C NMR, FTIR and XRD) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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