Solid-phase synthesis of CD40L mimetics

Alberto Bianco,* Sylvie Fournel, Sébastien Wieckowski, Johan Hoebeke, and Gilles Guichard*

Institut de Biologie Moléculaire et Cellulaire, UPR 9021 CNRS, Immunologie et Chimie Thérapeutiques, 15 Rue René Descartes, 67084 Strasbourg, France. Fax: 33 388 610680; Tel: 33 388 417088; E-mail: A.Bianco@ibmc.u-strasbg.fr; G.Guichard@ibmc.u-strasbg.fr

Electronic Supplementary Information

General

All reagents and solvents were obtained from commercial suppliers and used without further purification. 2-(3,5-Dimethoxy-4-formylphenoxy)ethyl polystyrene resin (0.62 mmol/g) was obtained from Novabiochem (Läufelfingen, Switzerland). Wang resin and amino acids were purchased from NeoMPS (Strasbourg, France). Peptides were synthesised manually on 5 ml fritted syringes or on a semi-automated synthesiser working under nitrogen flow.[1] Peptide H-Lys-Gly-Tyr-Tyr-Ahx-OH **4** was synthesised on a Wang resin using standard solid-phase procedures.[2] RP-HPLC analyses were carried out on a Macherey-Nagel C_{18} column (5 μm, 150 × 4.6 mm) using a linear gradient of A: 0.1% TFA in water and B: 0.08% TFA in acetonitrile, 5-65% B in 20 min at 1.2 mL/min flow rate. Chromatograms were recorded on a Varian ProStar 330 photodiode array detector. RP-HPLC purifications were carried out on a Macherey-Nagel C_{18} column (7 μm, 250 × 10 mm) using a linear gradient of A: 0.1% TFA in water and B: 0.08% TFA in acetonitrile, 5-65% B in 30 min at 6.0 mL/min flow rate. Chromatograms were recorded at 230 nm. MALDI-tof mass analysis was performed on a linear Protein-tof Bruker instrument using α-cyano-4-hydroxycinnamic as a matrix.

Abbreviations

Symbols and abbreviations for amino acids and peptides are in accord with the recommendations of the IUPAC-IUB Commission on Nomenclature (*J. Biol. Chem.* 1972, *247*, 977). Other abbreviations used are: AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; Bop, benzotriazole-1-yl-oxy-tris-(dimethylmino)-phosphonium hexafluorophosphate; *t*Bu, *tert*-butyl; DBU, 1,8-diazabicyclo[5,4,0]undecen-7-ene; DIC, diisopropylethylcarbodiimide; DEA, diethylamine, DIEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; EDC×HCl, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; Fmoc, 9*H*-fluoren-9-ylmethyloxycarbonyl; HATU, 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; MALDI-tof, Matrix Assisted Laser Desorption Ionization time-of-flight; Mtt, 4-methyltrityl; NMM, N-methyl morpholine; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

Synthesis

Boc-(D)Ala-OAll. Boc-D-Ala-OH (1.89 g, 10 mmol) was solubilised in DCM (70 ml) and the solution was cooled at 0° C. 1.5 ml of DBU (1.2 equiv.) were added. A solution of allyl bromide (0.72 ml, 1 equiv.) in ACN (10 ml) was dropped along 15 minutes. The solution was

stirred at room temperature for 21 hours. After evaporation of the solvent, the crude product was dissolved in AcOEt and washed with 5% $NaHCO_3$, water, 1N $KHSO_4$ and water. The organic phase was dried over Na_2SO_4 and evaporated. The product was recovered as oil in 73% yield. ^{1}H NMR (200 MHz, $CDCl_3$): δ 5.89 (m, 1H), 5.22 (m, 3H), 4.99 (m, 1H), 4.57 (d, 2H), 4.24 (m, 1H), 1.38 (s, 9H), 1.34 (d, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 173.04, 155.07, 131.68, 118.60, 79.86, 65.79, 49.30, 28.32, 18.69.

HCl×H-(D)Ala-OAll. Boc-D-Ala-OAll (1.67 g, 7.3 mmol) was solubilised in a 4N HCl in dioxane (5 ml) under argon. The solution was stirred for 30 minutes. After evaporation of the solvent, the product was obtained as a white solid in 90 % yield. ^{1}N NMR (200 MHz, $CDCl_3$) δ 5.89 (m, 1H), 5.26 (m, 2H), 4.68 (m, 2H), 4.26 (m, 1H), 1.70 (d, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 169.87, 131.12, 119.24, 66.86, 49.361, 16.09.

Fmoc-Lys(Mtt)-OAll. Fmoc-Lys(Mtt)-OH (625 mg, 1 mmol) was solubilised in DCM (5 ml) in the presence of HOBt (153 mg, 1 equiv.) and EDC×HCl (211 mg, 1.1 equiv.). After 5 minutes, allyl alcohol (68 µl, 1 equiv.) and DMAP (12 mg, 0.1 equiv.) were added. After 27 hours HOBt (76 mg, 0.5 equiv.), EDC×HCl (105 mg, 0.55 equiv.), allyl alcohol (34 µl, 0.5 equiv.) and DMAP (12 mg, 0.1 equiv.) were added. The solution was stirred for 44 hours under the dark. The solvent was evaporated and the crude product was dissolved in AcOEt and washed with 5% $NaHCO_3$, water, 1N $KHSO_4$ and water. The organic phase was dried over Na_2SO_4 and evaporated. The product was recovered as oil in 94% yield. ^{1}N NMR (200 MHz, $CDCl_3$) δ 7.7((m, 2H), 7.62 (m, 2H), 7.47 (m, 4H), 7.25 (m, 14H), 5.87 (m, 1H), 5.26 (m, 3H), 4.63 (m, 2H), 4.39 (m, 3H), 4.17 (m, 1H), 2.30 (s, 3H), 2.14 (m, 2H), 1.51 (m, 6H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.18, 155.85, 146.27, 143.75, 141.29, 131.49, 128.57, 127.74, 127.66, 127.03, 126.17, 125.04, 119.92, 118.91, 66.98, 65.90, 53.84, 47.18, 43.34, 32.70, 23.02, 20.89.

H-Lys(Mtt)-OAll. Fmoc-Lys(Mtt)-OAll (240 mg, 0.36 mmol) was solubilised in a solution of 5:8 DEA/DCM (13 ml). The solution is stirred for 5 hours. After evaporation of the solvent, the product is dissolved in Et_2O and extracted with 1N $KHSO_4$. The acid solution was basified with solid $NaHCO_3$ and the desired product extracted with AcOEt. After washing with water and drying over N_2SO_4, the solvent was evaporated affording an oily compound in quantitative yield. The product was used directly for the further reaction.

Reductive amination on the resin (5). 2-(3,5-Dimethoxy-4-formylphenoxy)ethyl polystyrene resin (100 mg, 62 µmol) was swollen in DMF (1.2 ml) in the presence of HCl×H-D-Ala-OAll (80 mg, 10 equiv.) and $NaBH_3CN$ (38 mg, 10 equiv.) The reaction was followed

using FT-IR and after 26 hours the resin was washed with DMF, DCM, MeOH and dried with Et_2O. The presence of the secondary amine on the resin was verified using chloranil test.[3]

Synthesis of cyclo-(L-Lys-D-Ala)$_3$ (3). Fmoc-Lys(Mtt)-OH (194 mg, 5 equiv.) was solubilised in dry DCM (1 ml) in the presence of collidine (115 µl, 14 equiv.) and activated with triphosgene (30 mg, 1.65 equiv.) for 1 minute. The solution is added to the resin **5** (62 µmol) previously functionalised with H-D-Ala-OAll (see reductive amination). The resin was shaken for 30 minutes and extensively washed with DCM, DMF and Et_2O. Negative chloranil test confirmed the completeness of the coupling. The dipeptide-resin conjugate **6** (62 µmol) was treated with $Pd(Ph_3)_4$ (143 mg, 2 equiv.) dissolved in 2 ml of DCM/AcOH/NMM 1850:100:50 for 6 hours under argon, affording **7**. H-Lys(Mtt)-OAll (159 mg, 5.8 equiv.) was solubilised in 1.5 ml of DMF and 600 µl of DCM with HATU (94 mg 4 equiv.), HOAt (34 mg, 4 equiv.), $CuCl_2$ (4.2 mg, 0.5 equiv.) and collidine (74 µl, 9 equiv.), and subsequently added to the resin **7** (62 µmol). The resin was shaken for 2 hours and washed with DMF, DCM and Et_2O, affording **8**. Fmoc was removed with 25 % piperidine in DMF (2×15 minutes) and Fmoc-D-Ala-OH (96 mg, 5 equiv.) activated with Bop (137 mg, 5 equiv.), HOBt (47 mg, 5 equiv.) and DIEA (162 µl, 15 equiv.) in DMF was coupled for 1 hour. The coupling was repeated a second time using the same conditions. After Fmoc cleavage, the same procedure was applied for the coupling of the subsequent Fmoc-Lys(Mtt)-OH and Fmoc-D-Ala-OH, affording **10**. Allyl group was removed with $Pd(Ph_3)_4$ (143 mg, 2 equiv.) dissolved in 2 ml of DCM/AcOH/NMM 1850:100:50 for 6 hours under argon, affording **11**. Fmoc was removed with 25 % piperidine in DMF (2×15 minutes), and the linear solid-supported peptide was cyclised head-to-tail in the presence of HOAt (34 mg, 4 equiv.), DIC (42 µl, 4.4 equiv.) in a 5/2 solution of DMF/DCM (2.1 ml) for 3 hours, affording **13**. Mtt groups were finally removed using a solution of 85 % DCM, 10 % TIS and 5 % TFA (2 ml) (3×2 minutes) , affording **14**.

Part of the resin was cleaved with a solution of 90 % TFA, 5 % water and 5 % TIS (3 ml) for 3 hours. After precipitation in cold Et_2O the crude peptide **3** was lyophilised on a mixture of water/AcOH (9/1), characterised by analytical HPLC and mass spectrometry, and purified using semi-preparative HPLC. MALDI-Tof (*m/z*) [$C_{27}H_{51}N_9O_6$] calcd. 597.75; found 620.54 $[M+Na]^+$.

Synthesis of ligand (1). Fmoc-Ahx-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Tyr(tBu)-OH (×2), Fmoc-Gly-OH et Fmoc-Lys(Boc)-OH (15 equiv.) activated with Bop (15 equiv.), HOBt (15 equiv.) and DIEA (45 equiv.) in DMF were coupled to the resin bound cyclo-(L-Lys-D-Ala)$_3$

along 1 hour. Each coupling was repeated twice. After each coupling step, Fmoc was removed using 25 % piperidine in DMF (2×15 minutes). After the last Fmoc cleavage, the loading was recalculated affording a value of 28 μmol of final crude product on the resin. The final cyclic hexapeptide **1** was removed from the resin with a solution of 90 % TFA, 5 % water and 5 % TIS (3 ml) for 3 hours. After precipitation in cold Et_2O the crude peptide was lyophilised on a mixture of water/AcOH (9/1). The product was controlled by analytical HPLC, mass spectrometry and purified using semi-preparative HPLC. The purity of crude **1** was around 50%. After lyophilisation, 11.3 mg of pure **1** were obtained (Yield: 16% based on the loading after the last Fmoc cleavage). MALDI-Tof (*m/z*) [$C_{123}H_{183}N_{27}O_{27}$] calcd. 2471.93; found 2473.12 $[M+H]^+$, 2495.35 $[M+Na]^+$.

Synthesis of ligand (2). Ligand **2** was isolated during HPLC purification of ligand **1.** The product was characterised by mass spectrometry. MALDI-Tof (*m/z*) [$C_{91}H_{139}N_{21}O_{20}$] calcd. 1847.21; found 1848.20 $[M+H]^+$, 1870.60 $[M+Na]^+$.

Surface plasmon resonance analysis

BIAcore™ 3000 (Biacore AB, Uppsala, Sweden) was used to evaluate the binding of CD40L mimetics to CD40. Flow cells of a Biacore AB CM5 Sensor Chip (Research Grade, Biacore AB) were pre-coated with a rabbit polyclonal antibody directed against murine Ig (RAM-Ig, Biacore AB, Uppsala, Sweden) using amine coupling at 30 μg/mL in 10 mM acetate buffer, pH 5.5 according to the manufacturer's protocol. The chip was then flushed with 1 M ethanolamine hydrochloride pH 8.5 (Biacore AB) and 50 mM HCl to eliminate unbound antibody. Generally *ca* 10,000 RU of RAM-Ig was obtained after immobilisation. Biosensor assays were performed at 25°C with HBS-EP buffer (10 mM Hepes, pH 7.4, containing 0.15 M NaCl, 3.4 mM EDTA and 0.005% (v/v) P20 surfactant (Biacore AB)) as running buffer. Capture of soluble human CD40-mIg fusion protein (hCD40:mIg; Ancell corporation, Bayport, MN), of mouse anti-huCD40 antibody (Pharmingen, San Jose, CA) and of LG11-2, an IgG2a murine monoclonal antibody directed against H2B histone used as irrelevant control, was performed on individual flow cells at a flow rate of 5 μL/min and at a concentration allowing to achieve equivalent protein mass binding.

Inhibition of human soluble CD40L binding to CD40: hCD40L:CD8 fusion protein (Ancell, Bayport, MN) was injected at 150 at a flow rate of 10 μL/min in the presence of various concentrations of CD40L mimetics, over both the control channel and the CD40 channel for 5 minutes and allowed to dissociate for 5 additional minutes. The channels were regenerated for

10 s with 50 mN HCl. Sensorgrams of ligands in the absence of hCD40L:CD8 were subtracted from the sensorgrams of ligands co-injected with hCD40L:CD8, and then substracted by the control sensorgrams. The inhibition capacity was estimated from the decrease in the initial linear association phase. Data were processed with the BIAevaluation v4.1 software (Biacore).

Measure of apoptosis

BL41 cells have been characterized as human Burkitt lymphomas. Cell death was evaluated either by measurement of a decrease in mitochondrial transmembrane potential ($\Delta\psi m$) associated with a reduction of the cationic dye 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (Interchim, Montluçon, France) uptake as demonstrated by flow cytometry, or by detection of phosphatidylserine externalisation by flow cytometry after co-labelling with annexin V-FITC and propidium iodide (PI). To analyse apoptosis by annexin V/PI labeling,[4] cells were resuspended in 100 µL of annexin V binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 5 µL of annexin V-FITC (Pharmingen, San Jose, CA) and 10 µL of propidium iodide (PI) (Molecular Probes) at 10 µg/mL and incubated at room temperature in the dark. After 20 min, 400 µL of annexin V binding buffer were added and cells were analysed by flow cytometry. To analyse apoptosis by $DiOC_6(3)$ uptake,[5] approximately 1×10^6 cells washed in PBS were resuspended in 300 µL of PBS containing 40 nM $DiOC_6(3)$ and incubated at 37° C for 30 minutes. Cells were then directly analysed by flow cytometry (FACScalibur; CellQuest v3.3). Results are expressed as percentage of specific apoptosis according to the following formula: % specific apoptosis = [(% of apoptotic treated cells - % of apoptotic control cells) × 100]/(100 - % of apoptotic control cells).

References

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