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PHOTOACTIVATABLE PEPTIDES BASED ON BMS-197525: A POTENT ANTAGONIST OF THE HUMAN THROMBIN RECEPTOR (PAR-1)

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Abstract: Photoactivatable analogs of the human thrombin receptor (PAR-1) antagonist, *N-trans*-cinnamoyl-*p*-fluoroPhe-*p*-guanidinoPhe-Leu-Arg-NH_2 (BMS-197525), were prepared with benzophenone substitutions in the N-terminal, Leu, or Arg position. The analogs retained antagonist activity (with reduced potency); the tritium-labeled isotopomers are potential photoaffinity labels for the receptor. C-Terminal extension of the analogs with ornithine(biotin) did not significantly alter antagonist potency.

α-Thrombin is a multifunctional serine protease intimately involved in regulating clotting processes, wound healing, and inflammatory responses. It exerts many of its cellular effects by proteolytically activating the tethered-ligand of the 7-transmembrane segment G protein-coupled thrombin receptor (PAR-1).[1] Thrombin receptor activating peptides (TRAP) with sequences derived from the tethered-ligand can activate the receptor independent of thrombin; the minimum peptide sequence that functions as an agonist is SFLLR-NH_2.[2] The identification of a single receptor responsible for many of the events that are initiated by thrombin has stimulated extensive ligand structure–activity relationship (SAR) studies aimed at generating therapeutically useful antagonists. Recently, a potent antagonist of the PAR-1 receptor, *N-trans*-cinnamoyl-*p*-fluoroPhe-*p*-guanidinoPhe-Leu-Arg-NH_2 (BMS-197525), was discovered.[3] It bound to the receptor with high affinity (K_d = 8 nM) and inhibited SFLLRNP-NH_2-induced platelet aggregation (IC_{50} = ca. 0.20 μM).

In general, to develop a potent receptor antagonist, it is useful to obtain information about the receptor amino acid to define the ligand binding site. This is routinely performed by site-directed mutations of residues that are postulated to influence ligand–receptor interactions. Photoaffinity labeling experiments also provide the opportunity to directly identify receptor residues that contact the ligand.[4] In these studies, a photoactivatable crosslinking moiety is introduced into the ligand and the resulting compound is used to covalently label the receptor. Purification of the photoadduct and subsequent biochemical and chemical processing can reveal the receptor fragment or even individual residues that are in the vicinity of the photoactivatable region of the ligand. The benzophenone photophore (BP) is commonly employed in the preparation of peptide-based photoaffinity labels because of its chemical stability and high crosslinking efficiency with low photoreactivity towards water.[4c] It is also commercially available as a protected amino acid or as a side chain reactive conjugating reagent.[4c,5]

PAR-1 photoaffinity labels based on the native SFLLRN-NH_2 agonist peptide have been prepared in our laboratory (manuscript in preparation) and by other researchers.[6] Although the photoactivatable analogs have

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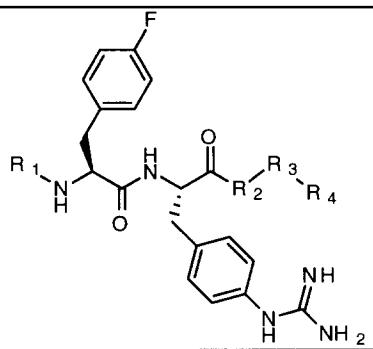
been shown to be biologically active, clear photolabeling of PAR-1 in cellular membranes has yet to be demonstrated. These results are likely to be due to the modest affinity of the native TRAP sequence (ca. 100 nM)[7] and to reduction in affinity arising from the photophore substitutions. To prepare photolabels with higher binding affinity, we developed a series of photoactivatable peptides based on the high affinity PAR-1 antagonist, BMS-197525 (Table 1). One analog was prepared by substituting the BP, *p*-benzoyldihydrocinnamide (BZDC)[5a], into the N-terminal position of the antagonist. Additional peptides were prepared by substituting *p*-benzoylphenylalanine (Bpa)[5b] for the Leu or Arg residue. In Table 1, photoactivatable R groups are indicated by boldface type. The SAR generated during BMS-197525 development indicated that substitutions at the *p*-fluoroPhe or *p*-guanidinoPhe position would significantly reduce potency.[3]

In addition to the crosslinker modifications, some of the peptides were prepared with a C-terminal ornithine to investigate different reporter groups that could be used to experimentally identify the ligand-receptor photoadducts. The ornithine was modified with three amine-reactive reagents: (1) propionate-N-hydroxysuccinimide (NHS) ester, a non-radioactive analog of the commercially available [^{3}H]propionate-NHS ester, (2) biotin-NHS ester, which can be used with sensitive avidin-based detection methods, and (3) 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), a structurally compact fluorophore. The use of these reporter groups can facilitate detection and analysis procedures in photolabeling experiments.[8] Each of the photoactivatable

Table 1. Physical characterization and biological activity of photoactivatable thrombin receptor antagonists.[a]



ID No.	R_1	R_2	R_3	R_4	HPLC[b] (min)	MALDI-MS (expected, Da)	IC_{50} (μM)[c]
1	TC	Leu	Arg	-	27.4	788.0 (787.7)	0.3 ± 0.1[d]
2	-	Leu	Arg	-	18.2	656.5 (655.8)	-
3	**BZDC**	Leu	Arg	-	29.7	891.1 (891.8)	3.1 ± 0.3
4	TC	**Bpa**	Arg	-	30.5	926.8 (926.6)	6 ± 1
5	TC	Leu	**Bpa**	-	34.8	882.3 (883.3)	13 ± 2
6	TC	**Bpa**	Arg	Orn	28.5	1039.5 (1039.0)	-
6P	TC	**Bpa**	Arg	Orn(propionyl)	30.4	1096.6 (1094.2)	3.1 ± 0.3
6B	TC	**Bpa**	Arg	Orn(biotin)	30.0	1265.8 (1269.1)	3.4 ± 0.3
6N	TC	**Bpa**	Arg	Orn(NBD)	32.5	1202.6 (1204.8)	~90
7	TC	Leu	**Bpa**	Orn	32.1	995.5 (995.5)	-
7P	TC	Leu	**Bpa**	Orn(propionyl)	34.5	1052.6 (1051.6)	9 ± 1
7B	TC	Leu	**Bpa**	Orn(biotinyl)	33.7	1221.8 (1226.6)	9.7 ± 0.9
7N	TC	Leu	**Bpa**	Orn(NBD)	37.0	1158.6 (1159.7)	NA@110

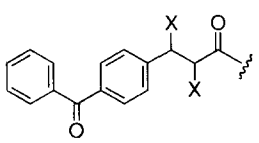
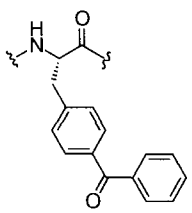
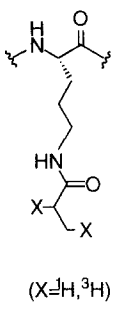
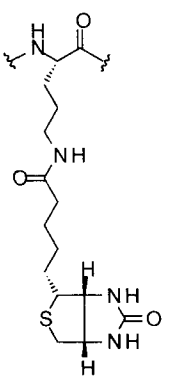
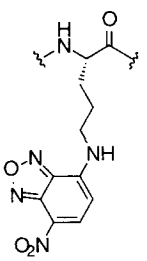
[a]Chemical abbreviations are TC = *trans*-cinnamoyl, BZDC = benzoyldihydrocinnamoyl, Bpa = L-4-benzoylphenylalanine and NBD = 7-nitrobenzo-2-oxa-1,3-diazole. The structures of the benzophenone and C-terminal compounds are shown in Table 2. All peptides are C-terminal carboxamides. [b]Semi-analytical HPLC conditions.[12] [c]Concentration required for 50% inhibition of platelet aggregation induced with SFLLRN-NH_2 (2.7 μM).[13] The values are an average of three separate experiments with a single platelet-rich plasma sample. The order of potency was reproducible in platelets from four separate donors. [d]Reported value is ca. 0.20 μM. [3] NA = not active.

antagonists was evaluated in a platelet aggregation assay to determine the effect of the alterations on biological activity.

Peptide Synthesis. All peptides were synthesized manually using FMOC protection chemistry and a E.I. du Pont RaMPs® multiple peptide synthesis system.[9] Each cartridge contained 200 mg of E.I. du Pont RapidAmide® resin (ca. 0.5 mequiv amine per g of resin) for synthesizing 0.1 mmol of peptide. Deprotection and 1-hydroxybenzotriazole/diisopropylcarbodiimide-based coupling were performed as described by the manufacturer. All coupling cycles utilized 2.5 equiv of FMOC-amino acid except *trans*-cinnamic acid, which was performed with 10 equiv. Synthesis of an FMOC version of *p*-(*N,N'*-bis-Boc-guanidino)Phe was required for peptide construction.[10] After the last coupling cycle, the peptides were deprotected and cleaved from the resin by adding TFA containing 5% thioanisole, 5% H_2O and 7.5% (w/v) crystalline phenol.[11] After 24 h at rt, the resin was removed by filtration through glass wool, and the peptides were isolated as described to give white powdered TFA salts (>95% pure) after HPLC purification.[12] Sequence details, HPLC retention times and MALDI-MS data for all of the peptides are shown in Table 1. The structural details of the crosslinkers are shown in Table 2.

Post-synthetic modifications were used to generate some of the photoaffinity labels.[13] Peptide acylations were performed using NHS esters. Thus, peptide **2** (5 mg), BZDC-NHS (1 equiv) and triethylamine (TEA) (15 equiv) were dissolved in DMF (400 μL) and kept overnight at rt. The product (**3**) was purified on a semi-analytical C-8 column.[12] Acylation of peptides **2**, **6**, and **7** (Table 1) with 1.0 equiv of the respective NHS ester resulted in the desired modification in greater than 80% yield. The addition of more acylating reagent resulted in overacylation, presumably at *p*-guanidinoPhe. The NBD modification of peptides **6** and **7** was performed by first dissolving NBD-Cl (10 mg, 50 μmol) in MeOH (400 μL) containing TEA (1 equiv). The NBD-Cl/TEA solution (2 equiv) was added to peptide **6** or **7** (5 mg) in DMF (200 μL). The reaction vials were vortexed, covered in foil, and kept at rt for 24 h. NBD-Cl did not appear to react with the *p*-guanidinoPhe amino groups under the reported conditions (data not shown); the fluorescent peptides were recovered in 30% chromatographic

Table 2. Structural features of the photoactivatable thrombin receptor antagonists.[a]

Cross-Linker Groups		C-Terminal Reporter Groups		
benzoyldihydrocinnamoyl (BZDC)	L-benzoylphenylalanine (Bpa)	Orn(propionyl)	Orn(biotin)	Orn(NBD)
 (X=H, ^{3}H)		 (X=H, ^{3}H)		

[a]Refer to Table 1 for the location within peptide sequences.

yield. HPLC retention times and MALDI-MS data for the modified peptides are shown in Table 1. The purity of these peptides was greater than 95% except for those derived from peptide **7**. These peptides were approximately 85% pure; the major contaminants were determined to be unreacted peptide and the bis-acylated peptide.

Biological Results and Discussion. The PAR-1 antagonist photoaffinity analogs were tested for their ability to inhibit SFLLRN-NH_2-induced platelet aggregation.[14] The apparent IC_{50} values of the antagonists differed by less than twofold between platelets from different donors, and the order of potency was reproducible in platelets from four donors. The IC_{50} values from one platelet donor are shown in Table 1. BMS-197525 (**1**) was a potent antagonist of SFLLRN-NH_2-induced platelet aggregation with an IC_{50} of 0.3 ± 0.1 μM. This is in agreement with the previously reported value of 0.20 ± 0.07 μM.[3] The BZDC substitution at the N-terminal position (**3**) resulted in a tenfold loss in activity. This result is consistent with the existing SAR data showing that bulky aryl groups at the N-terminal position reduce potency.[3] Bpa substitutions at the Leu or Arg position (**4**, **5**) resulted in a 20- and 60-fold loss in potency, respectively. These effects are similar to those that were observed for the corresponding substitutions in the PAR-1 agonist, SFLLRN-NH_2 (manuscript in preparation). If the relative potencies of tetrapeptide antagonists, **1**, **3**, **4**, and **5**, are considered to be proportional to their binding affinity, it would suggest that they bind with K_d values of 80 nM or less. Substitution was best tolerated at the N-terminal position. This analog is of particular interest because it could be used to identify the receptor residues that interact with the key N-terminal group of the antagonist. It has been suggested that these residues are important for determining the extent of agonist or antagonist activity of a PAR-1 ligand.[3] In addition, a tritiated version of this peptide can be easily prepared with [^{3}H]BZDC-NHS ester.[5a,15] Photolabeling experiments utilizing this label are currently in progress.

C-Terminal modification of the Bpa-containing analogs with ornithine followed by acylation with propionate-NHS ester (**6P**, **7P**) increased antagonist potency 1.4- to 2-fold as shown in Table 1. This is in agreement with SAR studies that showed that C-terminal extension of BMS-197525 (**1**) with Orn(propionyl) results in 2.2-fold increase in antagonist potency[3] and indicates that tritium can be introduced into the Bpa-containing antagonists with [^{3}H]propionate-NHS ester. Peptides **6** and **7** acylated with biotin-NHS (**6B**, **7B**) also demonstrated an increase in antagonist potency (Table 1) despite the size of the biotin group (Table 2). Apparently, the ligand binding site can accommodate the biotin modification without steric interference. This observation suggests a number of other reporter groups (i.e., fluorophores, ^{125}I/tyrosine, photocrosslinkers) could be coupled to the antagonist if they can be tethered by at least a four-carbon linker. The biotin modification is desirable since it can be used with sensitive avidin-based detection techniques. Preliminary photoaffinity labeling studies were attempted with biotinylated analogs, **6B** and **7B**, and a streptavidin-alkaline phosphatase detection method.[8c] We were not been able to identify a competitively labeled protein in whole platelet or CHRF-288 (megakaryoblastic cell type) cells, but new experimental methods are now being explored. The fluorescent NBD modification (**6N**, **7N**) was not well-tolerated at the C-terminus as indicated by the greater than 15-fold loss in activity (Table 1). This suggests the ligand binding site does not tolerate aromatic groups on the C-terminal ornithine side chain. The photoactivatable peptides did not inhibit irreversible induction of platelet aggregation induce by ADP (100 μM), precluding involvement of this alternate platelet aggregation mechanism.

The results described in this report outline the successful synthesis of a series of photoactivatable TR antagonists that contain BP photocrosslinkers and experimentally useful reporter groups. Further studies with

these peptides may elucidate the regions of the TR that are involved in ligand recognition and ultimately aid in the development of therapeutically useful TR antagonists.

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9. Wolfe, H. R.; Wilk, R. R. *Peptide Res.* **1989**, 2, 352. All FMOC-protected amino acids and supporting reagents were obtained from standard laboratory chemical suppliers. L-*p*-fluoroPhe was FMOC protected as described in Lapatsanis, L.; Milias, G.; Froussios, K.; Kolovos, M. *Synthesis* **1983**, 671.

10. *N,N'*-bis-Boc-guanylpyrazole was prepared as previously described (Wu, Y.; Matsueda, G. R.; Bernatowicz, M. *Synth. Commun.* **1993**, *23*, 3055. Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. *J. Org. Chem.* **1992**, *57*, 2497. Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. *Tetrahedron Lett.* **1993**, *34*, 3389) and purified by silica gel chromatography (Drake, B.; Patek, M.; Lebl, M. *Synthesis* **1994**, 579). L-*p*-AminoPhe.HCl (0.74 g, 3.2 mmol) was dissolved in 1 M sodium acetate (15 mL) and *N,N'*-bis-Boc-guanylpyrazole (1.0 g, 3.2 mmol) dissolved in acetone (15 mL) was added to the amino acid mixture. The resulting solution was adjusted to pH 4.5 with concd HCl and stirred overnight at rt. The white precipitate was collected, washed in a 50% ethyl acetate (EtOAc)/10% acetic acid (10 mL) mixture and diethyl ether (Et_2O) before drying under vacuum. Selective aryl amine modification was determined by the 0.3 ppm downfield shift of the aromatic resonances in the ^1H NMR spectra (Fahrenholz, F.;

Thierauch, K.-H. *Int. J. Peptide Protein Res.* **1980**, *15*, 323.). The resulting amino acid (1.15 g, 2.6 mmol) was suspended in 50% aq dioxane (28 mL) containing $NaHCO_3$ (0.65 g, 7.8 mmol). N-(9-fluorenylmethoxy-carbonyloxy)succinimide (FMOC-OSu) (1.31 g, 3.9 mmol) dissolved in dioxane (14 mL) was added to the solution and it was stirred overnight at rt. The dioxane was removed by rotary evaporation and water (30 mL) was added to the solution before it was acidified to pH 2.0 with solid $KHSO_4$. The thick gum precipitate was extracted into EtOAc, and the combined organic layers were washed with water and sat. NaCl, and then dried over $MgSO_4$. The EtOAc was removed and the protected amino acid was chromatography over silica gel using a $CH_2Cl_2/CH_3OH/NH_4OH$ (80/10/1) solvent system (R_f~0.23). The product was triturated in Et_2O/hexane, collected by filtration and dried to a constant weight (1.31 g, 81%): mp 128 °C (dec.); FABMS, *m/z* 643; ^1H NMR (300 MHz, $CDCl_3$) 1.29–1.51 (m, 18 H), 3.09 (br s, 2 H), 4.16–4.52 (m, 4 H), 5.51 (d, 1 H), 7.05–7.74 (m, 12 H), 10.18 (s, 1 H), 11.59 (br s, 1 H). An alternative method for the preparation of FMOC-*p*-(*N,N'*-bis-Boc-guanidino)Phe was described during the development of this procedure (Niu, J.; Lawrence, D. S. *J. Biol. Chem.* **1997**, *272*, 1493.).

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12. Peptides were purified by HPLC (LKB/Bromma 2152 system) on a semi-preparative C-8 column (Vydac, 10 × 250 mm) using 20 to 100% B in 55 min at 2 mL/min or a semi-analytical C-8 column (Zorbax, 4.6 × 250 mm) using 0 to 80% B in 40 min at 1 mL/min. Mobile phase A: 0% CH_3CN/0.06% trifluoroacetic acid (TFA); Mobile phase B: 80% CH_3CN/0.052% TFA. MALDI-MS was performed on a Bruker Protein-TOF® system using the manufacturer's instructions.

13. BZDC-NHS ester was prepared as described in reference 5a. Biotin-NHS ester was obtained from Pierce Chemical Co.(Rockford, IL). Propionate-NHS ester was prepared with the following protocol: Propionic anhydride (4.2 mL, 32.5 mmol), N-hydroxysuccinimide (2.0 g, 17.4 mmol) and conc. HCl (50 µL) were heated at reflux for 15 min. The brown liquid was cooled and extracted with hexane (2 x 40 mL); the phases were separated by low speed centrifugation. The lower layer (ca. 2.5 mL) was dissolved in ethyl acetate (30 mL), washed twice with sat. $NaHCO_3$ and twice with sat. NaCl, and dried over $MgSO_4$. The solvent was removed, and the oil was dissolved in anhydrous Et_2O (40 mL) and placed at -20 °C. The white crystals were collected by filtration, washed with diethyl ether (-20 °C), and dried under vacuum. The total product yield was 0.6 g (20%). mp 43–45 °C (lit. 44–46 °C, Stefanowicz, P.; Siemion, I. Z. *Polish J. Chem.* **1992**, *66*, 111.)

14. Antagonist inhibition of SFLLRN-NH_2-stimulated human platelet aggregation was determined in an end-point turbidity assay similar to the microtiter plate assay previously described (Natarajan, S.; Riexinger, D.; Peluso, M.; Seiler, S. M. *Int. J. Peptide Protein Res.* **1995**, *45*, 145.). Platelet-rich plasma (PRP) was obtained from five-day-old outdated platelet concentrates (University Blood Bank, The University at Stony Brook, Stony Brook, NY). The EC_{50} for SFLLRN-NH_2-induced platelet aggregation was between 1 and 2 µM for all donors tested. Antagonist peptide (1 to 3 µL of stock solutions in DMSO) was added to 450 µL of PRP and rocked for 1 min. Aggregation was induced with SFLLRN-NH_2 (6 µL of stock solution in H_2O, 2.7 µM total concentration) and the platelets were rocked for an additional 3 min. The 600 nm absorbance of the platelet sample was measured and used to determine the extent of irreversible aggregation. The experiments were performed in triplicate with four different donors. The results from peptides **1**, **3**, and **4** were verified at RWJPRI. The apparent IC_{50} values were determined by fitting the data to a dose–response curve with Graph Pad Prism software.

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