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LDV peptidomimetics equipped with biotinylated spacer-arms: Synthesis and biological evaluation on CCRF-CEM cell line

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ABSTRACT

The tripeptide Leu-Asp-Val (LDV) is known to bind $\alpha_4\beta_1$ integrin in leukemia cells. Here we have synthesized a LDV peptidomimetic equipped with a biotin-conjugated spacer-arm. Compound **9** acts as an inhibitor of the $\alpha_4\beta_1$ integrin in an adhesion assay using fluorescently labeled, $\alpha_4\beta_1$ integrin-expressing leukemia CCRF-CEM cells. Furthermore, when bound to neutravidin-coated plates, compound **9** could capture CCRF-CEM cells. Such biotin-conjugated LDV peptidomimetic may thus represent a novel tool for biotechnological applications using avidin interaction for leukapheresis or leukemia cell targeting.

The cellular adhesion receptor $\alpha_4\beta_1$, also known as Very Late Antigen-4 (VLA-4, or CD49d/CD29), is a member of the integrin family and is expressed on the surface of activated leukocytes, including leukemia cells.[1,2] It mediates cellular adhesion and activation through a variety of cell–cell and cell–matrix interactions that regulate leukocyte trafficking during inflammatory responses or cancer progression.[3,4] This receptor binds to two natural ligands, the vascular cell adhesion molecule-1 (VCAM-1),[5] expressed on endothelial cells in response to inflammatory cytokines, and the alternatively spliced portion of the type III connecting segment of the extracellular matrix protein fibronectin (Fn), known as the CS-1 region. The tripeptide Leu-Asp-Val (LDV) was identified as the minimum sequence of the CS-1 region of fibronectin necessary to bind $\alpha_4\beta_1$ integrin.[6] This sequence was the starting point in medicinal chemistry for the design of small molecules or peptidomimetics, antagonists of the $\alpha_4\beta_1$ integrin.[7,8] Indeed, inhibition of the interaction between the $\alpha_4\beta_1$ integrin and its ligands may prevent the recruitment and tissue infiltration of leukocytes and may thus be a potential treatment for inflammatory, cancer and autoimmune pathologies.[9,10] This approach has been validated with the approval of the humanized α_4 monoclonal antibody 'natalizumab' for the treatment of multiple sclerosis and Crohn's disease.[11]

In this study, we were interested in the development of biotin-conjugated LDV peptidomimetics for the detection/capture of leukemia cells expressing the $\alpha_4\beta_1$ integrin.[12] Combined with the extraordinary affinity between biotin and avidin/streptavidin ($K_D = 10^{-14}$ to 10^{-15} M),[13] LDV peptidomimetics could indeed enable the removal of malignant white blood cells in people with leukemia or hyperleukocytosis. Leukapheresis could also be useful when debulking of circulating leukocytes is advisable before lytic therapy. More generally, biotin-conjugated LDV peptidomimetics have the potential to be exploited to image in vivo $\alpha_4\beta_1$ integrin-expressing tumor cells or for the production of dedicated drug targeting systems.

We decided to synthesize small molecules that mimic the LDV motif recognized by the $\alpha_4\beta_1$ integrin. These molecules were equipped with spacer-arms to introduce a minimum spacing between the biotin and the peptidomimetic core and to favor the availability of the biotin/avidin binding without altering the peptidomimetic recognition by the integrin.[14]

We began our study by the synthesis of peptides **1** and **2** containing the active LDV sequence, recognized by the $\alpha_4\beta_1$ integrin, and a diaryl urea cap on the N-terminal function (Fig. 1). Lin et al. have previously demonstrated the possibility to increase the binding affinity towards this integrin by the introduction of a similar motif.[7] The C-terminal function was protected with a phenethyl amide or with a hydrophobic spacer-arm. The molecules **1** and **2** were prepared according to the classical peptide synthesis methods in solution (see Supplementary data).[7,15]

The activity of these two molecules towards the $\alpha_4\beta_1$ integrin was evaluated in a standard in vitro cell adhesion assay, consisting in the inhibition of leukemia cell adhesion on plates coated with fibronectin, the natural ligand of the $\alpha_4\beta_1$ integrin. The human

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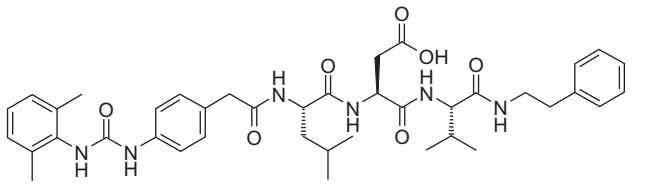

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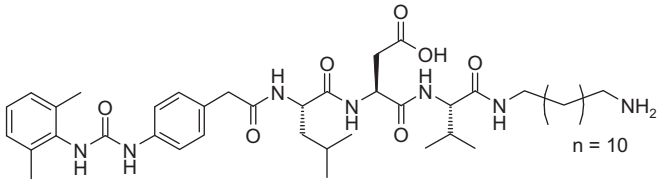


2
(TFA salt)

Figure 1. LDV modified peptides **1** and **2**.

T lymphoblast-like cell line CCRF-CEM expressing the $\alpha_4\beta_1$ integrin (derived from a child with acute lymphoblastic leukemia) was chosen for this assay among different leukemia cells; in preliminary experiments, attachment of this cell line to fibronectin was indeed specifically inhibited by anti-α4 or anti-β1 antibody. As a control, we also used quiescent human umbilical vein endothelial cells (HUVEC) which do not express $\alpha_4\beta_1$ integrin and the adhesion of which on fibronectin failed to be prevented by anti-$\alpha_4\beta_1$ integrin antibodies (not shown).

The pre-labeling of CCRF-CEM cells with calcein allowed tracking by fluorescence measurements, the number of adherent cells after 1 h incubation at 37 °C with increasing amounts of our molecules (see Supplementary data). The results showed an inhibition activity of the LDV modified peptides **1** and **2** at the micromolar level (Table 1) with a prominent activity of compound **2** (equipped with the spacer-arm) reaching an IC_{50} value of 2 μM. Interestingly, this activity was obtained without addition of Mn^{2+} divalent cations, a treatment necessary to activate $\alpha_4\beta_1$ integrin in normal lymphocytes.[16] This observation confirms the constitutive activation of $\alpha_4\beta_1$ integrin in CCRF-CEM as observed in many leukemia cells and thereby further supports the potential of LDV peptidomimetics to specifically interfere with leukemia cells.

From this encouraging result, that is, no loss of activity by the incorporation of a spacer-arm, we decided to synthesize small molecules that mimic the LDV peptide **2**. Indeed, peptidomimetics are usually preferred because of their better stability towards enzymatic hydrolysis and their easiness of synthesis, purification and characterization.

We have previously reported the synthesis of a series of good antagonists of the $\alpha_4\beta_1$ integrin, based on the methyl 3-(5-amino-2-hydroxyphenyl)propanoate template, such as the peptidomimetic **3** (Scheme 1)[15] structurally close to compounds of the Biogen group.[17] Starting from **3**, we envisaged the synthesis of the two peptidomimetics **6** and **7** equipped with α,ω-diamino spacer-arms. Our general synthetic approach to obtain the molecules **6** and **7** is outlined in Scheme 1 (see also Supplementary data). Two spacer-arms, featuring different hydrophilicities but the same number of carbon atoms, were introduced on compound **3**. An alkyl chain **4** and an oligoethylene glycol (OEG) **5** were chosen to study the effect of the nature of the linker on the adhesion of CCRF-CEM cells. Ethylene glycol oligomers are usually considered due to their hydrophilic character, repulsive effect versus non-specific adsorption of proteins and to improve the solubility of molecules.[18] Moreover, previous studies have shown that the introduction of a hexaethylene glycol chain (12 carbons) enabled to maintain the activity of our lead peptidomimetics, antagonists of the $\alpha_v\beta_3$ integrin.[19] The synthesis of the two linkers **4** and **5,** mono-protected at one end by a *tert*-butyl carbamate (Boc), were described in Supplementary data. [20,21]

The two different spacer-arms were coupled to the peptidomimetic core **3** after activation with COMU (1-[(1-(cyano-2-ethoxy-2-oxo-ethylideneaminooxy)-dimethylamino-morpholinomethylene)] methanaminium hexafluorophosphate) in the presence of *N*,*N*-diisopropylethylamine (DIPEA).[22] This coupling agent, chosen to facilitate the work up and the purification, gave the desired products with good to moderate yields (80% and 55% for compounds with the alkyl and the OEG chains, respectively). Then, simultaneous deprotection of the Boc carbamate and the *tert*-butyl ester with trifluoroacetic acid (TFA) afforded the target compounds **6** and **7** quantitatively.

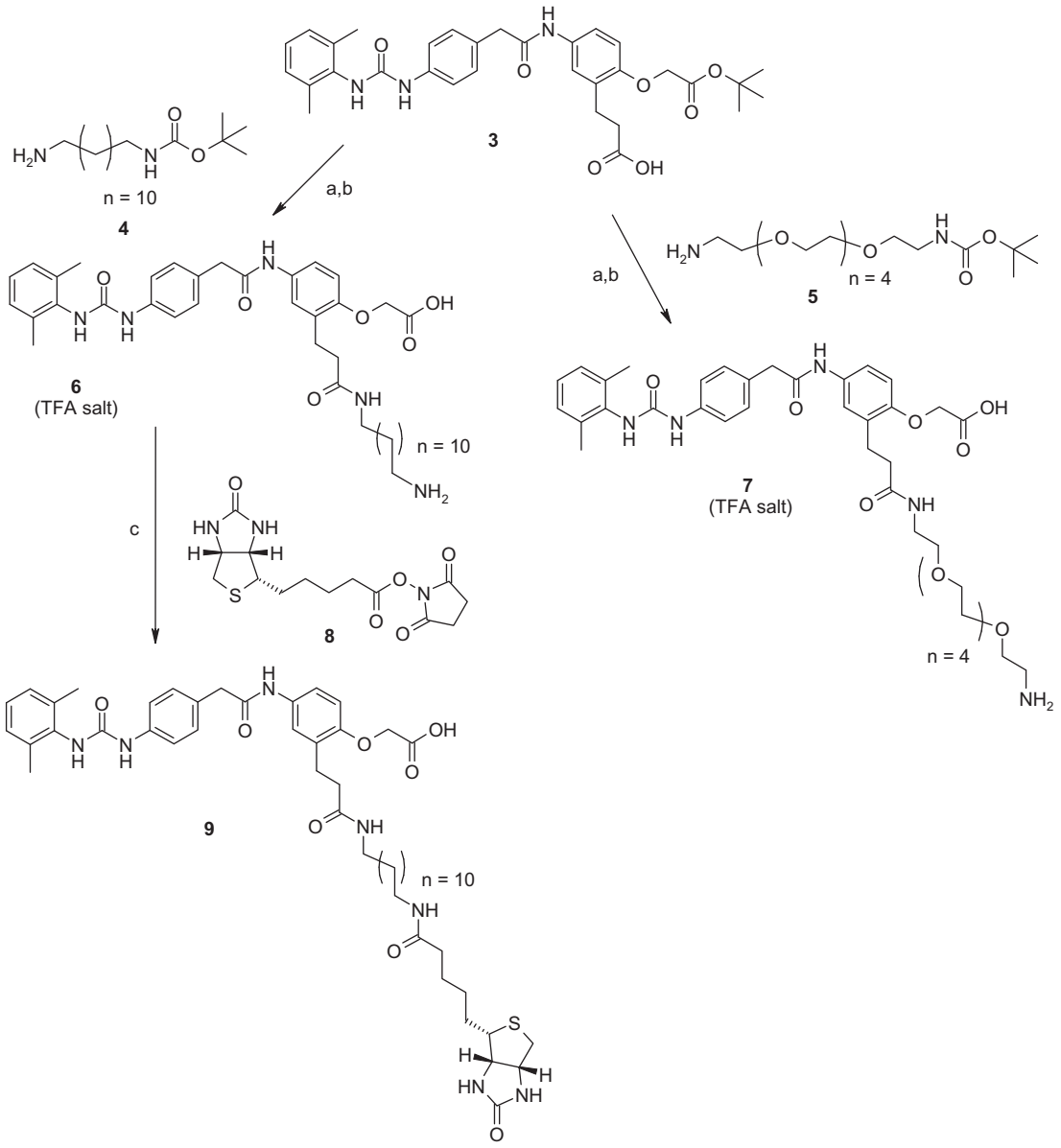
A reference molecule **11**, featuring the diaryl urea motif solely and the alkyl chain, was prepared by conventional chemistry, as described in Scheme 2 (see also Supplementary data). The diaryl urea molecule **10** was obtained by reaction of 2,6-dimethylphenyl isocyanate with *p*-aminophenylacetic acid (see Supplementary data).[23] The coupling of **10** and the spacer-arm **4** was then performed with PyBOP (benzotriazolyloxy-tris(pyrrolidino)-phosphonium hexafluorophosphate) as activation agent, and in presence of triethylamine, with a yield of 89%.[24] Lastly, reaction with trifluoroacetic acid gave quantitatively the compound **11**.

The inhibition activities of the peptidomimetics **6** and **7** on the CCRF-CEM cells adhesion were also determined (Table 1). Compound **6**, equipped with the alkyl chain, was a better inhibitor than compound **7** with an OEG chain ($p < 0.05$), revealing an important influence of the nature of the linker. Indeed, the reference compound **11** was also slightly more active than compound **7** (although not statistically significant, $p = 0.07$) and deletion of the spacer-arm in **11** resulted in a loss of activity (see compound **10**). The activity of compound **11** seems to result of the hydrophobic character of the linker**.** Moreover, peptidomimetic **6** was able to detach the CCRF-CEM cells which had previously adhered on fibronectin during 24 h, contrary to **7** (vs the inhibition of the

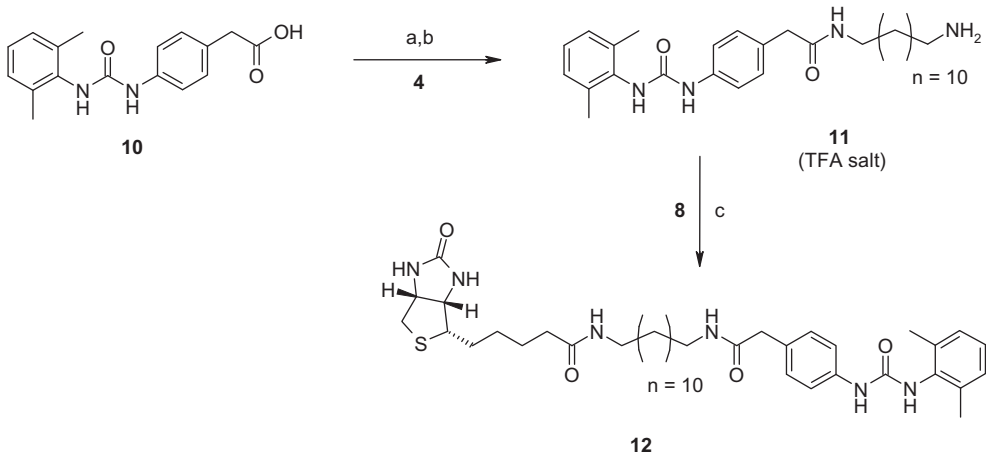
Table 1
Half-maximal inhibitory concentrations (IC_{50}) of the different compounds on the adhesion of $\alpha_4\beta_1$-expressing CCRF-CEM cells

Compound	Description	IC_{50}[a] (μM)
1	Peptide LDV with a phenethyl chain	36.3
2	Peptide LDV with an alkyl chain	2.6
3	Peptidomimetic LDV	68.4
6	Peptidomimetic LDV with an alkyl chain	123.0
7	Peptidomimetic LDV with an OEG chain	169.8
9	Biotinylated peptidomimetic LDV with an alkyl chain	14.1
10	Diaryl urea cap	>1000
11	Diaryl urea cap with an alkyl chain	151.4
12	Biotinylated diaryl urea cap with an alkyl chain	97.7

[a] Inhibition of adhesion of CCRF-CEM leukemia cells to fibronectin (10 μg/mL) was measured after 1h incubation at 37 °C in the presence or the absence of increasing doses of the listed compounds (1–500 μM) (see examples in Fig. S1).



Scheme 1. Reagents and conditions: (a) COMU, DIPEA, DMF; (b) TFA, DCM; (c) Et_3N, DMF.



Scheme 2. Reagents and conditions: (a) PyBOP, Et_3N, DMF; (b) TFA, DCM; (c) Et_3N, DMF.

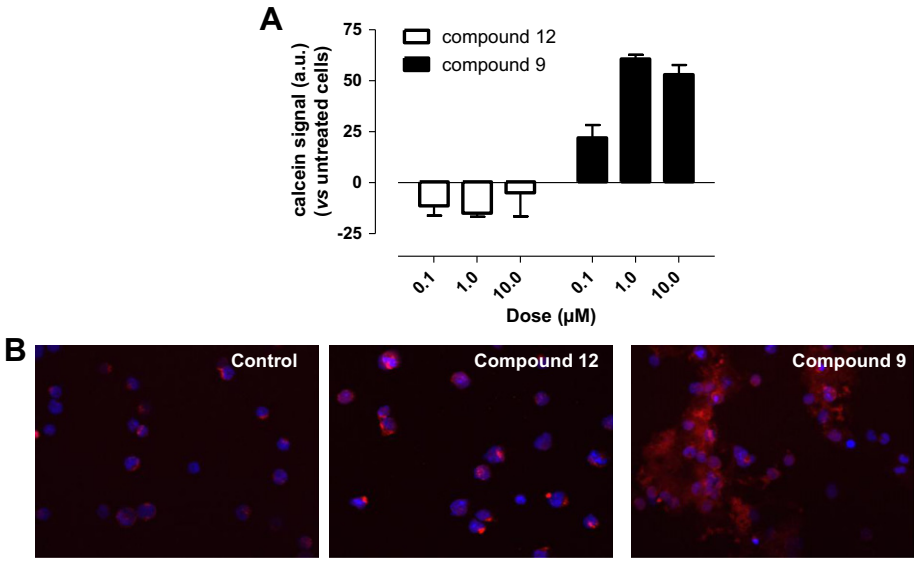


Figure 2. (A) Adhesion of calcein-labeled CCRF-CEM cells to biotinylated molecules **9** and **12**. (B) Pictures obtained after incubation of biotinylated molecules **9** and **12** with CCRF-CEM cells adhering on plates coated with fibronectin (10 μg/mL) and followed by the addition of Alexa 568-conjugated streptavidin (Control = not pre-treated, only addition of fluorescent streptavidin).

adhesion process itself as determined above). This observation supports the higher capacity of compound **6** to interfere with the $\alpha_4\beta_1$ integrin/fibronectin interaction (see Fig. S2).

Due to its better activity, peptidomimetic **6** was then chosen for the introduction of a biotin molecule on the amine end-function of the spacer-arm. Biotinylation of **6** and the reference **11** (Schemes 1 and 2) was performed in presence of triethylamine, by reaction with D-biotin **8** pre-activated in the form of a *N*-hydroxysuccinimidyl (NHS) ester (see Supplementary data).[25,26] The biotinylated molecules **9** and **12** were obtained with moderate yields (58% and 54% respectively).

Compound **9** was a good inhibitor of the adhesion of the CCRF-CEM cells to fibronectin with an IC_{50} value of 14 μM, comparatively to 98 μM for the reference **12** (Table 1). The addition of biotin improved the activity of the initial compounds **6** and **11**. The biotin can favor the solubility of the molecules in the testing conditions or induce physico-chemical effects.

A propidium iodide-based assay was further used to determine a possible toxicity of compounds **9** and **12**. For that purpose, CCRF-CEM cells were incubated for 24 h with increasing amounts of **9** and **12** and then stained with fluorescent propidium iodide to detect dead cells. At 200 μM (final concentration), the results did not show any significant cytotoxicity of these molecules (Fig. S3 and Supplementary data).

Interestingly, the adhesion of calcein-labeled CCRF-CEM cells was increased when neutravidin-coated plates were pre-treated with biotin-conjugated peptidomimetic **9**(Fig. 2A and Supplementary data). Saturation of the signal was however obtained with 1 μM of compound **9**. Of note, the negative calcein signal from plates treated with the reference compound **12** (i.e., lower than in control (untreated) conditions) suggests repulsion between this compound and the cells.

In another assay, adherent CCRF-CEM cells (on fibronectin coated plates), were allowed to interact with the biotin-conjugated molecules **9** and **12** at 100 μM concentration, for 24 h (Supplementary data). After extensive washing, fluorescent Alexa-conjugated streptavidin was then added to detect the presence of peptidomimetics on the cell surface (Fig. 2B). Only cells pre-treated with peptidomimetic **9** showed an extracellular fluorescent signal. An intracellular signal was observed in cells exposed or not to the molecules, reflecting the known unspecific binding of streptavidin to some cytosolic proteins.

In conclusion, we have described the synthesis of two LDV peptidomimetics (compounds **6** and **7**) equipped with different spacer-arms. The most active compound **6**, in cell adhesion assays, featured C_{12} alkyl chain as linker, which was then conjugated with biotin. The resulted biotinylated molecule **9** acts as a good inhibitor of the $\alpha_4\beta_1$ integrin-expressing CCRF-CEM cells adhesion on fibronectin (IC_{50} = 14 μM). Moreover, we have documented the capacity of this peptidomimetic to bind at the surface of the CCRF-CEM cells and to promote their adhesion on neutravidin-coated support. Thus, the interaction of biotin-conjugated compound **9** with streptavidin/avidin has the potential to be exploited in biotechnological applications aiming to label or capture leukemia cells expressing the $\alpha_4\beta_1$ integrin.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.078.

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