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Research paper

Evaluation of toxicity on epithelial and tumor cells of biaryl dipeptide tyrosines

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ABSTRACT

We report a method to obtain biaryl dipeptide tyrosine via Suzuki–Miyaura and alkynyl dipeptide tyrosine by Sonogashira cross-coupling reactions. Analysis of the biological action of biaryl dipeptide tyrosine 4d compound showed its ability to impair the metabolism and proliferation of SK-Mel-28 human melanoma lineage cells, independently of mitochondrial membrane depolarization, apoptosis and necrosis. Moreover, 4d compound did not cause toxicity to human umbilical vein endothelial cells (HUVEC), suggesting its toxic specificity to cancer cells.

1. Introduction

Structural component of proteins and peptides, amino acids have been used as building blocks in the synthesis of more complex molecules and biologically active compounds, this fact is due to the low cost of amino acids, high availability and low toxicity. In this sense the tyrosine, a nonessential amino acid, but proteinogenic, is one of the few aromatic amino acids and being the only carrier of a phenolic nucleus, *ortho* director for alkylation and acylation reactions it is also used in obtaining of biaryl subunit via cross-coupling reactions [1].

The functionalization of tyrosine cores is a synthetic strategy that will achieve unnatural peptides, assigning a higher biological potential of this class of compounds, as an example of peptides containing tyrosine residues with known biological activity, the ustiloxin D, a potent antimitotic agent, isolated from the fungus *Ustaliginoidea virens* and recently synthetically exploited by Hutton and co-workers [2], as well as valorphin, with antiproliferative properties against tumor cells [3] (Fig. 1).

Moreover, amino acid derivatives have been employed as tracers in Positron Emission Tomography (PET) to detect neoplasms [4,5]. In this context, tyrosine derivatives have been considered promising tracers candidates because they take into account the lengthened half-live of tyrosine in comparison to other amino acids, such as methionine [4,6,7]. Indeed, the efficacy of fluoralkyltyrosine compounds as tracers has been shown in experimental models and human cancer [8–13].

Melanoma is a malignancy of melanocytes which are pigment-producing cells found in the skin, iris and rectum. The rate of malignant melanoma has increased in the last few years and it is estimated that there will be more than 73,000 new cases in 2015 in the United States (National Cancer Institute of NIH, 2015). Melanoma has a high metastatic index and patients with stage IV melanoma have a poor prognosis, with a mean survival of 8–10 months [14,15]. Advanced stages of disease are resistant to established therapeutic approaches, including chemotherapy, surgical excision, radiotherapy and immunotherapy [15].

In this sense, there is great demand for methods that allow the synthesis of a variety of functionalized tyrosine dipeptide derivatives. Herein, we report an efficient and general access method for the synthesis of biaryl dipeptide tyrosine derivatives via the Suzuki–Miyaura reaction and 3-alkynyl dipeptide tyrosine via Sonogashira coupling. Furthermore, we show that the **4d** derivative had an anti-proliferative effect on the human melanoma cell line

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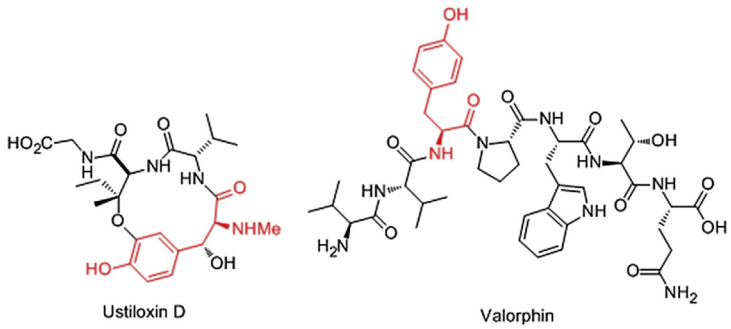


Fig. 1. Peptide tyrosine derivatives with known biological activity.

SK-Mel-28, with selectivity over normal human umbilical vein endothelial cells (HUVEC).

2. Results and discussion

2.1. Chemistry

Our strategy included the preparation of dipeptides **3a**–**c** through the formation of a peptide bond between tyrosine-methyl ester **1** and *N*-Boc-tyrosine **2**, using hydroxybenzotriazole (HOBt), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), triethylamine (TEA) and dichloromethane (DCM), in a 15 h reaction at room temperature. Using the same methodology it was possible to obtain the peptide **3a** in 57% yield with an iodine atom in the structure, as well as the peptides **3b** and **3c** containing one iodine atom in each aromatic ring, which were achieved in 48% and 57% yield respectively. From peptides core containing iodine we performed Suzuki–Miyaura or Sonogashira cross-coupling reactions [16,17].

In order to obtain an alkenyl dipeptide derivative, the peptide **3a** it was reacted with potassium trans-styryltrifluoroborate as substrate, using $Pd(OAc)_2$ in 10 mol%, K_2CO_3 as base in MeOH, the dipeptide **4a** was achieved in 89% yield. To obtain two different biarylic fragments, we prepared a Suzuki–Miyaura coupling reaction between the dipeptide **3b** and the potassium phenyltrifluoroborate salt, the reaction led the exclusive formation of desired cross-coupling product **4b** that after purification was isolated in 52% yield. Since iodine was still present in the peptide fragment, this allowed us to use a new cross-coupling reaction to get different biarylic subunits in peptides fragments. Therefore, dipeptide **4b** was reacted with potassium 3-thiophenetrifluoroborate salt under Suzuki–Miyaura conditions to give the compound **4c** in 42% yield.

The reactivity of the dipeptide **3b** was observed in the bis coupling reaction, when we used two equivalent of potassium 4-methoxyphenyltrifluoroborate salt and one equivalent of **3b** under appropriate conditions for Suzuki–Miyaura cross-coupling, just the compound **4d** was observed, after flash chromatography, the product could be obtained in 61% of yield.

The Suzuki–Miyaura products were obtained in the peptide fragments without phenol group protection; but when the Sonogashira cross-coupling reaction was performed, it was necessary to protect the phenol; the reaction without prior protection has led to the desired product in low yields. In this sense, using Pd(dppf) Cl_2CH_2Cl2 as a catalyst, CuI as a co-catalyst, TEA and THF, dipeptide **3c** and ethynyltrimethylsilane were reacted via a Sonogashira reaction to give the desired product **4e** in 89% yield in a single step (Scheme 1).

Peptide bond conditions: **2** (0.5 mmol) HOBt (1.1 eq), EDC (1.2 eq) **1** (0.5 mmol) TEA (1.2 eq), DCM, 15 h. Yield related to the isolated products.

2.2. Biological activity

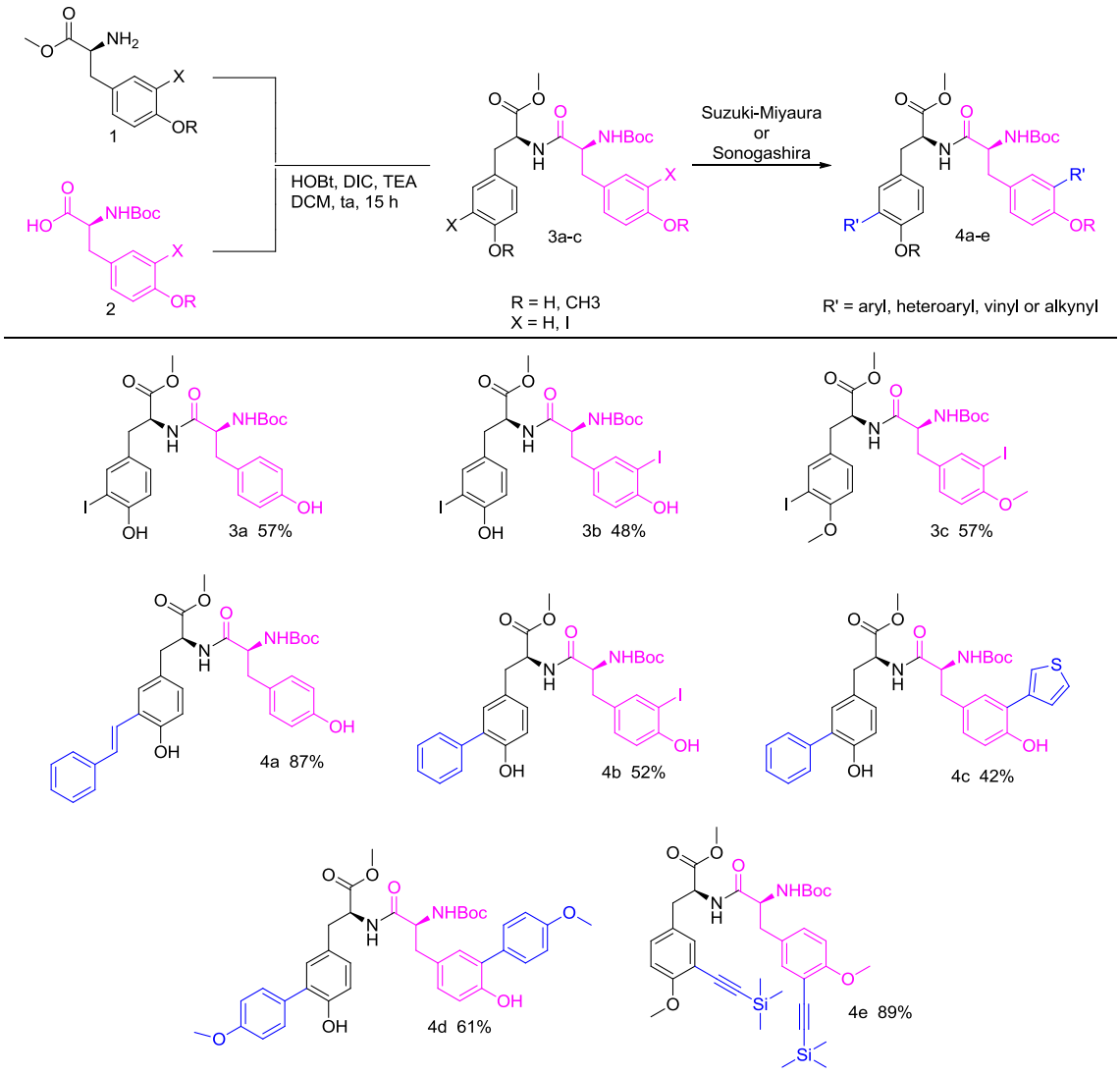
A MTT assay showed that a solution of **4d** impaired the metabolism of SK-Mel-28 cells but not HUVEC. This effect was observed 24 or 48 h after incubation of the cells with two concentrations of the **4d** solutions (Fig. 2). As results from MTT assays may indicate alterations of mitochondrial membrane potential, we further evaluated if **4d** solutions could affect mitochondrial membrane depolarization. The results showed equivalent depolarization in HUVEC or SK-Mel-28 cells treated with PBS, vehicle or **4d** solutions (Fig. 3). Nevertheless, the positive control valinomycin, caused depolarization in both HUVEC and SK-Mel-28 cells, indicating the efficacy of the experimental procedure (Fig. 3).

A reduction in SK-Mel-28 cell metabolism may lead to impaired cell proliferation. Therefore cell proliferation assays were performed to further examine the biological action of solution **4d**. As shown in Fig. 4, incubation of SK-Mel-28 cells with both concentrations of **4d** led to lower cell numbers than when cells were incubated with vehicle, and an equivalent number of DMSO-incubated cells (positive control) (Fig. 4). Moreover, as expected, the proliferation of **4d**-treated HUVEC was similar to that of vehicle-treated cells and higher than that of DMSO-treated cells (Fig. 4), indicating that **4d** did not affect HUVEC proliferation.

Apoptosis or necrosis impair cell metabolism; therefore, we investigated if **4d** could induce apoptosis or necrosis in SK-Mel-28 cells. Results showed that **4d** did not induce cell death in the concentrations evaluated, as the percentage of SK-Mel-28 or HUVEC in apoptosis, late apoptosis or necrosis was similar in vehicle-treated or **4d**-treated cells (Fig. 5).

Together, these results indicate that the biaryl dipeptide tyrosine derivative is toxic to melanoma but not to epithelial cells. SK-Mel-28 lineage was chosen to test the toxicity because it is a malignant melanoma cell line. Melanoma development is aggressive, especially in the metastatic form, and no efficient treatments have been proposed until now [18]. Therefore, novel or complementary pharmacological approaches are needed for the treatment of melanoma. The results from this study show that further biological tests should be conducted to determine if biaryl dipeptide tyrosine derivatives could be used as therapeutic agents. It is important to mention that **4d** selectivity to SK-Mel-28 cells over HUVEC strengthens our data, as the endothelium consists epithelial cells covering the luminal surface of all vessels, and therefore, it is the first barrier to chemical agents during tissue distribution from the blood circulation [19,20].

The mechanism involved in the selectivity of **4d** has not been identified, but it is possible that cancer cells may uptake the



Peptide bond conditions: **2** (0.5 mmol) HOBt (1.1 eq), EDC (1.2 eq) **1** (0.5 mmol) TEA (1.2 eq), DCM, 15 h. Yield related to the isolated products.

Scheme 1. Functionalization of tyrosine dipeptides.

compound with more effciency than epithelial cells, as cancer cells have more amino acids and increased protein metabolism [21,22]. Based in this phenotypic characteristic of cancer cells, tyrosine derivatives have been employed as tracers, as previously mentioned. Here we show, for the first time, that the tyrosine derivatives may work as teranostic compounds, with the ability to detect and induce mechanisms of cancer cell toxicity. These results pave the way for future studies to investigate the mechanism by which biaryl dipeptide tyrosine derivatives may be preferentially captured by cancer cells, leading to more selectivity as cytotoxic agents.

3. Conclusion

The combination of tyrosine fragments via peptide bond followed by functionalization via Suzuki–Miyaura and Sonogashira cross-coupling reactions afforded structurally and biologically interesting dipeptide compounds. Moreover, for the first time, the toxicity of a biaryl dipeptide tyrosine derivative was demonstrated in a melanoma cell lineage, but not to non-tumoral epithelial cells, suggesting that these compounds may have selective cytotoxic potential.

4. Experimental section

4.1. Chemistry

All of the starting materials were commercial grade and used without further purification. ^{1}H NMR ^{13}C NMR and spectra were recorded on a Bruker DPX 300 at 300 MHz and 75 MHz, respectively, using $CDCl_3$. Chemical shifts are reported in *ppm*, referenced to the solvent signal of $CDCl_3$ or tetramethylsilane (TMS) as the internal reference. Data are reported as follows: chemical shift (δ), multiplicity, coupling constant (*J*) in Hertz and integrated intensity. Abbreviations to denote the multiplicity of a particular signal are: s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), sex

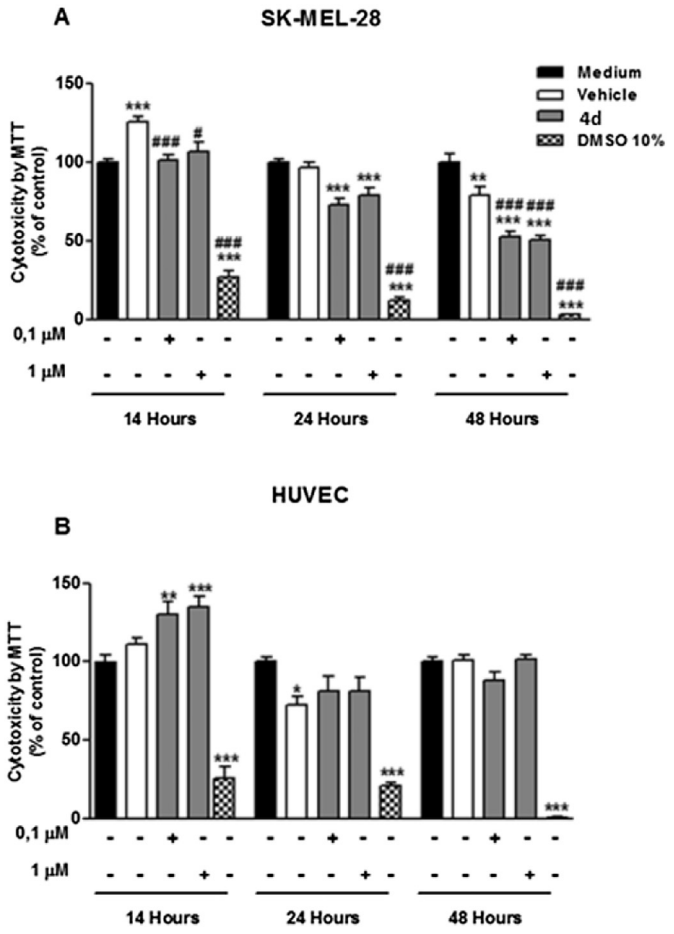


Fig. 2. Effect of biaryl dipeptide tyrosine derivative (compound **4d**) on human umbilical vein endothelial cell (HUVEC) and melanoma cell (SK-Mel-28) metabolism. 1×10^3 cells/well of HUVEC (A) or SK-Mel-28 cells (B) were incubated with medium R10 (control), vehicle (0.1% DMSO), or **4d** (0.1 or 1 μM) for 14, 24, or 48 h. Cell metabolism was assessed using a MTT assay and values are represented as mean ± SEM of three independent assays. Data are expressed as % of control group. 10% DMSO was used as positive control. Significant differences from R10 (control) are $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ assessed by two-way ANOVA followed by the Tukey's test.

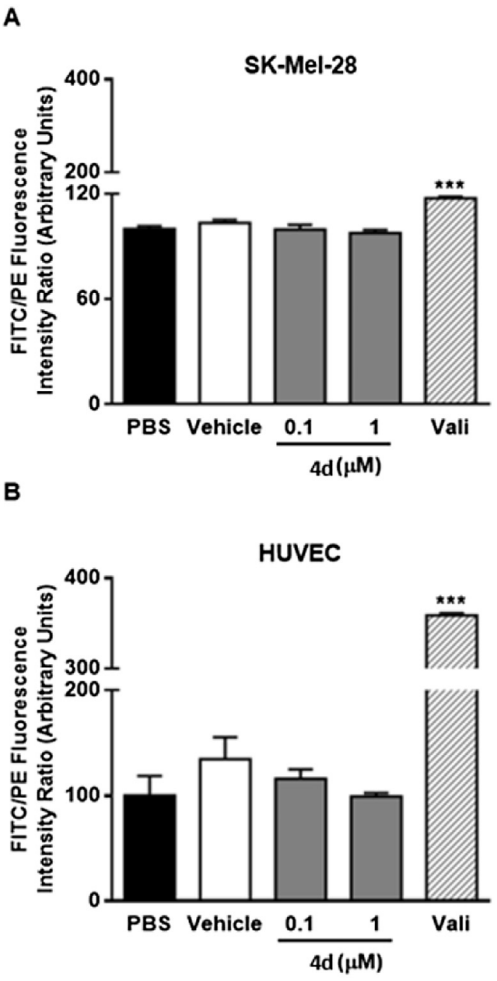


Fig. 3. Effect of biaryl dipeptide tyrosine derivative (compound **4d**) on human umbilical vein endothelial cell (HUVEC) and melanoma cell (SK-Mel-28) mitochondrial membrane potential. 1×10^4 cells/well of SK-Mel-28 (A) or HUVEC (B) were incubated with medium R10 (control), vehicle (0.1% DMSO), or **4d** (0.1 or 1 μM) for 48 h and the mitochondrial membrane potential was determined using JC-1 (1.5 μg/mL) by flow cytometry. Cells were treated with valinomycin (200 μM) for 24 h as a positive control. Values are represented as mean ± SEM of two independent assays. Significant differences from R10 (control) are $^{*}p < 0.05$, and $^{***}p < 0.001$ assessed by one-way ANOVA followed by the Tukey's test.

(sextet) and m (multiplet). Column chromatography was performed using silica gel (230–400 mesh). Thin Layer Chromatography (TLC) was performed using silica gel UV254, 0.20 mm thickness. High-Resolution Mass Spectra were obtained using a high-resolution ESI-TOF mass spectrometer Shimadzu LCMS-IT-TOF.

4.1.1. General procedure for dipeptide formation

A solution of **2** (140 mg, 0.5 mmol) in dry DCM was cooled to 0 °C and 1-hydroxybenzotriazole (1.1 eq) was added, followed by EDC (1.2 eq). After 1 h, the mixture was allowed to warm to room temperature at which point compound **1** (179 mg, 0.5 mmol) dissolved in DMF was added, followed by TEA (1.2 eq). The reaction mixture was stirred for 15 h. Then the reaction mixture was diluted with DCM and washed with a saturated solution of NH_4Cl (3×20 mL). The organic phase was collected, dried with $MgSO_4$, filtered and the solvent was removed under vacuum. The product was purified by silica flash chromatography and eluted with ethyl acetate/hexane 5:5.

The product **3a** was obtained as a white solid. Yield 166 mg (57%). Melting point 83–84 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34 (s, 1H), 6.97 (d, $J = 8.0$ Hz, 2H), 6.82 (d, $J = 8.3$ Hz, 1H), 6.76 (s, 1H), 6.71 (t, $J = 7.9$ Hz, 2H), 6.57 (d, $J = 7.7$ Hz, 1H), 5.16 (s, 1H), 4.72 (d, $J = 7.0$ Hz, 1H), 4.28 (s, 1H), 3.68 (s, 3H), 2.99–2.85 (m, 4H), 1.40 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.59, 171.35, 155.67, 155.26, 154.53, 139.13, 130.73, 130.39, 129.32, 115.75, 115.19, 85.01, 53.43, 52.50, 37.54, 36.51, 28.28; HRMS (ESI-TOF) *m/z,* calcd. for $C_{24}H_{29}IN_2O_7 + H^+$: 585.1097, found: 585.1094.

Product **3b** was obtained as a yellow oil. Yield 170 mg (48%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.49 (d, 1H, J 1.8 Hz), 7.36 (d, 1H, J 1.8 Hz), 7.25–7.20 (m, 2H), 7.00 (d, 1H, J 7.9 Hz), 6.86 (dd, 1H, J 1.8, 8.3 Hz), 6.78 (d, 1H, J 8.0 Hz), 6.66 (d, 1H, J 7.7 Hz), 5.19 (d, 1H, J 7.2 Hz), 4.74 (dd, 1H, J 6.0, 13.0 Hz), 4.47 (d, 1H, J 7.8 Hz), 4.30 (s, 1H), 3.69 (s, 3H), 3.02–2.89 (m, 4H), 1.41 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.35, 157.55, 155.57, 154.78, 154.67, 139.28, 130.72, 130.62, 129.83, 129.13, 115.31, 115.26, 84.93, 60.55, 53.48, 52.64, 42.34, 28.32, 23.46, 14.19; HRMS (ESI-TOF) *m/z,* calcd. for $C_{24}H_{28}I_2N_2O_7 + H^+$: 710.2973, found: 710.2965.

Product **3c** was obtained as a white solid. Yield 210 mg (57%). Melting point 132–134 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.61 (s, 1H), 7.44 (s, 1H), 7.18–7.10 (m, 1H), 6.96 (dd, J = 8.3, 1.8 Hz, 1H),

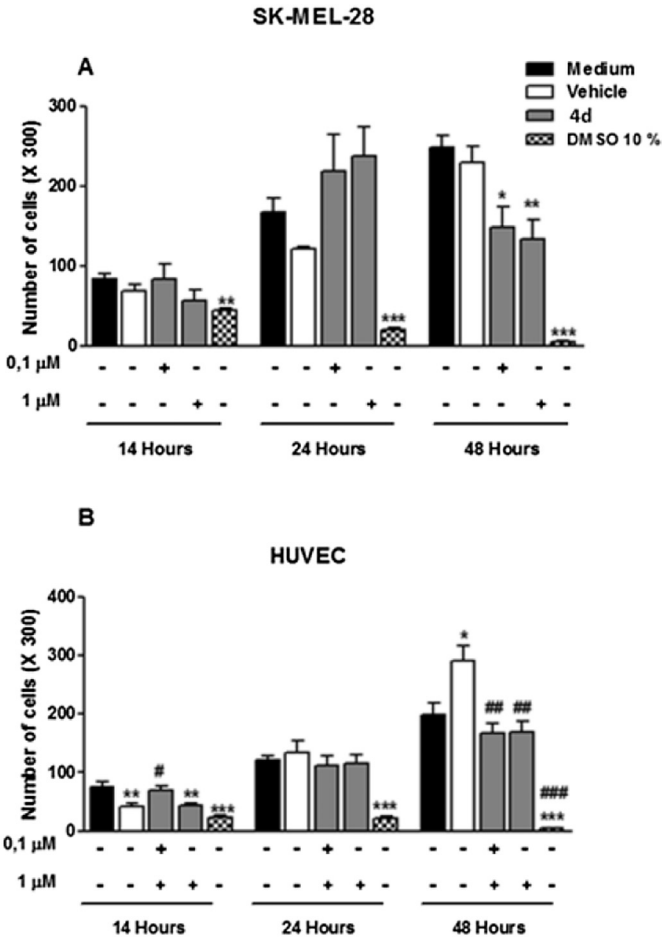


Fig. 4. Effect of biaryl dipeptide tyrosine derivative (compound **4d**) on human umbilical vein endothelial cell (HUVEC) and melanoma cell (SK-Mel-28) proliferation. 1×10^4 cells/well of HUVEC (A) or SK-Mel-28 cells (B) were incubated with medium R10 (control), vehicle (0.1% DMSO), or **4d** (0.1 or 1 μM) for 14, 24, or 48 h and cell proliferation was determined using a trypan blue assay by optical microscopy. Values are represented as mean ± SEM of three independent assays. 10% DMSO was used as positive control. Significant differences from R10 (control) are $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ assessed by one-way ANOVA followed by the Tukey's test.

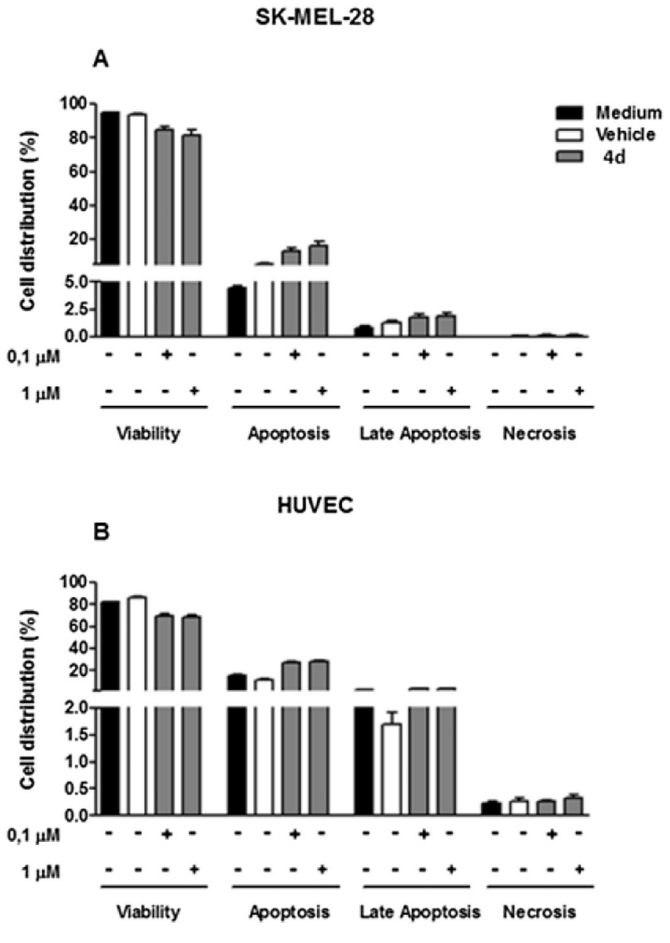


Fig. 5. Effect of biaryl dipeptide tyrosine derivative (compound **4d**) on human umbilical vein endothelial cell (HUVEC) and melanoma cell (SK-Mel-28) viability. 1×10^4 cells/well of HUVEC (A) or SK-Mel-28 cells (B) were incubated with medium R10 (control), vehicle (0.1% DMSO), or **4d** (0.1 or 1 μM) for 48 h and cell viability was determined using Annexin-V (1:100) and propidium iodide (0.5 μg/10 μL) by flow cytometry. Values are represented as mean ± SEM of three independent assays. No significant differences were found.

6.76–6.61 (m, 3H), 5.18 (d, $J = 8.0$ Hz, 1H), 4.75 (q, $J = 5.9$ Hz, 1H), 4.40–4.27 (m, 1H), 3.82 (s, 6H), 3.69 (s, 3H), 2.97 (q, $J = 7.0$, 6.4 Hz, 4H), 1.40 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.30, 170.82, 157.20, 157.12, 155.28, 140.10, 140.05, 130.75, 130.43, 130.28, 129.86, 111.01, 110.87, 86.07, 85.86, 80.23, 56.39, 56.32, 55.72, 53.32, 52.45, 36.90, 36.54, 28.31; HRMS (ESI-TOF) *m/z,* calcd. for $C_{26}H_{32}I_2N_2O_7 + H^+$: 739.0377, found: 739.0375.

4.1.2. General procedure for Suzuki–Miyaura coupling

K_2CO_3 (207 mg, 1.5 mmol) was added to a two-necked 25 mL round-bottom flask under a nitrogen atmosphere containing $Pd(OAc)_2$ (11.23 mg, 10 mol%), potassium trifluoroborate salt (0.6 mmol), dipeptide **3a** or **3b** (0.5 mmol) and MeOH (3 mL). The reaction mixture was stirred and heated to 60 °C. TLC was used to monitor the 10-h reaction. The reaction mixture was then diluted with ethyl acetate and washed with a saturated solution of NH_4Cl (3×20 mL). The organic phase was collected, dried with $MgSO_4$, filtered and the solvent was removed under vacuum. The product was purified by silica flash chromatography and eluted with ethyl acetate/hexane 5:5.

The product **4a** was obtained as a white solid. Yield 243 mg (87%). Melting point 133–134 °C. ^{1}H NMR (300 MHz, MeOH) δ 7.51 (d, $J = 7.5$ Hz, 2H), 7.38 (d, $J = 7.5$ Hz, 1H), 7.31 (t, $J = 7.6$ Hz, 2H), 7.21 (d, $J = 7.3$ Hz, 1H), 6.99 (d, $J = 8.0$ Hz, 2H), 6.91 (d, $J = 8.3$ Hz, 1H), 6.74 (d, $J = 8.2$ Hz, 1H), 6.66 (d, $J = 8.1$ Hz, 2H), 4.67 (t, $J = 6.8$ Hz, 1H), 4.22 (s, 1H), 3.67 (s, 3H), 3.12–2.65 (m, 4H), 1.33 (s, 9H). ^{13}C NMR (75 MHz, MeOH) δ 174.32, 173.26, 157.21, 155.26, 139.58, 131.35, 130.36, 129.62, 129.12, 128.80, 128.46, 128.23, 127.46, 125.77, 124.82, 116.87, 116.21, 80.74, 57.60, 55.27, 52.74, 37.96, 30.42, 28.68. HRMS (ESI-TOF) *m/z,* calcd. for $C_{32}H_{36}N_2O_7 + H^+$: 561.2601, found: 561.2597.

Product **4b** was obtained as a colorless oil. Yield 171 mg (52%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37–7.35 (m, 5H), 6.88 (d, 1H, *J* 7.5 Hz), 6.82 (s, 1H), 6.77–6.76 (m, 2H), 6.68 (d, 1H, *J* 8.2 Hz), 6.35 (d, 1H, *J* 7.5 Hz), 5.78 (s, 1H), 4.95 (s, 1H), 4.73–4.67 (m, 1H), 4.17 (s, 1H), 3.61 (s, 3H), 2.96–2.93 (m, 2H), 2.81–2.79 (m, 2H), 1.32 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.52, 170.83, 155.35, 154.33, 151.93, 139.10, 137.12, 131.08, 130.82, 130.19, 129.68, 129.06, 128.38, 127.75, 127.53, 116.21, 115.17, 85.30, 52.46, 42.38, 37.19, 37.01, 28.27, 23.45; HRMS (ESI-TOF) *m/z,* calcd. for $C_{30}H_{33}IN_2O_7 + H^+$: 661.1411, found: 661.1407.

Product **4c** was obtained as a colorless oil. Yield 64 mg (42%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36–7.28 (m, 7H), 7.22–7.19 (m, 1H), 7.07 (s, 1H), 6.86–6.79 (m, 2H), 6.76 to −6.67 (m, 3H), 6.30 (d, 1H, *J* 7.5 Hz), 6.11 (s, 1H), 5.75 (s, 1H), 4.96 (s, 1H), 4.69–4.65 (m, 1H), 4.22

(s, 1H), 3.55 (s, 3H), 2.95–2.75 (m, 4H), 1.30 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.52, 171.07, 152.12, 151.90, 137.51, 137.13, 131.02, 130.59, 129.68, 129.29, 129.05, 129.03, 128.36, 128.09, 127.71, 127.56, 126.05, 123.05, 116.41, 116.17, 60.44, 53.44, 52.30, 42.34, 37.18, 28.25, 23.45, 14.19; HRMS (ESI-TOF) *m/z*, calcd. for $C_{34}H_{36}N_2O_7S + H^+$: 617.2321, found: 617.2318.

Product **4d** was obtained as a colorless oil. Yield 102 mg (61%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35 (d, 4H, *J* 8.4 Hz), 7.02–6.96 (m, 6H), 6.87–6.79 (m, 4H), 6.42 (d, 1H, *J* 7.6 Hz), 5.70 (s, 1H), 5.04 (s, 1H), 4.80–4.74 (m, 1H), 4.32 (s, 1H), 3.84 (s, 6H), 3.65 (s, 3H), 2.99–2.88 (m, 4H), 1.40 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.53, 171.05, 159.22, 159.20, 151.93, 131.17, 130.99, 130.21, 129.31, 128.31, 128.06, 128.02, 127.54, 116.08, 116.01, 114.53, 114.48, 55.34, 53.44, 52.28, 42.32, 28.25, 23.46; HRMS (ESI-TOF) *m/z*, calcd. for $C_{38}H_{32}N_2O_9 + H^+$: 671.2969, found: 671.2960.

4.1.3. General procedure for Sonogashira cross-coupling

Dipeptide **3c** (74 mg, 0.1 mmol), $Pd(dppf)Cl_2CH_2Cl_2$ (16.2 mg, 0.02 mmol), and CuI (38 mg, 0.2 mmol) were added to a dried flask under nitrogen atmosphere. Freshly distilled THF (4 mL) was added using a syringe, then Na_2CO_3 (21 mg, 0.2 mmol) was added and the resulting solution was stirred for 10 min. Ethynyltrimethylsilane (0.6 mmol) was added slowly. The resulting solution was stirred at 60 °C. The reaction was monitored by TLC. The mixture was poured into 10 mL saturated NH_4Cl, and then extracted with ethyl acetate (15 mL) three times. The organic layer was combined, dried with $MgSO_4$, filtered and the solvent was evaporated. The resulting residue was purified by silica gel chromatography and eluted with ethyl acetate/hexane.

The product **4e** was obtained as a yellow oil. Yield 60.3 mg (89%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.53 (qd, J = 7.6, 4.9, 3.2 Hz, 2H), 7.30–7.23 (m, 3H), 7.13–7.09 (m, 1H), 6.92 (dd, J = 8.5, 2.3 Hz, 1H), 6.75 (d, J = 8.7 Hz, 1H), 4.93 (s, 1H), 4.75 (q, J = 6.4 Hz, 1H), 4.26 (d, J = 7.4 Hz, 1H), 3.86 (s, 6H), 3.70 (s, 3H), 3.06–2.86 (m, 4H), 1.43 (s, 9H), 0.26 (s, 18H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.24, 170.64, 159.42, 159.36, 135.16, 134.78, 134.66, 130.77, 130.62, 128.25, 127.42, 112.38, 111.00, 110.80, 100.97, 98.64, 60.30, 55.85, 55.79, 53.26, 52.23, 36.87, 28.19, 0.00. HRMS calcd. for $C_{36}H_{50}N_2O_7Si_2 + H^+$: 679.3350. Found: 679.3355.

4.2. Cell culture and treatment

Human skin malignant melanoma cells (SK-Mel-28) and human umbilical vein endothelial cells (HUVEC) were maintained in RPMI 1540 medium containing 10% fetal bovine serum (Vitrocell Embrolife, Campinas, São Paulo, Brazil) at 37 °C with 5% CO_2. When cells reached confluency, they were trypsinized (Vitrocell embrolife, Campinas, São Paulo Brazil), centrifuged (600 × g for 10 min), counted in a haemocytometer, plated, and incubated with culture medium for 24 h. After adhesion, the culture medium was replaced with a solution of **4d** (0.1 or 1 μM) or vehicle (0.1% DMSO). **4d** solution was prepared from a DMSO stock solution (10 mM) and stored at room temperature in dark conditions. Dilutions were made in complete culture medium just before the experiments.

4.3. Cell metabolism

The MTT assay is a colorimetric assay used to quantify cell metabolism. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma–Aldrich; St Louis, MO, USA) is a yellow compound that is soluble in water and converted to formazan crystal, a violet insoluble product in water, by the action of mitochondrial reductases in active cells. 1×10^3 cells/well SK-Mel-28 or HUVEC were prepared and treated as described above and incubated with a solution of **4d** or vehicle for 14, 24 or 48 h, at 37 °C and 5% CO_2. Subsequently, cells were washed and incubated with 100 μL of MTT solution (0.5 mg/mL in RPMI media) for 3 h in the dark at 37 °C and 5% CO_2. After incubation, the MTT solution was removed and cells were washed with PBS buffer (300 μL). 200 μL DMSO was then added to each well and. the plate was mixed for 5 min at 500 rpm. The optical density was read by spectrophotometry at 570 nm. 10% DMSO was employed as positive control. The percentage of viable cells was calculated by comparing the absorbance of treated and untreated cells (incubated with only RPMI, corresponding to 100% viability).

4.4. Cell death

A flow cytometry-based method using Annexin V (Becton and Dickinson, San Jose, CA, USA) and Propidium Iodide dye (PI; Sigma–Aldrich, St Louis, MO, USA) was employed to determine the ability of a solution of **4d** to induce apoptosis or necrosis, respectively. Annexin-V binds to phosphatidylserine exposed on apoptotic cell membranes and PI stains necrotic cells, which lose plasma membrane integrity. Briefly, 1×10^4 cells/well were plated and incubated for 48 h. Thereafter, cells were washed with PBS, trypsinized, centrifuged (600 × g, 10 min) and incubated with FITC-conjugated Annexin V diluted in binding buffer (1:100) for 30 min. After this period, 0.5 μg/10 μL PI in binding buffer was added and 10,000 events were acquired in a flow cytometer (FACS Canto, Becton and Dickinson, San Jose, CA, USA). The percentage of apoptotic (FITC positive and PI negative), necrotic (FITC negative and PI positive), late apoptotic (FITC and PI positive) and living cells (FITC and PI negative) was quantified.

4.5. Cell proliferation

After cell plating (1×10^4 cells/well) and adhesion, Trypan Blue dye (Sigma–Aldrich, St Louis, MO, USA) was used to quantify cell proliferation of SK-Mel-28 or HUVEC after incubation with a solution of **4d** or vehicle for 14, 24 or 48 h. A haemocytometer was used to count the number of cells. Results are expressed as absolute number of counted cells.

4.6. Mitochondrial membrane potential assay

5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (denoted JC-1) is a cationic lipophilic dye used as an indicator for monitoring membrane potential [23]. This molecule enters mitochondria in monomeric form. At an excitation wavelength of 490 nm, the molecule has an emission wavelength of 527 nm. However, depending on the membrane potential, JC-1 forms aggregates are associated with a large shift in emission (590 nm). Thus, the color of the dye changes reversibly from green (read in FITC channel) to greenish orange (read in PE channel) as the mitochondrial membrane becomes more polarized [24]. 1×10^4 cells/well SK-Mel-28 or HUVEC were plated and after adhesion were incubated with a solution of **4d** or vehicle for 48 h. After this period, cells were washed with PBS and incubated with JC-1 (1.5 μg/mL, Life Technologies, Walthan, MA, USA) for 30 min at 37 °C and 5% CO_2. Thereafter, cells were washed again, trypsinized, centrifuged (600 × g, 10 min) and transferred to flow cytometry tubes. 10,000 events were acquired in a flow Cytometer (FACS Canto, Becton and Dickinson, San Jose, CA, USA) at 488 nm (excitation) and 529 and 590 (emission for monomeric form and aggregate form, respectively). For the control, cells were treated with medium RPMI 1540 or 200 μM Valinomycin (positive control; Sigma–Aldrich, St Louis, MO, USA) for 30 min before incubation with JC-1 dye. Data are expressed as the ratio of mean of green fluorescence intensity to red fluorescence intensity.

4.7. Statistics analysis

All data are expressed as mean ± standard error of the mean (SEM). Comparisons were performed using student's *t* test when two groups were compared. ANOVA test followed by Tukey's test was performed when three or more groups were compared. Significant difference was considered with $p < 0.05$.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.02.062.

References

[1] L. Feliu, M. Planas, Cyclic peptides containing biaryl and biaryl ether linkages, Int. J. Pep. Res. Ther. 11 (2005) 53–97;
A.F.M. Noisier, M.A. Brimble, C–H functionalization in the synthesis of amino acids and peptides, Chem. Rev. 114 (2014) 8775–8806;
L. Mendive-Tapia, S. Preciado, J. García, R. Ramón, N. Kielland, F. Albericio, R. Lavilla, New peptide architectures through C–H activation stapling between tryptophan–phenylalanine/tyrosine residues, Nat. Commun. 6 (2015) 1–9.

[2] A.L. Brown, Q.I. Churches, C.A. Hutton, Total synthesis of ustiloxin D utilizing an Ammonia–Ugi reaction, J. Org. Chem. 80 (2015) 9831–9837.

[3] E. Blishchenko, O. Sazonova, A. Surovoy, S. Khaidukov, Y. Sheikine, D. Sokolov, I. Freidlin, M. Philippova, A. Vass, A. Karelin, V. Ivanov, Antiproliferative action of valorphin in cell cultures, J. Pept. Sci. 8 (2002) 438–452.

[4] O.S. Fredorova, O.F. Kuznetsova, S.V. Shatik, M.A. Stepanova, Y.N. Bellokon, V.I. Maleev, R.N. Krasikova, 18F-Labeled tyrosine derivatives: synthesis and experimental studies on accumulation in tumors and abscesses, Russ. J. Bioorg. Chem. 35 (2009) 306–314.

[5] R.O. Robinson, C.D. Ferrie, M. Capra, M.N. Maisey, Positron emission tomography and the central nervous system, Arch. Dis. Child. 81 (1999) 263–270.

[6] B. Palumbo, T. Buresta, S. Nuvoli, A. Spanu, O. Schillaci, M.L. Fravolini, I. Palumbo, SPECT and PET Serve as molecular imaging techniques and in vivo biomarkers for brain metastases, Int. J. Mol. Sci. 15 (2014) 9878–9893.

[7] C. Plathow, W. Weber, Tumor cell metabolism imaging, J. Nucl. Med. 49 (2008) 43–63.

[8] T. Rodt, C.V. Falck, R. Halter, K. Ringe, H.O. Shin, M. Galanski, J. Borlak, In vivo microCT quantification of lung tumor growth in SPC-raf transgenic mice, Front. Biosci. (Landmark Ed. 14 (2009) 1939–1944.

[9] A. Habermeier, J. Graf, B.F. Sandhöefer, J.-P. Boissel, F. Roesch, E.I. Closs, System l amino acid transporter LAT1 accumulates O-(2-fluoroethyl)-l-tyrosine (FET), Amino Acids 47 (2015) 335–344.

[10] V. Dunkl, C. Cleff, G. Stoffels, N. Judov, S. Sarikaya-Seiwert, I. Law, L. Bøgeskov, K. Nysom, S.B. Andersen, H.J. Steiger, G.R. Fink, G. Reifenberger, N.J. Shah, H.H. Coenen, K.J. Langen, N. Galldiks, The usefulness of dynamic O-(2-18F-fluoroethyl)-L-tyrosine PET in the clinical evaluation of brain tumors in children and adolescents, J. Nucl. Med. 56 (2015) 88–93.

[11] N. Galldiks, G. Stoffels, C. Filss, M. Rapp, T. Blau, C. Tscherpel, G. Ceccon, V. Dunkl, M. Weinzierl, M. Stoffel, M. Sabel, G.R. Fink, N.J. Shah, K.-J. Langen, The use of dynamic O-(2-18F-fluoroethyl)-L-tyrosine PET in the diagnosis of patients with progressive and recurrent glioma, Neuro-Oncology 17 (2015) 1293–1300.

[12] P. Lohmann, H. Herzog, E.R. Kops, G. Stoffels, N. Judov, C. Filss, N. Galldiks, L. Tellmann, C. Weiss, M. Sabel, H.H. Coenen, N.J. Shah, K.-J. Langen, Dual-time-point O-(2-[18F]fluoroethyl)-L-tyrosine PET for grading of cerebral gliomas, Eur. Radiol. 25 (2015) 3017–3024.

[13] P.M. Rosenschold, J. Costa, S.A. Engelholm, M.J. Lundemann, I. Law, L. Ohlhues, S. Engelholm, Neuro-Oncology 17 (2015) 757–763.

[14] C.M. Balch, J.E. Gershenwald, S.J. Soong, J.F. Thompson, M.B. Atkins, D.R. Byrd, A.C. Buzaid, A.J. Cochran, D.G. Coit, S. Ding, A.M. Eggermont, K.T. Flaherty, P.A. Gimotty, J.M. Kirkwood, K.M. McMasters, M.C. Mihm Jr, D.L. Morton, M.I. Ross, A.J. Sober, V.K. Sondak, Final version of 2009 AJCC melanoma staging and classification, J. Clin. Oncol. 27 (2009) 6199–6206.

[15] Cancer Research UK [home page on internet]. London: Cancer Research UK [updated 2015 Oct; cited 2015 Oct]. Available from: http://www.cancerresearchuk.org/. (accessed Oct, 2015).

[16] S.N.S. Vasconcelos, C.S. Barbeiro, A.N. Khan, H.A. Stefani, Synthesis of biphenyl tyrosine via cross-coupling suzuki-miyaura reaction using aryltrifluoroborate salts, J. Braz. Chem. Soc. 26 (2015) 765–774.

[17] S.N.S. Vasconcelos, A. Shamim, B. Ali, I.M. de Oliveira, H.A. Stefani, Functionalization of protected tyrosine via Sonogashira reaction: synthesis of 3-(1,2,3-triazolyl)-tyrosine, Mol. Divers. (2016), http://dx.doi.org/10.1007/s11030-015-9642-y online version of this article.

[18] D. Schadendorf, Melanoma, Nat. Rev. Dis. Prim. (2015) 1–20;
C. Garbe, T.K. Eigentler, U. Keilholz, A. Hauschild, J.M. Kirkwood, Systematic review of medical treatment in melanoma: current status and future prospects, Oncologist 16 (2011) 5–24.

[19] Y. Komarova, A.B. Malik, Regulation of endothelial permeability via paracellular and transcellular transport pathways, Annu. Rev. Physiol. 72 (2010) 463–493.

[20] B.E. Sumpio, J.T. Riley, A. Dardik, Cells in focus: endothelial cell, Int. J. Biochem. Cell. Biol. 34 (2002) 1508–1512.

[21] L. Wang, W. Qu, B. Lieberman, K. Ploessl, H.F. Kung, Synthesis and in vitro evaluation of 18F labeled tyrosine derivatives as potential positron emission tomography (PET) imaging agents, Bioorg. Med. Chem. Lett. 20 (2010) 3482–3484.

[22] L. Wang, W. Qu, B. Lieberman, K. Ploessl, H.F. Kung, Synthesis, uptake mechanism characterization and biological evaluation of 18F labeled fluoroalkyl phenylalanine analogs as potential PET imaging agents, Nucl. Med. Biol. 38 (2010) 1–23.

[23] M. Reers, T.W. Smith, L.B. Chen, J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential, Biochemistry 30 (1991) 4480–4486.

[24] A. Cossarizza, M. Baccarani-Contri, G. Kalashnikova, C. Franceschi, A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), Biochem. Biophys. Res. Com. 197 (1993) 40–45.