Solid-phase synthesis of CD40L mimetics†

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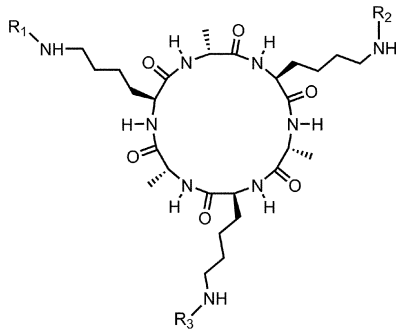
The C_3-symmetric molecule 1 has been previously shown to mimic CD40 ligand (CD40L) homotrimers and to display effector functions. This molecule consists of a cyclic hexapeptide core containing the repetition of the D-Ala-L-Lys motif. The side chains of the lysine residues have been modified by appending the CD40L-derived sequence ^{143}Lys-Gly-Tyr-Tyr^{146} *via* a 6-aminohexanoic acid residue as a spacer. The present report describes a general solid-phase synthesis approach to 1 and related trimeric architectures. In addition, their CD40 binding properties as well as their effector functions have been evaluated.

The interaction between CD40, a member of the tumour necrosis factor receptor (TNF-R) superfamily, and its ligand CD40L is key to the differentiation and activation of antigen presenting cells (APCs) as well as T and B lymphocytes.[1] Blockade or activation of this pathway represents a potentially useful approach to immunomodulate T-cell-mediated diseases or to enhance anti-infective/anti-tumour host defence, respectively.[2,3] Signalling through CD40 and other TNF-R members involves ligand-induced receptor trimerisation and the formation of a 3 : 3 hexameric ligand–receptor complex.[4] In this context, we have described rationally designed CD40L mimetics (<3 kDa) that can both interact with CD40 and display effector functions.[5] The design of these small molecules was based on the crystal structure of CD40L homotrimers and a homology model of the CD40L/CD40 complex.[6,7] The three-fold axis of the hexameric complex suggested to conceive trimeric architectures with C_3 symmetry.

Large cyclic peptides can be considered as excellent cores to assemble multivalent ligands.[8] The preparation of this type of structure is highly flexible and is amenable to solid-phase synthesis.[8] For the design of CD40L mimetics we focused on macrocylic peptides with rigid ring conformation and three arms at the twelve, four and eight o'clock positions that could promote radial distribution of a short CD40 binding segment. Crystallographic studies have shown that cyclic hexapeptides consisting of alternated L- and D-amino acid residues meet these criteria and are able to adopt the expected symmetric C_3 spatial arrangement.[9–11] For this reason, we prepared a series of molecules based on the cyclo-(L-Lys-D-Ala)$_3$ core structure (Fig. 1).



1, R_1, R_2, R_3 = H-Lys-Gly-Tyr-Tyr-Ahx
2, R_1, R_2, = H-Lys-Gly-Tyr-Tyr-Ahx; R_3 = H
3, R_1, R_2, R_3 = H
4, H-Lys-Gly-Tyr-Tyr-Ahx-OH

Fig. 1 Molecular structures of CD40L mimetics.

The synthesis of molecules **1**–**3** was performed entirely on a solid support.[12] We followed the strategy for the head-to-tail cyclisation on polymer beads using Fmoc/*t*-Bu chemistry.[13,14] We initially functionalised 2-(3,5-dimethoxy-4-formylphenoxy)ethyl polystyrene resin with the D-Ala residue protected at the carboxylic group as an allyl ester under reductive amination conditions (Scheme 1).[13] The secondary amine **5** was coupled with Fmoc-L-Lys(Mtt)-OH, activated with triphosgene in the presence of collidine.[15] Because dipeptide esters made of heterochiral residues are particularly prone to fast 2,5-diketopiperazine formation,[16] we did not attempt to remove the Fmoc group of **6**. Instead, the synthesis was continued towards the C-terminus. The allyl group (All) was removed by treatment with Pd(Ph$_3$)$_4$ and H-L-Lys(Mtt)-OAll was coupled to give tripeptide **8**. At that stage, the elongation was continued from the N-terminus to give hexamer **10**.[17] The N- and C-protections of the linear peptide were subsequently removed and cyclisation to **13** was achieved by activation with DIC (diisopropylethylcarbodiimide) and HOAt (1-hydroxy-7-azabenzotriazole). The Mtt (4-methyltrityl) groups on the three lysine side chains were removed in mild acid conditions and the synthesis of **1** was terminated using Fmoc/*t*-Bu chemistry and standard coupling procedures.† 6-Aminohexanoic acid (Ahx) was inserted as a spacer to assure a favourable interaction between the CD40L-derived ^{143}Lys-Gly-Tyr-Tyr^{146} loop sequence and the residues of the CD40 receptor. After the cleavage from the resin, the crude compound **1** was obtained in good yield and fairly good purity (Fig. 2A). After purification by semi-preparative RP-HPLC (Fig. 2B), we isolated ligand **1** in 16% yield, subsequently characterised by MALDI-tof.

During the initial solid-phase peptide synthesis (SPPS) optimisation phase, we were able to identify and separate the ligand **2** (Fig. 1), which was not expected and presented one missing sequence in one of the arms due to an incomplete removal of Mtt groups (Scheme 1). This dimeric analogue of **1** lacking

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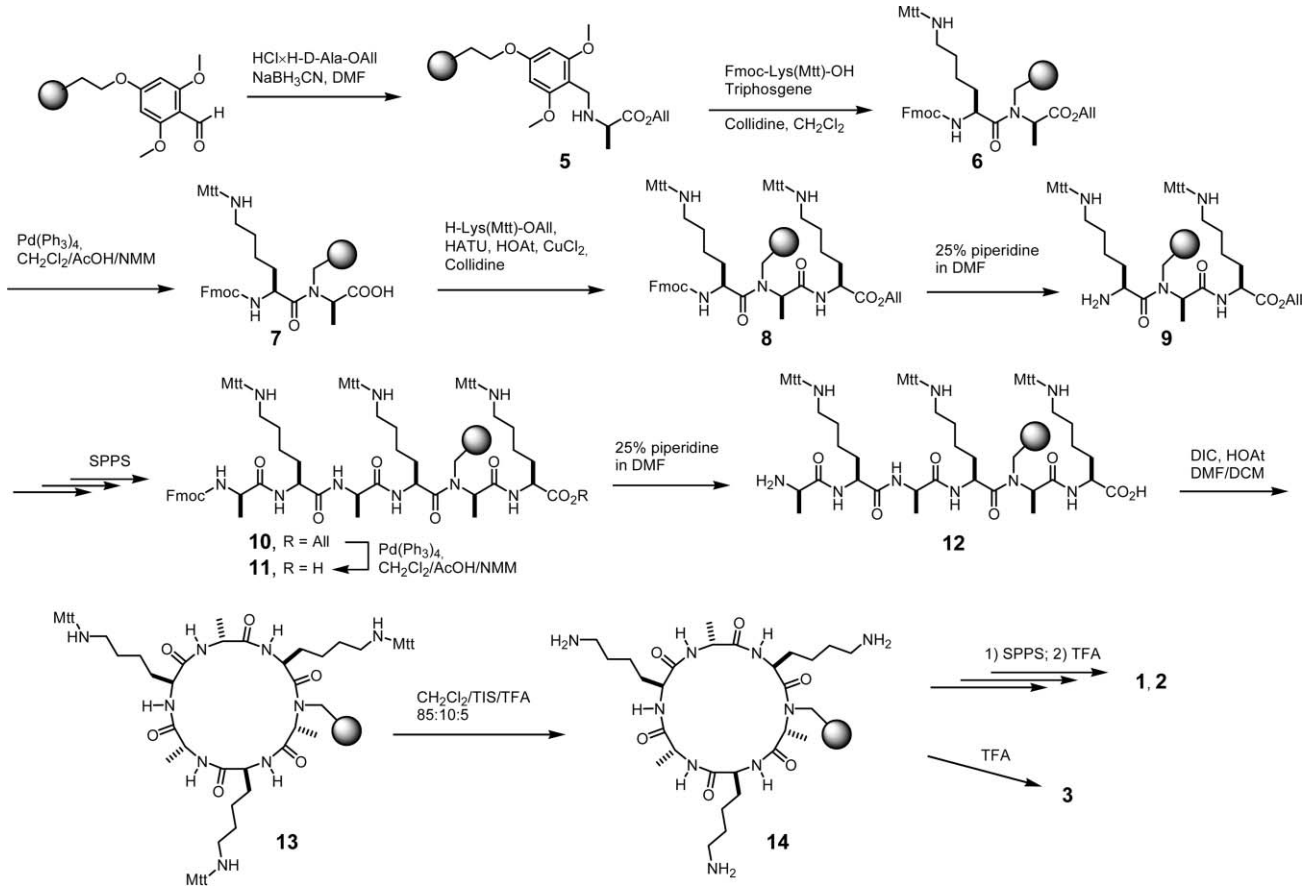

Scheme 1 Solid-phase synthesis of CD40L mimetics.

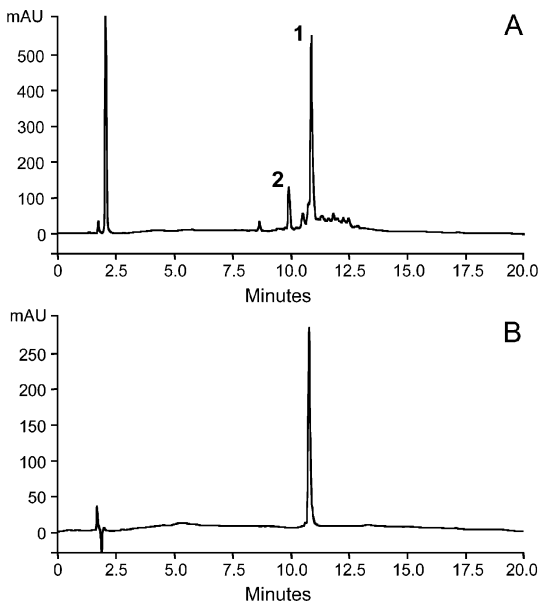

Fig. 2 RP-HPLC chromatograms of crude (A) and purified (B) **1**.

C_3 symmetry is potentially useful to evaluate the importance of valency in the interaction of CD40L mimetics with CD40 and to investigate possible cooperativity effects.[18] Finally, the circular core **3** was obtained directly by treating part of the cyclohexapeptide resin **14** with TFA (Scheme 1).

The capacity of the CD40L mimetic **1** to interact with CD40 was previously evaluated with surface plasmon resonance (SPR).[5] SPR experiments showed a concentration-dependent competition with binding of recombinant CD40L to CD40. The mean inhibitory concentration of **1** corresponded to 78 nM.[5] Here we have measured the inhibitory activity of dimeric ligand **2**. The measurements were performed by immobilising the recombinant CD40 onto the sensor chip and subsequently injecting a solution of 0.15 μM of CD40L alone, mixed to 0.1 or 1 μM of **2**.† As a control, we have tested in the same experimental trimeric ligand **1** at 1 μM. Fig. 3 shows the sensorgrams of the competition

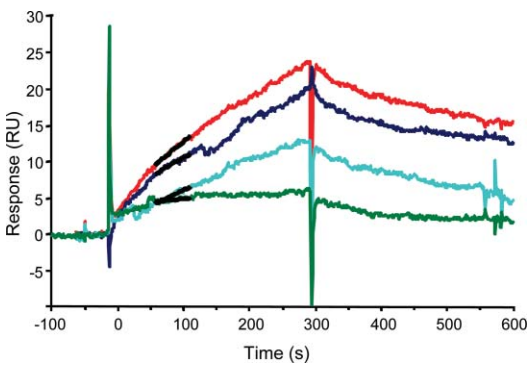

Fig. 3 Inhibition of CD40L binding to CD40 by ligand **1** and **2**. Red: 0.15 μM CD40L alone; blue: addition of 0.1 μM of **2**; cyan: addition of 1 μM of **2**; green: addition of 1 μM of **1**.

assay. Although ligand **2** is missing one CD40 binding motif compared to **1**, it still displays an inhibitory effect at the highest concentration. In contrast to **1**, however, the inhibition by **2** at 1 μM is not complete (about 40%). This result highlights how the concomitant presence of the three CD40 binding motifs is critical for an efficient interaction with CD40. We have formerly shown that ligand **3**, where the three H-Lys-Gly-Tyr-Tyr-Ahx-OH sequences were completely absent, was unable to block the formation of the CD40/CD40L complex.[5]

Finally, we investigated whether or not ligand **2** displays effector function. Our cellular assay was based on the property of Burkitt lymphoma cells to enter apoptosis after CD40 ligation.[19] As previously described,[5] compound **1** as well as recombinant soluble CD40L (data not shown) induced a high level of apoptosis of BL41 Burkitt lymphoma cells in a dose-dependent manner (Fig. 4). On the contrary, the core structure **3** and the short Lys-Gly-Tyr-Tyr-Ahx linear peptide **4**,† used as negative controls, had no effect (Fig. 4). Interestingly, compound **2** induced significant apoptosis, suggesting that it could bind to CD40 and trigger an apoptotic signalling. However, the apoptosis generated by compound **2** was reduced significantly compared with trimeric compound **1**, showing the importance of C_3 symmetry in CD40L signalling. This result is in agreement with the SPR data (Fig. 3), showing a reduced inhibition capacity of ligand **2** in comparison to **1**.

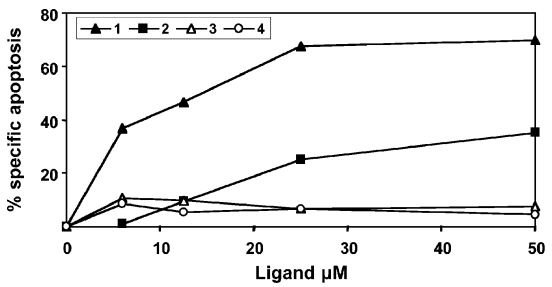

Fig. 4 Induction of apoptosis by compound **1** and **2**. BL41 cells (5×10^5 cells mL^{-1}) were incubated with compound **1**, **2**, **3** and **4** at the indicated concentrations for 16 h. Apoptosis was then measured by detection of phosphatidylserine externalisation by flow cytometry after co-labelling with annexin V-FITC and propidium iodide (PI). Results are expressed as percentage of specific apoptosis (see ESI†).

In conclusion, we have successfully developed a strategy for the solid-phase total synthesis of CD40L mimetics. As this approach is highly versatile, the design of new combinatorial ligands containing different peptide sequences involved in the interaction with CD40 as well as a structure–activity relationship should be greatly facilitated. In addition, this method can be extended to the conception of other types of mimetics involved in protein/protein interactions within the family of TNF-R, to whom CD40 belongs.

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