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Synthesis of labeled curcumin derivatives as tools for *in vitro* blood brain barrier trafficking studies

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Successful drug delivery to the brain is crucial for the therapy of many neurological disorders, including Alzheimer's disease. A stable tritiated pyrazole curcumin derivative, exhibiting ability to interact with Aβ peptide, has been synthesized to allow tracking studies in an *in vitro* model of blood brain barrier.

Keywords: blood brain barrier (BBB); trafficking studies; curcumin pyrazole derivatives; synthesis

Introduction

Alzheimer's disease is the most common cause of dementia among the elderly population.[1–5] This pathology is characterized by an overproduction of amyloid beta (Aβ) peptides and by their aggregation to form oligomer fibrils and plaques within the brain. One important aspect in the treatment of neurological disorders is the successful delivery of drugs to the brain. To achieve this task, the drug must be able to pass or to be carried through the blood brain barrier (BBB). Among all the natural Aβ ligands, curcumin, a major component of the yellow curry spice turmeric, was selected for its known ability to inhibit Aβ peptide aggregation both *in vitro* and *in vivo*.[6] However, curcumin has very high instability and very low solubility in physiological conditions.[7–10] These problems could be addressed by using different strategies, such as employing stable derivatives. In a project devoted to the generation of nanoparticles for the diagnosis and therapy of Alzheimer's disease, we synthesized different stable pyrazole curcumin derivatives for both the surface decoration and the inclusion inside these nanodevices. We studied the ability of these stable derivatives to interact with Aβ peptides (data not published). Among them, derivative **3** (Scheme 1) was selected as a compound suitable for the surface decoration of preformed nanoliposomes[11] or for their incorporation into nanoparticles. In order to track these compounds both *in vitro* and *in vivo*, we synthesized labeled compounds **4** and **5**. Radiolabeled compound **5** was incorporated into nanoliposomes and used to investigate the ability of the nanoparticles to overcome the BBB in an *in vitro* model. Promising preliminary results were obtained.

Result and discussion

[$^3H_2$]Curcumin derivative **5** was used for the preparation of a device aimed at the study of molecule BBB trafficking in an *in vitro* model of BBB. For this purpose, the radiolabeled molecule was used as such or incorporated in different types of nanoliposomes, and the ability of the different devices to overcome the BBB was measured, affording interesting preliminary results. The labeled compound was synthesized, involving a deprotonation reaction in 85% yield, with a specific activity high enough for the purpose.

The synthesis of the deuterium- and tritium-labeled compounds **4** and **5**, respectively, is shown in Scheme 1. Curcumin (1,1-diferuloylmethane) is present in a keto–enol tautomerism in water, with enol as the active component.[12] With this information, we synthesize an enol-form analog, treating the natural compound with ethyl 2-hydrazinylacetate hydrochloride in refluxing toluene, with trifluoroacetic acid (TFA) as catalyst. This reaction afforded the pyrazole derivative **1** in good yield (87%). Carboxylic acid **2** was obtained from **1** by basic hydrolysis of the ethyl ester in a quantitative yield. This compound is the starting material for the following functionalization with standard amide coupling procedure. Derivative **3** was obtained from **2** through coupling with propargylamine in 68% yield. In a project dedicated to the design of different types of nanoparticles, compound **3** is very important because, in addition to being incorporated into particles such as liposomes, it may be linked to their surface by chemoselective ligation procedures, such as Huisgen 1,3 cycloaddition.[11] With this possibility, we set up the tritium labeling of this compound, setting up the experimental protocol with its non-radioactive isotope, which allowed us to evaluate both the isotopic exchange and the yield of the reaction. Compound **3** was treated with freshly prepared lithium di-*iso*-propyl amide to obtain the removal of both the protons in α to the amide position and the acetylene proton; we ran this reaction in anhydrous solvent under inert atmosphere. The reaction mixture containing the dianionic species was treated with a 20-fold excess of [$^2H_2$]water, obtaining in 88% yield the desired isotopic exchange product

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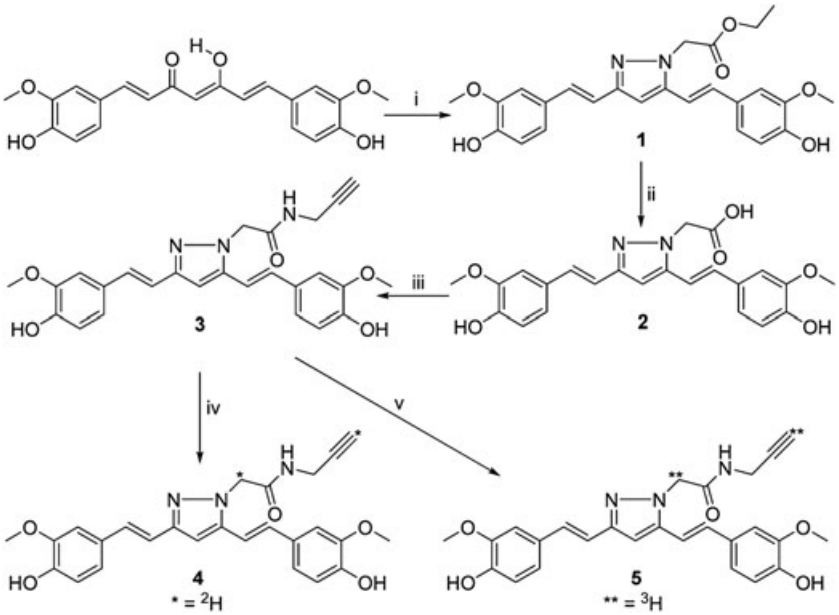

Scheme 1. Reagents and conditions: (i) ethyl 2-hydrazinyl acetate hydrochloride, TEA, TFA, toluene, reflux, 2.5 h; (ii) KOH 1.8 M methanolic solution, room temperature, overnight, 87% (over two steps); (ii) propargylamine, TBTU, HOBT, TEA, DMF dry, room temperature, overnight, 63%; (iii) *n*-BuLi, iPr_2NH, THF dry, 0 °C→room temperature, [$^2H_2$]water, 0.5 h, 88%; (iv) *n*-BuLi, iPr_2NH, THF dry, 0 °C→room temperature, [$^3H_2$]water, 0.5 h, 85%, 405 mCi/mmol.

4, with an isotopic exchange of 57% at the acetylene proton and 33% at the proton α to the amide position, as demonstrated by mass spectral and NMR analyses. This procedure affords stable labeling, since in physiological condition no isotopic exchange is possible. This deuterated compound was also useful for the incorporation of the labeled molecule inside the nanoliposomes without loss of any amount of radioactive material.

Using this labeling procedure, we performed the synthesis of the radiolabeled compound **5**. After the treatment with freshly prepared lithium di-*iso*-propyl amide, we added 100 μL of 250 μCi [$^3H_2$] water to obtain the radiolabeling of the starting curcumin derivative **3**. After 30 min, the desired compound was precipitated by acidifying the mixture with 5% $HCl_{(aq)}$ to pH < 2. The solid was filtered and re-dissolved with 5% $NaOH_{(aq)}$ to remove all the radioisotopes linked to the phenolic hydroxyl group of the two ferulic moieties.

The acidification with 5% $HCl_{(aq)}$ to pH < 2 and the filtration of the precipitate provided compound **5** in 85% chemical yield and 6% overall radiochemical yield, which was calculated from the starting activity of the commercial [$^3H_2$]water. The radiolabeling efficacy was much lower with respect to the deuterium labeling due to the different isotope enrichment of the labeling reagent ([$^2H_2$]water 99% pure, [$^3H_2$]water 2.5 mCi/mL),

Further attempts to optimize the reaction were set aside because of accelerated delivery timeline as requested by the customer group for the radioactive molecule. The tritium labeling was acceptable to deliver the target molecule with an activity high enough for *in vitro* studies. In order to increase the radiochemical activity of **5**, which could be important for other applications such as in surface decoration of nanoliposomes, [$^3H_2$]water with higher starting activity should be employed.

Experimental

All commercial chemicals were purchased from Sigma-Aldrich. All chemicals were used without further purification. All required anhydrous solvents were dried with molecular sieves for at least 24 h prior to use. [$^3H_2$]Water, 2.5 mCi/g, was obtained from Perkin-Elmer, Boston, MA. Thin layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merck) with detection under UV light when possible, or by charring with a solution of $(NH_4)_6Mo_7O_{24}$ (21 g), $Ce(SO_4)_2$ (1 g), concentrated H_2SO_4 (31 mL) in water (500 mL) or with an ethanol solution of ninhydrin or with Dragendorff' spray reagent.[13] Flash-column chromatography was performed on silica gel 230–400 mesh (Merck). ^{1}H and ^{13}C NMR spectra were recorded at 25 °C, unless otherwise stated, with a Varian Mercury 400-MHz instrument. Chemical shift assignments, reported in parts per million, were referenced to the corresponding solvent peaks. Mass spectra were recorded on a QTRAP system with ESI source, while HRMS were registered on a QSTAR elite system with a nanospray ion source. Beta counting was done with a Packard Tri-Carb liquid scintillation counter.

(*E*)-Ethyl 2-[3,5-bis(4-hydroxy-3-methoxystyryl)-1*H*-pyrazol-1-yl]acetate (1)

To a solution of curcumin (1.000 g, 2.7 mmol, 1 eq) in dry toluene (28 mL) was added ethyl 2-hydrazinylacetate hydrochloride (0.839 g, 5.4 mmol, 2 eq), triethylamine (0.753 mL, 5.4 mmol, 2 eq), and TFA (0.1 mL, 1.3 mmol, 0.5 eq), and the reaction was held at reflux for 2.5 h. The reaction was monitored by TLC. The reaction mixture was then cooled to room temperature, diluted with CH_2Cl_2, and washed with 5% $HCl_{(aq)}$ twice. The organic phase was dried over Na_2SO_4 and evaporated in vacuum to afford the crude product, which was further purified by flash chromatography (CH_2Cl_2/isopropanol 98:2) to afford the desired product as a yellow solid (1.058 g, 87%). ^{1}H NMR (400 MHz, DMSO d_6) δ ppm 7.22–6.70 (m, 11H, CHAr and conjugated double bond), 5.16 (s, 2H $CH_2C{=}O$), 4.13 (q, J = 7.05 Hz, 2H, OCH_2CH_3), 3.82–3.77 (m, 6H, OCH_3), 1.19 (t, J = 7.12 Hz, 3H, OCH_2CH_3). ^{13}C NMR (100 MHz, DMSO d_6) δ 168.32 (C=O), 150.02, 147.86, 147.27, 146.68, 143.35 (5 CqAr), 132.25, 129.79 (2 CHAr), 128.49, 128.06

(2 CqAr), 120.81, 120.01, 117.75, 115.57, 111.66, 110.24, 109.58, 98.63 (8 CHAr and conjugated double bond), 61.11 (CH_2), 55.76, 55.59 (2 CH_3), 50.30 (CH_2), 14.09 (CH_3). MS (ESI) calcd for $[M+H]^+$ 451.48; found $[M+H]^+$ 451.60.

(*E*)-2-[3,5-Bis(4-hydroxy-3-methoxystyryl)-1*H*-pyrazol-1-yl] acetic acid (2)

Compound **1** (3.000 g, 6.66 mmol, 1 eq) was dissolved in a methanolic solution of KOH 1.8 M (70 ml). The solution was left under magnetic stirring at room temperature overnight. The reaction was monitored by TLC. The solvent was evaporated in vacuum and purified by flash chromatography (eluent: ethyl acetate/methanol gradient from 9:1 to 7:3) to afford the final product (2.813 g, quant.). ^{1}H NMR (400 MHz, CD_3OD) δ ppm 7.23–6.72 (m, 11H, CHAr and conjugated double bond), 4.93 (s, 2H, $CH_2C{=}O$), 3.85–3.79 (m, 6H, OCH_3). ^{13}C NMR (100 MHz, DMSO) δ 170.17 (C=O), 147.85, 147.16, 146.58, 142.80 (4 CqAr), 131.54, 129.13 (2 CHAr), 128.62, 128.17 (2 CqAr), 120.61, 119.87, 118.08, 115.58, 112.31, 110.08, 109.53, 98.38 (8 CHAr and conjugated double bond), 55.74, 55.58 (2 CH_3), 51.58 (CH_2). MS (ESI) calcd for $[M+H]^+$ 423.43; found $[M+H]^+$ 423.4.

(*E*- 2-[3,5-Bis(4-hydroxy-3-methoxystyryl)-1*H*-pyrazol-1-yl]-*N*-(prop-2-yn-1-yl)acetamide (3)

HObt (0.017 g, 0.123 mmol, 1.23 eq) and TBTU (0.039 g, 0.12 mmol, 1.2 eq) were added to a solution of compound **2** (0.042 g, 0.1 mmol, 1 eq) in dry DMF (1 mL). The reaction mixture was left in the dark at room temperature under magnetic stirring for 15 min. Propargylamine (0.013 mL, 0.2 mmol, 2 eq) and triethylamine (0.07 mL, 0.5 mmol, 5 eq) were added, and the reaction was left in the dark at room temperature under magnetic stirring overnight. The reaction was monitored by TLC. The solvent was evaporated in vacuum. The crude product was diluted with CH_2Cl_2 and washed three times with water. The organic phase was dried over Na_2SO_4 and evaporated in vacuum to afford the crude product, which was further purified by flash chromatography (eluent: petroleum ether/ethyl acetate gradient from 3:7 to ethyl acetate) to give the desired product as an amorphous powder (0.031 g, 68%). ^{1}H NMR (400 MHz, CD_3OD) δ ppm 7.38–6.73 (m, 11H, CHAr and conjugated double bond), 5.12 (s, 2H $CH_2C{=}O$), 4.04 (s, 2H, CH_2NH), 3.96–3.83 (m, 6H, OCH_3), 2.63 (s, 1H, CH). ^{13}C (100 MHz, $CDCl_3$) δ ppm 169.31 (C=O) 152.55, 149.33, 148.03, 145.63 (4 CqAr), 135.08, 132.22 (2 CHAr), 130.75, 130.13 (2 CqAr), 122.07, 121.40, 118.27, 116.46, 112.40, 111.08, 110.70, 100.52 (8 CHAr and conjugated double bond), 80.27 (Cq), 72.42 (CH), 56.64, 56.55 (2 CH_3), 52.66 (CH_2), 29.71 (CH_2). MS (ESI) calcd for $[M+H]^+$ 460.49; found $[M+H]^+$ 460.4.

[$^2H_2$](*E*)-2-[3,5-Bis(4-hydroxy-3-methoxystyryl)-1*H*-pyrazol-1-yl]-*N*-(prop-2-yn-1-yl)acetamide (4)

A 1.6 M (in hexane) solution of *n*-BuLi (0.830 mL, 1.33 mmol, 6 eq) was added to a solution of iPr_2NH (0.188 mL, 0.136 g, 1.33 mmol, 6 eq) in dry THF (0.5 mL) at 0°C. After 30 min, a solution of compound **3** (0.102 g, 0.23 mmol, 1 eq) in dry THF (3.0 mL) was added. The reaction was then warmed to room temperature and monitored by TLC. [$^2H_2$]Water (0.092 mL, 0.092 g, 4.6 mmol, 20 eq) was added to obtain the isotopic exchange. The solution was acidified to pH<2 with 5% $HCl_{(aq)}$, obtaining a pale yellow suspension. The solid was filtered and dissolved with 5% $NaOH_{(aq)}$ to remove all the residues of mobile deuterium atoms. After 30 min, the solution was acidified to pH<2 with 5% $HCl_{(aq)}$, and a yellow suspension was obtained. The solid product was filtered, affording pure compound **4** (0.090 g, 88%). ^{1}H NMR (400 MHz, CD_3OD) δ ppm 7.38–6.73 (m, 11H, CHAr and conjugated double bond), 5.13 (m, 1.3H $CH_2C{=}O$), 4.04 (s, 2H, CH_2NH), 3.96–3.83 (m, 6H, OCH_3), 2.63 (m, 0.43H, CH). ^{13}C (100 MHz, $CDCl_3$) δ ppm 169.31 (C=O) 152.55, 149.33, 148.03, 145.63 (4 CqAr), 135.08, 132.22 (2 CHAr), 130.75, 130.13 (2 CqAr), 122.07, 121.40, 118.27, 116.46, 112.40, 111.08, 110.70, 100.52 (8 CHAr and conjugated double bond),80.27 (Cq), 72.42 (CH), 56.64, 56.55 (2 CH_3), 52.66 (CH_2), 29.71 (CH_2). MS (ESI) calcd for $[M+H]^+$ 460.49, $[[^2H]M+H]^+$ 461.49, $[[^2H_2]M+H]^+$ 462.49; found $[M+H]^+$ 460.4, $[[^2H]M+H]^+$ 461.4, $[[^2H_2]M+H]^+$ 462.4; the highest peak is the one with two deuterium atoms at 462.4 (experimental). On the basis of the ^{1}H NMR and mass spectra, we can assume that we obtained a 33% isotopic exchange of the proton in α to the amide position and a 57% isotopic exchange of the acetylene proton.

[$^3H_2$](*E*)-2-[3,5-Bis(4-hydroxy-3-methoxystyryl)-1*H*-pyrazol-1-yl]-*N*-(prop-2-yn-1-yl)acetamide (5)

A 1.6 M (in hexane) solution of *n*-BuLi (0.275 mL, 0.44 mmol, 10 eq) was added to a solution of iPr_2NH (0.062 mL, 0.044 g, 0.44 mmol, 10 eq) in dry THF (0.5 mL) at 0°C. After 30 min, a solution of compound **3** (0.020 g, 0.044 mmol, 1 eq) in dry THF (1.0 mL) was added. The reaction was then warmed to room temperature and monitored by TLC. [$^3H_2$]H_2O (2.5 mCi/mL, 0.100 mL, 250 μCi) was added for radiolabeling. The solution was acidified to pH< 2 with 5% $HCl_{(aq)}$, and a pale yellow suspension was obtained. The solid was filtered and dissolved with 5% $NaOH_{(aq)}$ to remove all the residues of mobile tritium atoms. After 30 min, the solution was acidified to pH<2 with 5% $HCl_{(aq)}$, obtaining a yellow suspension. The solid product was filtered, obtaining pure compound **5** (0.017 g, 85%) with an activity of 15 μCi (0.88 μCi/mg or 405 μCi/mmol).

Conclusions

To achieve our goal of generating a radiolabeled pyrazole curcumin analog, we evaluated a synthetic route employing a cold starting material. We worked with [$^2H_2$]water as source of deute rium atoms for the isotopic exchange in compound **3**. After some good preliminary results were obtained for both the synthesis and the incorporation in nanoliposomes, labeled [$^3H_2$]curcumin derivative **5** was synthesized in 50% overall yield, using [$^3H_2$] water as source of radioactivity. The incorporation of ^{3}H atoms was effective, and the resulting specific activity was sufficient for the trafficking studies carried out using an *in vitro* BBB model.

The present sequence is a cost-effective and efficient route to label this stable and highly functionalizable Aβ ligand.

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References

[1] R. Brookmeyer, S. Gray, C. Kawas, *Am. J. Public Health* **1998**, *88*, 1337.
[2] R. Brookmeyer, M. M. Corrada, F. C. Curriero, C. Kawas, *Arch. Neurol.* **2002**, *59*, 1764.
[3] D. J. Selkoe, *Neuron* **2001**, *32*, 177.

[4] C. L. Masters, R. Cappai, K. J. Barnham, V. L. Villemagne, *J. Neurochem.* **2006**, *97*, 1700.
[5] S. S. Sisodia, P. H. St George-Hyslop, *Nat. Rev. Neurosci.* **2002**, *3*, 281.
[6] F. S. Yang, G. P. Lim, A. N. Begum, O. J. Ubeda, M. R. Simmons, S. S. Ambegaokar, P. P. Chen, R. Kayed, C. G. Glabe, S. A. Frautschy, G. M. Cole, *J. Biol. Chem.* **2005**, *280*, 5892.
[7] M. Bernabe-Pineda, M. T. Ramirez-Silva, M. Romero-Romo, E. Gonzadlez-Vergara, A. Rojas-Hernandez, *Spectrochim. Acta A* **2004**, *60*, 1091.
[8] Y. J. Wang, M. H. Pan, A. L. Cheng, L. I. Lin, Y. S. Ho, C. Y. Hsieh, J. K. Lin, *J. Pharm. Biomed. Anal.* **1997**, *15*, 1867.
[9] H. H. Tonnesen, J. Karlsen, *Z. Lebensm. Unters. For.* **1985**, *180*, 132.
[10] H. H. Tonnesen, J. Karlsen, *Z. Lebensm. Unters. For.* **1985**, *180*, 402.
[11] S. Mourtas, M. Canovi, C. Zona, D. Aurilia, A. Niarakis, B. La Ferla, M. Salmona, F. Nicotra, M. Gobbi, S. G. Antimisiaris, *Biomaterials* **2011**, *32*, 1635.
[12] D. Yanagisawa, N. Shirai, T. Amatsubo, H. Taguchi, K. Hirao, M. Urushitani, S. Morikawa, T. Inubushi, M. Kato, F. Kato, K. Morino, H. Kimura, I. Nakano, C. Yoshida, T. Okada, M. Sano, Y. Wada, K. Wada, A. Yamamoto, I. Tooyama, *Biomaterials* **2010**, *31*, 4179.
[13] K. Thoma, R. Rombach, E. Ullmann, *Sci. Pharm.* **1964**, *32*, 216.