Rational Optimization of a Short Human P-selectin-binding Peptide Leads to Nanomolar Affinity Antagonists*

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P-selectin plays an important role in the development of various diseases, including atherosclerosis and thrombosis. In our laboratory we recently identified a number of specific human P-selectin-binding peptides containing a Glu-Trp-Val-Asp-Val consensus motif, displaying a low micromolar affinity for P-selectin ($IC_{50} = 2$ μM). In search of more potent antagonists for P-selectin, we have optimized the EWVDV pentapeptide core motif via a two-step combinatorial chemistry approach. A dedicated library of peptide derivatives was generated by introducing seven substituents at the N and C termini of the motif. In particular, pentapeptides with gallic acid or 1,3,5-benzenetricarboxylic acid substituents at the N terminus proved to be considerably more potent inhibitors of P-selectin binding than the parental peptide. After removal of the N-terminal glutamic acid from the core sequence, which appeared to be replaceable by a carboxamide function without loss of affinity, a second library was synthesized to map the chemical moieties within the gallic acid or 1,3,5-benzenetricarboxyl acid groups responsible for the enhanced P-selectin binding. Moreover, by varying the length and rigidity of the connective spacer, we have further optimized the spatial orientation of the N-terminal substituent. The combined use of phage display and subsequent combinatorial chemistry led to the design of a number of gallic acid-containing peptides with low nanomolar affinity for P-selectin both under static and dynamic conditions ($IC_{50} = 15.4$ nM). These small synthetic antagonists, which are equally as potent as the natural ligand P-selectin glycoprotein ligand-1, are promising leads in anti-atherothrombotic therapy.

P-selectin, a cell adhesion molecule involved in the initial attachment and "rolling" of leukocytes across the inflamed vessel wall (1–4), plays a key role in atherosclerosis. In fact, P-selectin deficiency in mice has been shown to reduce atherosclerotic lesion formation (5). Also, P-selectin activation induces hypercoagulance of platelets and mediates platelet-monocyte aggregation and has thus been associated with thrombosis (6–9). Therefore, intervention in P-selectin-mediated processes is an attractive therapeutic entry.

The endogenous ligand for P-selectin is P-selectin glycoprotein ligand-1 (PSGL-1),[1] a 220-kDa disulfide-linked homodimeric sialomucin (10). P-selectin binding to PSGL-1 proceeds via a short N-terminal amino acid sequence of the latter, containing three sulfated tyrosines and a sialyl Lewis X (sLeX, Neu5Acα2–3Galβ1–4[Fucα1–3]GlcNAc) moiety (11–14). Most P-selectin antagonists are carbohydrate derivatives of the sLeX structure (15). However, as compared with PSGL-1, these glycosides are relatively poor and unselective P-selectin inhibitors, because binding to the other selectin family members (E- and L-selectin) is equally affected.

In our lab we recently identified, through the use of phage display, a number of human P-selectin-binding peptides containing an EWVDV pentapeptide consensus motif (16). Binding of these peptides to human P-selectin was calcium-dependent and highly specific over E- and L-selectin. With its IC_{50} of 8 μM, the stripped pentapeptide already appeared to be much more potent than most of the sLeX-derived carbohydrate ligands. For therapeutic purposes, however, the affinity of an antagonist has to be in the low nanomolar range. We obtained this affinity by tetrameric exposure of the EWVDV peptide on streptavidin ($IC_{50} = 2$ nM). However, streptavidin-peptide complexes are rather inadequate for *in vivo* use, and smaller synthetic leads are pharmaceutically much more interesting. For this reason an optimization of the core sequence was performed.

In this paper, we describe the rational design of potent monomeric P-selectin antagonists using a combinatorial chemistry strategy with the consensus motif (E)WVDV as core sequence. A structure-activity study yielded a number of peptide derivatives that are equally as potent as the natural ligand PSGL-1.

EXPERIMENTAL PROCEDURES

Materials—Fmoc protected amino acids, 1-hydroxybezotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), and 4-hydroxymethylphenoxyacetic acid (HMPA) were purchased from Nova Biochem (Läufelingen,

[1] The abbreviations used are: PSGL-1, P-selectin glycoprotein ligand-1; sLeX, sialyl Lewis X; Fmoc, *N*-(9-fluoroenyl)methoxy-carbonyl; HOBt, 1-hydroxybezotriazole; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; BOP, benzotriazol-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate; HMPA, 4-hydroxymethylphenoxyacetic acid; Dipea, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; TM11-PO, tetrameric complex of biotinylated TM11 with streptavidin peroxidase; HPLC, high performance liquid chromatography; Boc, *N*-(*t*-butoxycarbonyl); DDE, 4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; CHO, Chinese hamster ovary; CHO-P, CHO cells expressing P-selectin; PAA, polyacrylamide; Le^a, Lewis A; AM, acetoxymethyl.

Switzerland), except Fmoc-4-aminobenzoic acid, Fmoc-(4-aminomethyl)-benzoic acid, and Fmoc-tranexamic acid, which were from Neosystem (Strasbourg, France). Trifluoroacetic acid, *N*,*N*-diisopropylethylamine (Dipea), dichloromethane, dichloroethane, *N*,*N*-dimethylformamide (DMF), piperidine, and 1-methyl-2-pyrrolidine were of peptide grade and purchased from Biosolve (Valkenswaard, The Netherlands). 4-Dimethylaminopyridine was obtained from Janssen (Beersse, Belgium). *N*,*N'*-dicyclohexylcarbodiimide, hydrazine monohydrate, and all of the carboxylic acids were obtained from Acros (Hertogenbosch, The Netherlands). *N*-Methylmorpholine was obtained from Fluka (Buchs, Switzerland). Tentagel S-NH$_2$ (0.26 mmol/g) was from Rapp Polymer (Tübingen, Germany). Triisopropylsilane, goat anti-human IgG (Fc-specific), and bovine serum albumin were from Sigma-Aldrich. TM11-biotin (biotin-CDVEWVDVSSLEWDLPC) was synthesized by Dr. Van der Zee (Department of Immunology, Utrecht, The Netherlands). Human P-selectin/IgG-Fc and human E-selectin/IgG-Fc chimeras were kindly provided by Drs. Appelmelk and Van Dijk (Free University of Amsterdam, Amsterdam, The Netherlands). Human L-selectin and mouse P-selectin were purchased by R&D Systems Europe, Ltd. (Abingdon, UK). Streptavidin peroxidase was obtained from Amersham Biosciences, and biotin-PAA-Le^a-SO$_3$H was from Synthesome (München, Germany). 3,3′,5,5′-Tetramethylbenzine/H$_2$O$_2$ was obtained from Pierce. RPMI 1640 medium, Dulbecco's modified Eagle's medium, fetal calf serum, and penicillin/streptomycin were obtained from Bio-Whittaker Europe (Verviers, Belgium).

Chemical Synthesis of Resin-bound Core Sequence 9—Core sequence 9 FmocHN-Glu(OtBu)-Trp(Boc)-Val-Asp(OtBu)-Val-Lys-(DDE)-γ-aminobutyric acid-HMPA-resin was synthesized on an Applied Biosystems 9050 peptide synthesizer (Warrington, UK) using standard Fmoc chemistry. In short, Tentagel S-NH$_2$ (load 0.26 mmol/g) was provided with HMPA as a linker, resulting in resin 8. Fmoc-γ-aminobutyric acid-OH (10 eq) was attached to the HMPA resin 8 under the agency of *N*,*N'*-dicyclohexylcarbodiimide (5 eq) and 4-dimethylaminopyridine (0.5 eq). All other amino acids, with acid-labile side chain protection if necessary, were attached by coupling in the presence of HOBt/TBTU/Dipea (4, 4, and 8 eq, respectively). After coupling, the resin was washed with DMF, *iso*-propanol, and diethyl ether and subsequently dried.

Synthesis of Library 10—The solid phase synthesis of library 10 was performed using a Flexchem© system (Robbins Scientific). After removal of the N-terminal Fmoc group of sequence 9 by 20% piperidine in DMF, the resin was washed (in DMF) and dried. The resin was distributed in 10-mg quantities over a solvent-resistant 48-well filter plate. After washing with DMF, a mixture of the desired carboxylic acid (40 eq), BOP (20 eq), HOBt (20 eq), and *N*-methylmorpholine (100 eq) was added (total volume, 300 μl), and the suspended resin was incubated for 3 h. Subsequently, the resin was washed with DMF and incubated three times for 3 min with hydrazine monohydrate (2% in DMF) to remove the DDE group. After washing with DMF, a mixture of the second carboxylic acid, BOP, HOBt, and *N*-methylmorpholine (same amounts as described above) was added and once again incubated for 3 h. Peptides, which were only modified at the C-terminal lysine (peptides HP01–HP07), were first *N*-bocylated with di-*tert*-butyl-dicarbonate (0.25 M) and Dipea (0.125 M) in 1-methyl-2-pyrrolidine to protect the N-terminal amine function. After removal of the solvent, the peptides were cleaved off from the resin with a trifluoroacetic acid, triisopropylsilane, and water mixture (95:2.5:2.5, v/v/v). Each sample was lyophilized and stored at −20 °C until use.

Synthesis of Peptides 11–13, Library 14, and Peptides 27–33—Peptides 11–13, library 14, and peptides 27–33 were prepared in the same manner as described for library 10 from resin bound core sequence FmocHN-Trp(Boc)-Val-Asp(OtBu)-Val-HMPA-resin. Peptide 11 was acetylated using acetic anhydride (0.25 M) and Dipea (0.125 M) in 1-methyl-2-pyrrolidine.

Preparation of Samples and Determination of Peptide Concentration—Lyophilized peptides were dissolved in ammonia (100 mM, 100 μl) and aqueous ammonium bicarbonate solution (5 mM, 400 μl). The peptide concentration was determined spectrophotometrically at 280 nm (tryptophan; ε = 5.5 mM^{-1} cm^{-1}). Absorptions were corrected for the absorption coefficient of the introduced carboxylic acid(s). Compound purity was checked randomly (~10% of all compounds) by HPLC analysis on a C8 or C18 reversed phase column (Alltech, Breda, The Netherlands) using an acetonitrile/water gradient with 0.1% trifluoroacetic acid at 280 nm and by matrix-assisted laser desorption ionization mass spectrometry. Compounds in Tables I and II were all purified by HPLC analysis (>90%) and analyzed by mass spectrometry.

Competition Assay with TM11-PO or Biotin-PAA-Le^a-SO^3—The peptides were assayed for their ability to inhibit TM11-PO binding to

human P-selectin (16) or biotin-PAA-Le^a-SO$_3$H binding to human and mouse P-selectin and human E- and L-selectin (17). TM11-PO, a tetrameric TM11/streptavidin peroxidase complex, were freshly prepared by incubating streptavidin peroxidase (8.4 μl, 2.0 μM) and TM11-biotin (biotin-CDVEWVDVSSLEWDLPC, 1.5 μl, 190 mM) for 2 h at room temperature in assay buffer. For competition studies, a 96-well microtiter plate (high binding, flat bottom; Costar, Corning, NY) was coated overnight at 4 °C with 10 μg/ml goat anti-human IgG in coating buffer (50 mM NaHCO$_3$, pH 9.6). Subsequently, the wells were washed with assay buffer (20 mM HEPES, 150 mM NaCl, 1 mM CaCl$_2$, pH 7.4) and incubated for 1 h at 37 °C with blocking buffer (3% bovine serum albumin in assay buffer). After washing with assay buffer, the wells were incubated for 2 h at 37 °C with human P-selectin/IgG-Fc (0.3 μg/ml). Subsequently, the wells were washed with assay buffer and incubated for 1 h at 4 °C with the TM11-PO complex or biotin-PAA-Le^a-SO$_3$. The wells were washed six times with washing buffer (0.1% Tween 20 in assay buffer). 3,3′,5,5′-Tetramethylbenzine/H$_2$O$_2$ was added, and the wells were incubated at room temperature for 15 min. The reaction was halted by the addition of 2 M H$_2$SO$_4$, and the absorbance was measured at 450 nm.

HL60 Adhesion Assay—HL60 cells were fluorescently labeled by incubation for 30 min at 37 °C with 5 μM calcein-AM (Molecular Probes, Leiden, The Netherlands) in RPMI. These cells (50,000/well) were added to cultured CHO cells expressing P-selectin (CHO-P cells, cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 5 mM nonessential amino acids, 5 mM L-glutamine, and 20,000 units of penicillin/streptomycin), seeded in 96-well plates in the presence or absence of the P-selectin antagonists (1h, 4 °C) (16). After gentle washing with RPMI, CHO-P-associated fluorescence was measured (λ$_{exc}$ = 485, λ$_{em}$ = 530 nm).

Flow Chamber and Perfusion Studies—Dynamic interactions between HL60 cells and CHO-P cells monolayers grown onto glass coverslips coated with 30 μg/ml collagen (collagen S type I; Roche Diagnosis, Brussel, Belgium) were analyzed in a parallel plate perfusion chamber as described (18), with some modifications. The coverslip constituted the bottom of the chamber, and the actual chamber was formed by a 254-μm height silicon rubber gasket designed with a conically shaped flow path, thus resulting in a 3-fold increase of wall shear rate from the inlet of the chamber to the outlet. Calcein-AM-labeled HL60 cells suspended in RPMI (0.5 × 10^6/ml) were perfused at 37 °C with an inverted syringe pump (Harvard Instruments, South Natick, MA) at a flow rate of 1 ml/min. By mounting the flow chamber on the table of an inverted epifluorescence microscope (Diaphot; Nikon, Melville, NY) coupled to a Cohu CCD video camera (COHU Inc., San Diego, CA), HL60 cells translocation over CHO-P monolayers were observed at wall shear rates of 300 and 600 s^{-1}, in the presence or absence of P-selectin antagonists added to HL60 suspensions 2 min before the onset of perfusion. Real time movies of 12 s (10 images/s), recorded at random positions in the flow path corresponding to chosen wall shear rates were stored into the memory of an attached computer and digitized with a Scion LG3 frame grabber (Scion Corp, Frederick, MD). The velocity of HL60 cells rolling over the CHO-P cells was determined by measuring the distance traveled by the HL60 cells during at least 1 s of flow, using the NIH Image program, version 6.1.

RESULTS

In pursuit of P-selectin antagonists for intervention in inflammatory diseases such as atherosclerosis, a cysteine-constrained phage-displayed peptide library was screened for P-selectin binders (16). A number of positive clones, including TM11, were identified and sequenced for their peptide insert (Table I). Comparison of the peptides for sequence homology revealed a pentapeptide consensus motif, which was established to be critical for human P-selectin binding by subsequent truncation and alanine scanning: EWVDV. With its low micromolar affinity, we argued that the therapeutic potential of the EWVDV peptide would greatly benefit from further optimization studies.

Instead of conventional replacement of individual amino acids by naturally occurring amino acids, we preferred introduction of new chemical entities within the EWVDV core sequence by acylation of available amine groups to enhance the affinity for P-selectin. This flexible strategy enables the introduction of an infinite range of substituents and already has been shown

effective for the optimization of an SH2 binding peptide by Yeh *et al.* (19). Although Yeh *et al.* used a vast peptide library ($\sim 10^3$ peptides) for screening, we considered a stepwise optimization protocol on the basis of dedicated libraries of approximately 100 EWVDV analogues to be at least equally effective and more practical. In the first screening step we have addressed the effect of substituting the N and C termini of the EWVDV motif with seven different moieties (Fig. 1, *acyl moieties 1–7*), resulting in a library of 63 compounds.

The N-terminal amine group within the peptide was readily available for coupling to the carboxylic acids after removal of the protecting Fmoc group. To enable modification at the C terminus, however, a DDE-protected lysine was introduced behind the last valine of the core peptide. The DDE group can

be selectively removed with 2% hydrazine without affecting the acid-labile side chain protecting groups (20), thus allowing independent introduction of substituents at either site of the peptide. Starting from Tentagel S-NH$_2$ resin with an acid-labile HMPA linker (resin 8), core sequence 9 was synthesized using HOBt/TBTU/Dipea couplings. The first amino acid, γ-aminobutyric acid, was introduced to increase the distance between the C-terminal carboxylic acid and the amine function of the lysine. After the introduction of carboxylic acids 1–7, library 10 was cleaved from the resin by incubation with a

TABLE I
Peptides and their affinities for human P-selectin as determined by competition enzyme-linked immunosorbent assay

Peptide	Sequence	IC$_{50}$[a]
		μM
TM11	CDVE**WVDV**SSLEWDLPC	2[b]
	E**WVDV**	8[b]
	D**WVDV**	16[b]
	K**WVDV**	28[b]
	A**WVDV**	27[b]
	WVDV	>1000[b]
11	Ac–**WVDV**	27
12	GA–**WVDV**	0.037
13	GA–E**WVDV**	0.031

[a] The results are the averages of at least three experiments at eight different concentrations of peptide as determined in TM11-PO competition ELISA (see "Experimental Procedures").
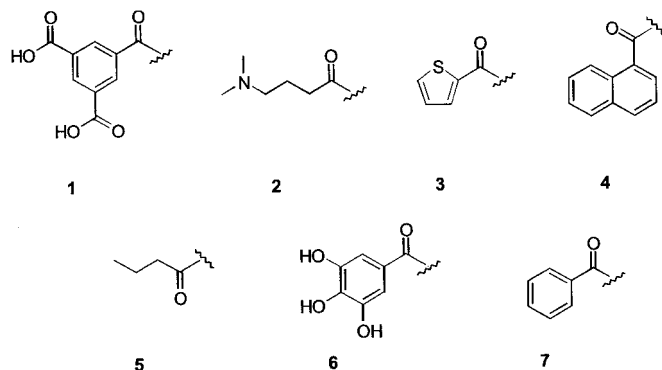[b] From Molenaar *et al.* (16).

FIG. 1. **Chemical structures of acyl moieties introduced to core sequence 10.**
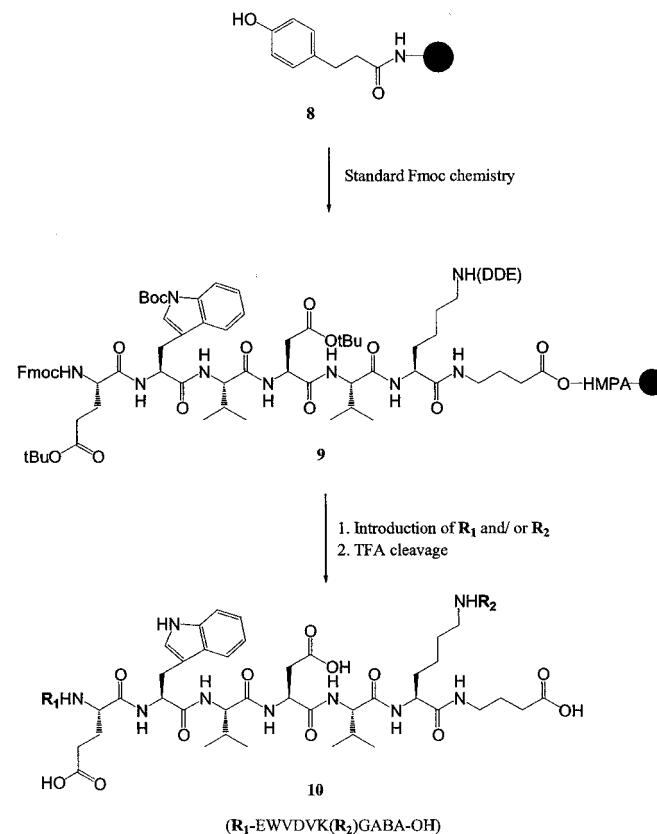

FIG. 2. **Solid phase synthesis of library 10.** Core sequence 10 was synthesized using standard Fmoc chemistry on Tentagel S-NH$_2$ resin with HMPA linker (sequence 9). The core peptide was then derivatized at the N terminus (after removal of the Fmoc) or on the C terminus (after selective removal of the DDE group by 2% hydrazine) with seven different carboxylic acids (see Fig. 1). The peptides were liberated from the resin by trifluoroacetic acid cleavage, resulting in library 10. *tBu*, *tert*-butyl; *TFA*, trifluoroacetic acid.

FIG. 3. **Ability of peptides HP10-HP77 to inhibit ligand binding to human P-selectin.** The crude peptides were tested in a competition assay for their ability to displace binding of TM11-PO to human P-selectin. Each *bar* represents the binding of TM11-PO in the presence of peptide (5 μM, $n = 3$–5 $\pm$ S.E.). The peptides are coded HP*ij*, where *i* and *j* refer to the acyl moiety attached to the N and C termini, respectively. The N/C-terminally unmodified amino group is indicated by 0.
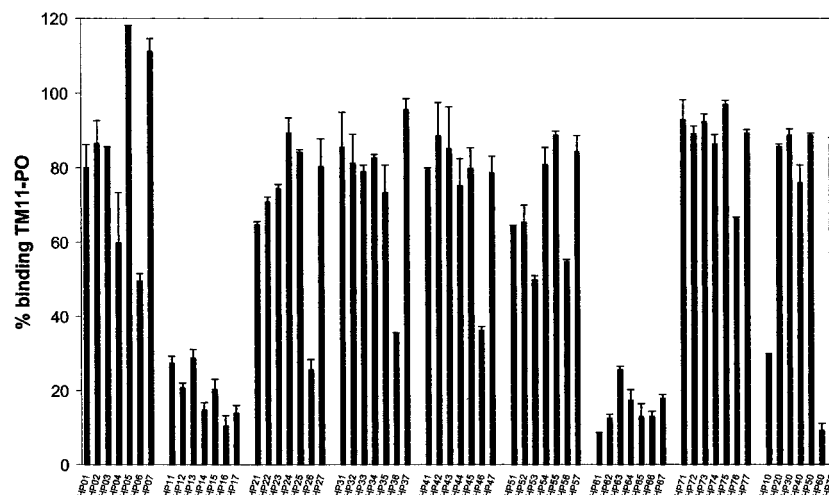
trifluoroacetic acid/triisopropylsilane/H$_2$O mixture (Fig. 2). To elucidate peptides with enhanced P-selectin binding as compared with the EWVDV core, all of the crude peptides were tested below the IC$_{50}$ of the EWVDV peptide (IC$_{50}$ = 6 μM) at 5 μM in a competition assay for binding TM11-PO to P-selectin (Fig. 3). This complex was previously shown to be a potent and specific ligand for P-selectin (16). Peptides with N-terminal 1,3,5-tricarboxylic acid (acyl moiety 1) or gallic acid (acyl moiety 6) substituents were found to be most effective in inhibiting P-selectin binding: > 90% as compared with only 35% for the unsubstituted reference. The C-terminal counterparts, peptides HP01 and HP06, were considerably less effective, whereas the disubstituted peptides HP11 and HP66 were equally as potent as the N-terminal monosubstituted peptides HP10 and HP60 (peptides are coded HPij, where i and j refer to the acyl moieties attached to the N and C termini, respectively; N/C-terminally unmodified amino group is indicated by 0). Therefore, for the second optimization step we shifted our attention to the N-terminal substitution, thus obviating the introduction of a potentially perturbing lysine group at the C-terminal end.

Earlier observations already suggested that the N-terminal amide function rather than the complete glutamic acid moiety is necessary for potent P-selectin binding (16). Peptides KWVDV and AWVDV were equally potent inhibitors of P-selectin binding, but the absence of an N-terminal amide function (WVDV) led to a complete loss in binding capacity (Table I). To conclusively demonstrate the involvement of the amide group in P-selectin binding, we have synthesized Ac-WVDV (Peptide 11). Indeed, Ac-WVDV was found to inhibit P-selectin binding at a similar potency as the core sequence EWVDV. Likewise, when gallic acid (acyl moiety 6), the most potent substituent of the first library, was attached directly to the α-amine group of WVDV (peptide 12), this had similar potency as when the longer EWVDV core was used instead (peptide 13) (IC$_{50}$ = 37 *versus* 31 nM, respectively).

On the basis of the above findings, a new library of 42 substituted WVDV peptides was designed (Fig. 4A, *library 14*). This dedicated library served two purposes. First, the length and flexibility of the linker between the N-terminal substituent group and the WVDV motif were varied to optimize the spatial orientation of the substituent. Carboxylic acids R$_3$ were attached directly to the α-amino group of the WVDV core or via a glycine or aminobutyric acid spacer (linkers R$_4$). Second, to be able to pinpoint the actual groups within 1,3,5-benzene carboxylic acid and gallic acid responsible for the enhanced affinity of peptides HP10 and HP60, several carboxylic acids resembling these carboxylic acids were introduced at the N-terminal amine (carboxylic acids R$_3$).

In addition, a range of other anionic substituents was introduced to mimic the negatively charged tyrosine sulfates and the neuraminic acid of sLeX of PSGL-1. These moieties have been shown to be crucial for high P-selectin binding, possibly through occupation of a second binding site on P-selectin (12, 21, 22). Because a number of peptides from library 10 could displace TM11-PO binding above 90% at a 5 μM concentration, the peptides from library 14 were tested at 1 μM. At this concentration, L-cysteic acid-derived (acyl moiety 15) and 5-sulfosalicyclic acid-derived (acyl moiety 16) peptides gave up to 40% inhibition of TM11-PO binding, which is comparable with the 1,3,5-benzenetricarboxylic acid-derivatized peptides (acyl moiety 1) (Fig. 4B). Attachment of nitroaryl (acyl moiety 17) or fluoroaryl groups (acyl moiety 18) resulted in peptides equally as potent as EWVDV, displaying a nonsignificant 5% inhibition at this concentration.
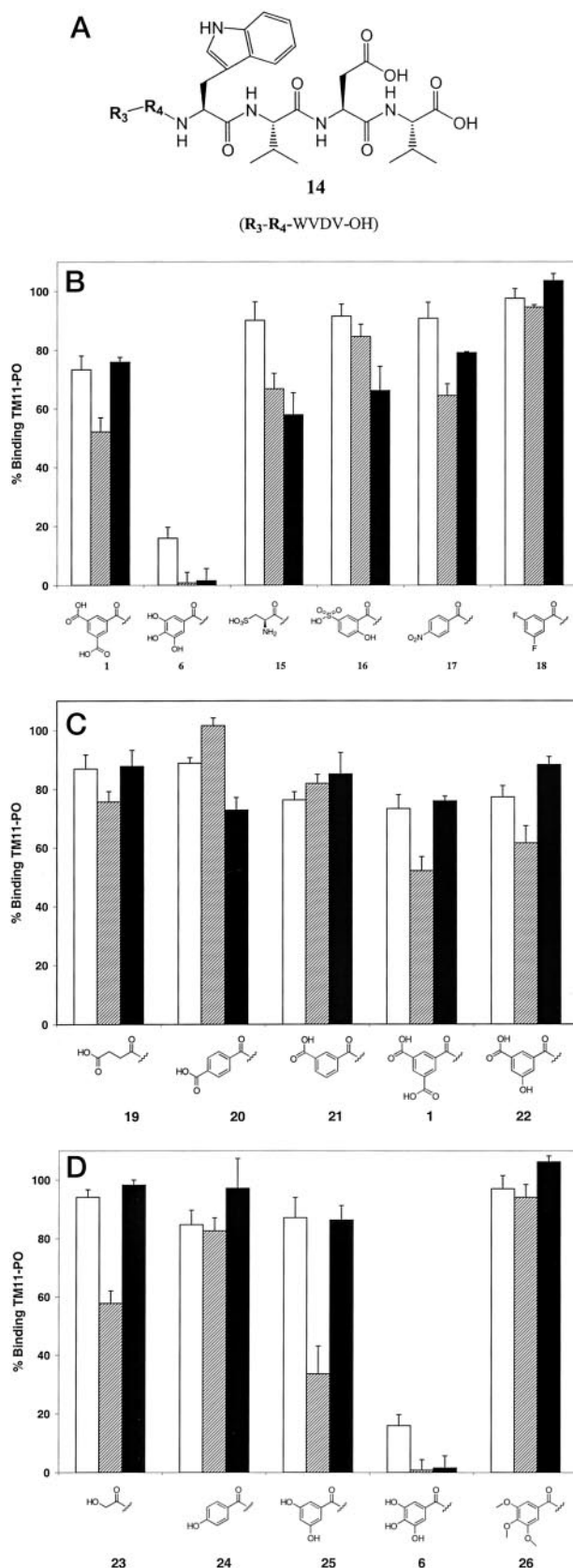
Removal of one of the carboxyl acids of substituent 1, by

FIG. 4. **Chemical structure and biological activity of library 14.** *A*, chemical structure of compounds from library 14. *B–D*, members of crude peptide library 14 (1 μM) were tested in a competition assay of TM11-PO binding to human P-selectin. Three different spacers between the acyl moiety and the WVDV core sequence were used (R$_4$): no spacer (*white bars*), a glycyl spacer (*hatched bars*), and amino butyric spacer (*black bars*). The chemical structures of the introduced acyl modifications (R$_1$) are depicted *below* these bars.

TABLE II
Gallic acid-derivatized peptides and their IC_{50} for human P-selectin as determined by competition enzyme-linked immunosorbent assay

Peptide	Structure	IC_{50} (nM)[a]
12		37.1
27		18.3
28		15.4
29		62.9
30		250
31		>1000
32		590
33		580

[a] The values are the means of at least three experiments at eight different concentrations of peptide as determined in TM11-PO competition enzyme-linked immunosorbent assay (see "Experimental Procedures").
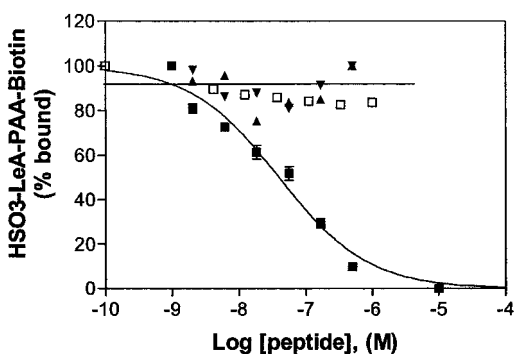

FIG. 5. **Competition of biotin-PAA-Le^a-SO$_3$ binding to human P-selectin (■), mouse P-selectin (□), human L-selectin (▲), and human E-selectin (▼) by peptide 28.** The wells were coated with each selectin (0.3 μg/ml) and incubated with 0.33 μg/ml biotin-PAA-Le^a-SO$_3$ with or without peptide 28 as described in under "Experimental Procedures." The values represent the means of triplicate experiments ± S.E.

introduction of carboxylic acids 19–21, did not considerably reduce the affinity for P-selectin, regardless of the position and flexibility of the remaining groups (Fig. 4*C*). Replacement of the carboxylic acid by an hydroxyl group (carboxylic acid 1 by 22) did not influence P-selectin binding either, suggesting that the gain in affinity is mediated by hydrogen bridging rather than electrostatic interactions. Contrary to the effect of the carboxylic acids, the number of exposed hydroxyls appear to be critical for its affinity, because monobenzoic acid-derivatized (acids 23 and 24) and dihydroxybenzoic acid-derivatized (acid 25) peptides were much less effective than the trihydoxylated counterparts (1) (Fig. 4*D*). Importantly, P-selectin binding was completely abolished after conversion of the hydroxyls into
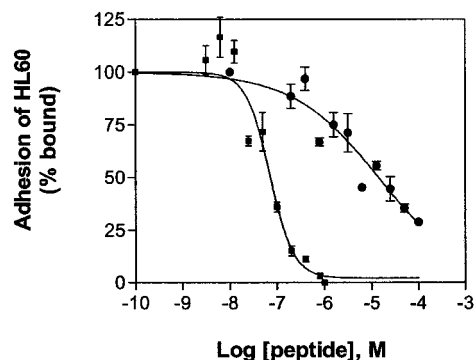

FIG. 6. **Competition of HL60 cell binding to CHO-P cells by peptide 28 (■) or EWVDV (●).** CHO-P cells seeded in 96 wells were incubated with calcein-labeled HL60 cells in the presence of peptide 28 or EWVDV. The values represent means of triplicate experiments ± S.E.
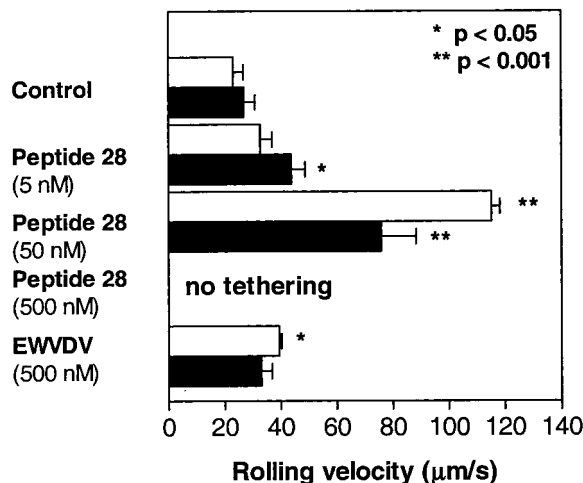

FIG. 7. **HL60 rolling velocities in flow chamber perfusion experiments.** The velocity (μm/s) of calcein-AM-labeled HL60 cells rolling over CHO-P cell-coated coverslips at wall shear rates of 300 s^{-1} (*white bars*) and 600 s^{-1} (*black bars*), in the absence (control) or presence of peptide 28 (5, 50, and 500 nM) or EWVDV (500 nM)) was determined from real time movies. For each condition the velocity of at least 20 individual HL60 cells was measured in three to nine independent perfusion assays. The values represent the means ± S.E. of rolling velocities. *no tethering* means a too short contact time (< 0.1 s) between HL60 cells and CHO-P monolayers to allow velocity measurement.

methyl ethers (via introduction of acid 26).

The spacer length R$_4$ between the core motif and the substituent was of little influence. IC_{50} values for the peptides lacking an intermediate spacer or having a glycyl or amino buryrate spacer (peptides 12, 27, and 28, respectively) ranged from 37.1 to 15.4 nM (Table II). Further elongation of the spacer, however, to an amino hexanoate (C-6) (peptide 29) caused a significant decrease in affinity (IC_{50} = 62.9 nM).

Because flexible spacers confer the advantage of minimal conformational constraints but at the same time cause a maximal loss in entropy after binding, it is preferable to insert a more rigid spacer between the substituent and the core motif, when possible. To investigate the effect of spacer flexibility on the affinity of the substituted peptides for P-selectin, we therefore introduced a number of equally sized, more rigid analogues. A cyclic L-proline linker (peptide 30) led to a 5-fold reduction of the IC_{50} to 250 nM. Use of a linear 4-amino benzoate spacer, which is conformationally locked and only allows axial rotation of the gallic acid moiety (peptide 31), let to a complete loss in affinity (>1000 nM). Interestingly, binding is partially recovered when inserting an additional CH$_2$ group between linker and gallic acid (peptide 32), regardless of

whether the cyclohexyl group is plain aromatic (phenyl; peptide 32) or chair/boat configured (cyclohexyl; peptide 33).

Introduction of the gallic acid moiety did not influence the specificity of the peptides. Peptide 28, the most potent antagonist of this series, did not displace biotin-PAA-Le^a-SO$_3$H binding to either mouse P-selectin or human E- and L-selectin (Fig. 5). This PAA-based conjugate of sulfated Le^a was reported to bind with low nanomolar affinity to all selectins (17).

Finally, we investigated peptide 28 for its ability to inhibit HL60 cell adhesion to P-selectin-transfected CHO cells (CHO-P cells) under static (Fig. 6) and flow conditions (Fig. 7). Monocyte-derived HL60 cells have a high expression of PSGL-1 (10, 23) and are adherent to CHO-P cells. Peptide 28 was found to inhibit HL60 cell adhesion with an EC$_{50}$ of 74 nM, indicating that it is an effective inhibitor of human P-selectin in a more physiological setting. Under flow conditions, the peptide significantly increased the rolling velocity of HL60 cells at concentrations as low as 50 nM, indicating a reduced interaction between PSGL-1 and P-selectin. Surprisingly, at 500 nM tethering to and rolling of HL60 cells along P-selectin expressing cells could be observed. This is in sharp contrast to the unmodified EWVDV peptide, which was unable to affect the rolling velocity or cell adhesion at the same concentration.

DISCUSSION

The use of phage display in ligand discovery has been shown to be very effective over the last decade, in particular for ill-defined targets (24, 25). Despite its apparent promise for the identification of peptide motifs as initial leads, phage display-based drug design has a number of setbacks. Derived leads often display affinity in the micromolar to millimolar range, which precludes direct use in a therapeutic setting. In addition phage display does not allow the introduction of unnatural amino acid derivatives or post-translational modifications. A conventional strategy to increase the affinity of the peptide ligands involves the systematic replacement of amino acids after having identified a minimal effective motif via alanine scanning and truncation studies (26, 27). An integrated approach in which lead peptides are optimized using combinatorial organic chemistry on the other hand will pave the way for non-amino acid modifications of the peptide leads and will greatly increase the number of possible substituents.

The most convenient way to introduce these modifications and to generate large compound libraries involves the use of solid phase combinatorial chemistry. However, the synthesis of large peptide libraries puts serious demands on purification and screening of compounds to render the process viable. Small dedicated libraries of compounds with a stepwise approach may likely be equally effective for enhancing target affinity.

In this study we have taken a phage display-derived peptide (16) as a starting point for organic chemical optimization. This EWVDV pentapeptide was shown to specifically bind to P-selectin and antagonized HL60 adhesion to P-selectin-transfected cells. In a first library, seven different substituents were introduced by acylation of primary amine groups at either the N or C terminus. Affinity testing of the crude peptides, at a concentration just below the IC$_{50}$ of the parental EWVDV peptide to reduce the number of hits (5 μM), revealed that N-terminal modification with 1,3,5-tricarboxybenzoic acid (carboxylic acid 1) or gallic acid (carboxylic acid 6) were most effective in inhibiting P-selectin binding (>90% inhibition *versus* 30% for the underivatized peptide). C terminus modifications had little to no influence on the affinity of the core peptide.

The core peptide could even be reduced to WVDV after iden-

tification that the N-terminal amide rather than the glutamic acid was imperative for P-selectin binding. However, when a negatively charged Glu or Asp before the WVDV was present, P-selectin binding was slightly improved compared with other uncharged amino acids like Ala and Lys. Negatively charged groups within PSGL-1, *i.e.* tyrosine sulfates and the neuraminic acid of sLeX, are found to be crucial for P-selectin binding (11–14). This might imply that negatively charged amino acids Glu and Asp interact with P-selectin at a site proximal to the actual sLeX-binding site. Indeed the existence of a second binding pocket has already been speculated upon in a number of reports (12, 21, 22), although solid evidence still remains to be provided. The derivatization of the core peptide with the gallic acid substituent, again the best substituent in our second library, would also allow occupation of both binding sites, thus explaining the considerably increased affinity.

Of the introduced spacers, the elongated aminobutyric acid spacer performed best (peptide 28, IC$_{50}$ = 15.4 nM). Introduction of longer or more rigid spacers led to a considerable loss in affinity, indicating that the spatial orientation of the terminal gallic acid group with respect to the peptide is critical. Peptide 28 was also tested for its ability to antagonize HL60 adhesion to P-selectin under both static and dynamic conditions. PSGL-1-mediated adhesion to P-selectin was impaired at 50 nM and even completely blocked at 500 nM.

In conclusion, we show in this study that stepwise optimization of peptide leads through dedicated small peptide libraries is a very efficient strategy. The affinity of the P-selectin binding sequence (E)WVDV was increased almost 800-fold via the introduction of a gallic acid moiety at the N terminus, as was shown in different testing systems. Thus, the combined use of phage display and subsequent combinatorial chemistry led to the design of P-selectin antagonists with nanomolar affinity. These small synthetic antagonists, which are equally potent as the natural ligand P-selectin glycoprotein ligand-1, may be promising leads in athero-thrombotic therapy.

For further *in vivo* use we plan to alter the pharmacokinetic profile of these compounds. Shifting our attention from peptides to peptidomimetics would also decrease the susceptibility to proteases. This work is currently in progress.

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Rational Optimization of a Short Human P-selectin-binding Peptide Leads to Nanomolar Affinity Antagonists

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