Probe Report

Title: Inhibitors of Platelet Integrin αIIbβ3

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Chemistry Center Name & PI: NCGC & Christopher P. Austin

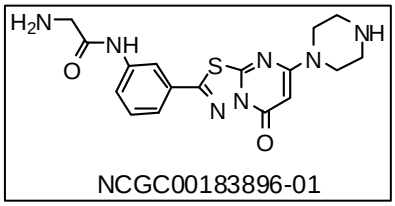
Assay Submitter & Institution: Barry Coller, Rockefeller University

PubChem Summary Bioassay Identifier (AID): 2663

Abstract:

Continued examination of substituted 5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-ones as inhibitors of the platelet αIIbβ3 receptor, resulted in the optimized agent ML165 (CID 44820665, NCGC00183896-01). This agent represents the most potent non-RGC mimetic inhibitor of the αIIbβ3 receptor, and due to its unique biding mechanism, offers a novel tool to study this receptor. Appropriate aqueous solubility and stability was found for this agent.

Probe Structure & Characteristics:



ML165

PubChem CID: 44820665
Internal ID: NCGC00183896-01
IUPAC Name: 2-amino-N-(3-(5-oxo-7-(piperazin-1-yl)-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-2-yl)phenyl)acetamide
Chemical Formula: $C_{17}H_{19}N_7O_2S$
Exact Mass: 385.1321

CID/ML#	Target Name	IC_{50}/EC_{50} (nM) [SID, AID]	Anti-target Name(s)	IC_{50}/EC_{50} (μM) [SID, AID]	Fold Selective*	Secondary Assay(s) Name: IC_{50}/EC_{50} (nM) [SID, AID]
44820665/ ML165	αIIbβ3 receptor	Platelet Adhesion: 1100 [89449681, 2634]	αVβ3 receptor	>100 [89449681, 2628]	>100 fold	Platelet Aggregation: 163 [89449681, 2639]

Recommendations for the scientific use of this probe:

The αIIbβ3 receptor plays a vital role in both hemostasis and thrombosis, with deficiency of the receptor leading to Glanzmann thrombasthenia, and uncontrolled activation of the receptor producing thrombosis and blood vessel occlusion in animal models and humans.[1-3] Current inhibitors of this key integrin receptor include a monoclonal antibody fragment and several RGD peptide mimetics.[4,5] Use of these agents can be problematic, as they engage the β3 subunit MIDAS metal ion and are capable of priming the receptor into an artificial activation conformation. This probe does not bind to the β3 subunit MIDAS metal ion as judged by molecular dynamic simulation, and will be useful for studying selective inhibition of the αIIbβ3 receptor.

1 Introduction

The understanding and control of integrin receptors have furthered our appreciation of numerous biochemical processes and formed the foundations for several clinical successes[1-3]. The αIIbβ3 receptor plays a critical role in hemostasis and thrombosis, and agents that antagonize this platelet-specific receptor are useful antithrombotics[1-3]. The study of αIIbβ3 has lead to an improved understanding of platelet interactions with other platelets and the vasculature. Several agents are capable of inhibiting the αIIbβ3 receptor, including abciximab, a derivative of a monoclonal antibody and an FDA approved agent for adjunctive therapy of coronary interventions to treat diseased blood vessels. While the success of abciximab has proven the principle of anti-αIIbβ3 directed therapeutics, it and the other drugs in the same class (eptifibatide and tirofiban), have several liabilities, including lack of oral bioavailability[4,5]. Several oral αIIbβ3 antagonists have been developed based on mimicry of the RGD peptide motif, but these do not prevent thrombotic events and may even increase their frequency. This paradoxical effect has been ascribed to their ability to induce the activated conformation of the receptor and initiate ligand binding and platelet aggregation[6]. Thus, the development of orally bioavailable small molecules capable of antagonizing αIIbβ3 without inducing an 'activation' conformation of the receptor would be of great value in patient populations at risk of thrombosis-related events. Specifically, a small molecule antagonist of the αIIbβ3 receptor that does not coordinate the β3 subunit MIDAS metal ion is hypothesized to exclusively antagonize the receptor without inducing the 'activation' conformation associated with abciximab, eptifibatide, tirofiban and RGD mimetics.

The Coller laboratory at the Rockefeller University utilized a platelet adhesion assay to identify RUC-1, a novel small molecule with a core thiadiazolo-pyrimidinone heterocycle that inhibited αIIbβ3 with an IC_{50} of approximately 10μM[7]. Importantly, this αIIbβ3 antagonist did not inhibit αVβ3, suggesting that it interacted only with the αIIb. This binding modality is supported by modeling studies and represents a novel mode of inhibition that engages a key aspartate residue (D224) but does not bind to residues of β3 or the MIDAS metal ion. Moreover, RUC-1 *in vitro* and *in vivo* studies demonstrated inhibition of platelet aggregation and anti-thrombotic effects in mice whose platelets solely express human αIIb and murine β3, but not in

mice expressing murine αIIbβ3 or murine αIIb and mouse β3. Finally, unlike the RGD mimetic drugs, RUC-1 does not induce the same conformational changes in the receptor as judged by the binding of β3 conformation-specific monoclonal antibodies. These results firmly establish RUC-1 as a novel lead agent. The rational of this project was to optimize RUC-1 to a more potent agent without engaging the β3 subunit MIDAS metal ion.

A chemical probe for this project is defined as a small molecule compound that inhibits the αIIbβ3 receptor with an IC_{50} of 1μM or less in the platelet aggregation assay. The probe must be active in the primary platelet adhesion assay as well. Probe identity and purity needs to be verified by resynthesis and/or repurification, followed by LC-MS QC.

2 Materials and Methods

2.1 Assays

PubChem AID	Type	Target	Conc. Range	Samples Tested
2634	Confirmatory platelet adhesion (% inhibition)	αIIbβ3	20mM, 100mM	32
2639	Confirmatory platelet aggregation (IC50)	αIIbβ3	50nM – 20μM	32
2628	Selectivity	αVβ3	20mM	2
2663	Summary	αIIbβ3	NA	2

Platelet Adhesion Assay [AID 2634]

Assay details and protocol.

The platelet adhesion assay was conducted by the Coller laboratory (Rockefeller University) by a modification of a published assay[7]. Thirty microliters of human fibrinogen (50 μg/ml) in Tris/saline (100mM NaCl, 50mM Tris/HCl, pH 7.4; American Diagnostica, Stamford, CT) were added to black, clear-bottom, untreated polystyrene, non-sterile 384-well microtiter plates (Corning no. 3711; Acton, MA). After incubating at 22°C for 1 hour, plates were washed 3 times with Tris/Saline, and wells were then blocked with HBMT (138mM NaCl, 12mM $NaHCO_3$, 10mM HEPES, 2.7mM KCl, 0.4mM NaH_2PO_4, 0.1% glucose, 0.35% BSA, pH 7.4) for at least 1 hour. An additional wash was performed using HBMT with 1mM $MgCl_2$ and 2mM $CaCl_2$. Calcein-labeled platelets (final concentration 1 x 10^{11}/L) were treated with compounds (final concentrations of 100μM, 30μM, 10μM or 1μM) at 22°C for 20 minutes. Thirty microliters of platelets were then added to the wells. After 1 hour of adhesion, wells were washed 3 times with HBMT-1mM $MgCl_2$/2mM $CaCl_2$, and the plates were read by a fluorescent microtiter plate reader (Envision;Perkin Elmer) to detect calcein fluorescence (490 nm excitation and 515 nm emission). Positive controls consisted of wells containing platelets without compounds. Negative

controls were wells containing platelets and known inhibitors of αIIbβ3, including mAbs 7E3 and 10E5, and EDTA.

Assay Summary

This project is a reassignment from the pilot phase of the MLPCN and includes the optimization of RUC-1 and the rescreening of the current MLSMR. Rescreening of the primary assay (utilizing a similar protocol) has not been performed yet.

Identification of Lead/Rational Probe Design

RUC-1 was identified during the pilot phase of the MLPCN.

Confirmatory Assay and Activity for NCGC00183896-01/CID44820665/ML165 and selected analogues.

The platelet aggregation assay was conducted by the Coller laboratory by modification of a published assay[7]. Citrated platelet-rich plasma (PRP), generated by the centrifugation of whole blood at 650g for 4 minutes at 22°C, was incubated in aggregometer cuvettes with compounds (final concentrations of 100μM, 30μM, 10μM or 1μM) or controls for 15 minutes at 37°C. After 30 seconds in the aggregometer (Kowa AF-10E; Tokyo, Japan) at 37°C with stirring, ADP (5 – 20μM) was added and the light transmittance was measured for 8 minutes. The initial slope of aggregation was used to generate an IC_{50}.

Anti-target assay(s): αVβ3 CS1 Cell Adhesion assays

An assay was used to determine the selectivity of αIIbβ3 inhibitors by measuring their ability to block the αVβ3 receptor. The assay detects the binding of cells expressing human αVβ3 to vitronectin and was performed by the Coller laboratory as described[7]. Polystyrene 96-well microtiter plates (Nunc) were coated with vitronectin (5 μg/ ml) or fibrinogen (50 μg/ ml) for 1 hour, and blocked with HBMT (138mM NaCl, 12mM $NaHCO_3$, 10mM HEPES, 2.7mM KCl, 0.4mM NaH_2PO_4, 0.1% glucose, 0.35% BSA, 1mM $MgCl_2$, pH 7.4) for at least 1 hour. CS1 cells expressing αVβ3 or HEK 293 cells expressing αIIbβ3 were resuspended in HBMT containing either 1mM MgCl2 or 2mM CaCl2/1mM MgCl2, respectively. Cells were treated with compounds or controls for 15 minutes at 22°C. CS1 cells expressing αVβ3 or HEK 293 cells expressing αIIbβ3 were added to plates coated with vitronectin or fibrinogen, respectively, and

were allowed to adhere for 1 hour at 37°C. Adherent cells were quantified by their endogenous acid phosphatase activity on p-nitrophenyl phosphate as described in Law *et al*., 1999.

2.2 Probe Chemical Characterization

The synthesis of RUC-1 was restrictive, which limited the study of SAR to selected alterations. However, this synthesis allowed several critical modifications, including alteration at the piperizine ring, the ethyl moiety and the 6-position of the 5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-one ring system. Additionally, molecular modeling suggested a hydrogen-bond between the piperizine ring and the key Asp residue D224 of αIIb was a critical interaction for binding (Figure 1). Further, the ethyl moiety appeared to be solvent exposed and did not make key contacts with the β3 MIDAS metal ion.

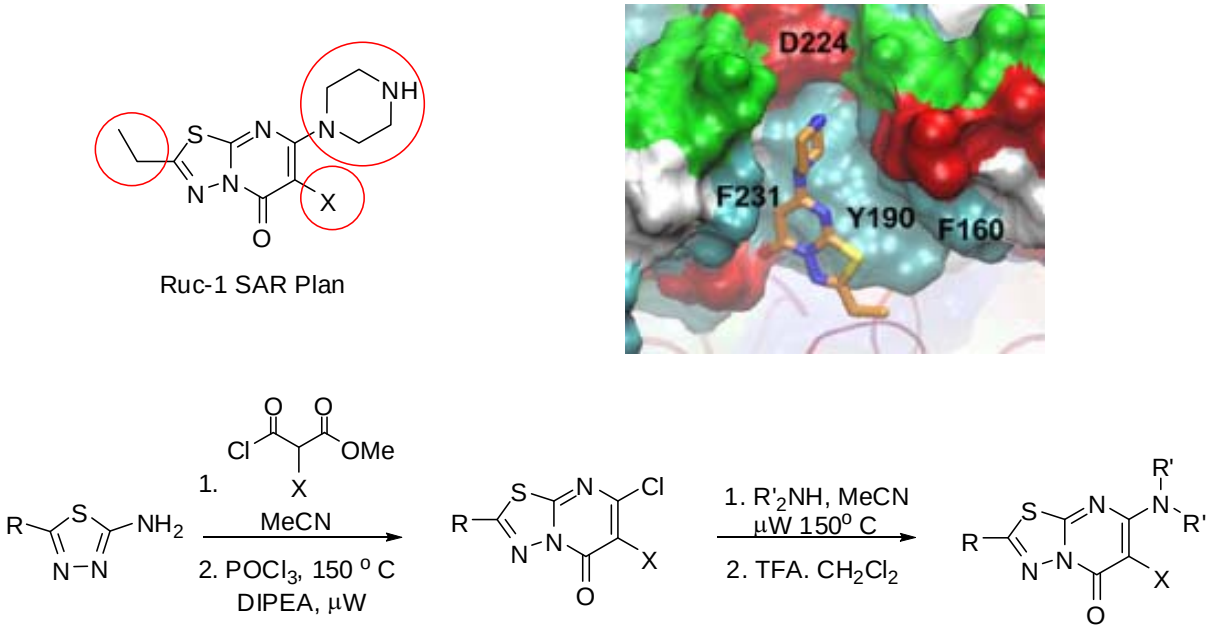


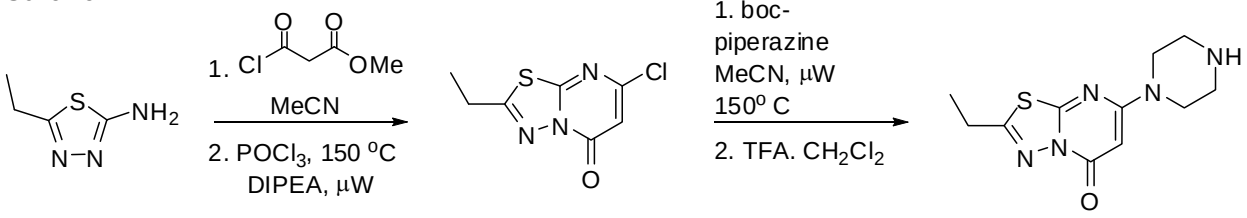
Figure 1. SAR plan for RUC-1 optimization, molecular docking of the RUC-1 interaction with αIIb and the synthetic plan for selected analogues.

2.3 Probe Preparation

The synthesis of RUC-1 involves a cyclization of 5-ethyl-1,3,4-thiadiazol-2-amine (**1**) with methyl 3-chloro-3-oxopropanoate and treatment with $POCl_3$ to yield 7-chloro-2-ethyl-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-one (**2**)(Scheme 1). This intermediate is then reacted with

mono-Boc protected piperazine and TFA-mediated Boc deprotection to complete the synthesis of 2-ethyl-7-(piperazin-1-yl)-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-one (Ruc-1, **3**).

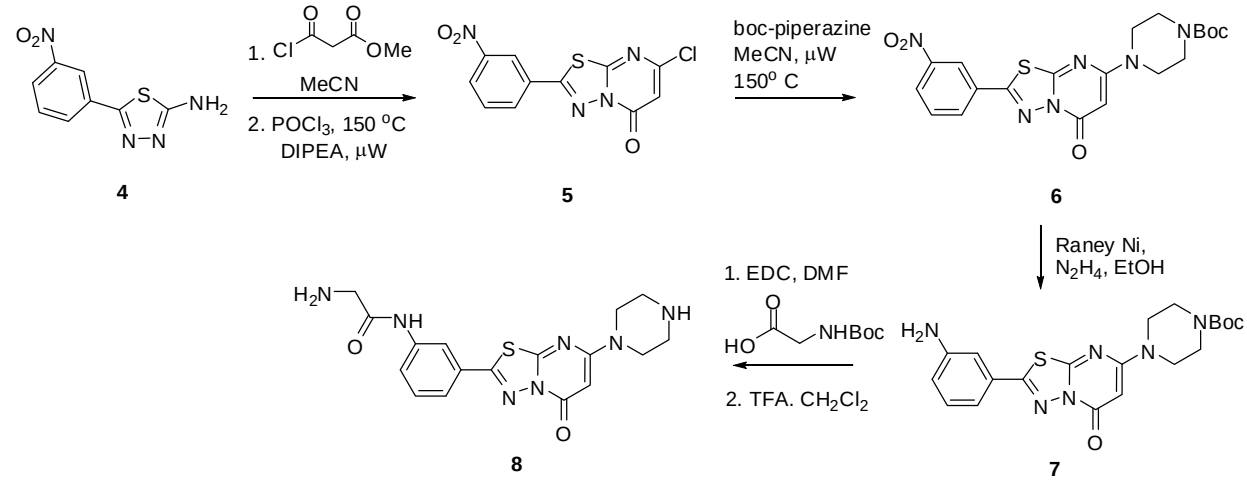
Scheme 1



1 **2** **3**

The synthesis of the probe compound NCGC00183896-01/CID 44820665/ML165 was accomplished utilizing a similar strategy (Scheme 2). 5-(3-nitrophenyl)-1,3,4-thiadiazol-2-amine (**4**) and methyl 3-chloro-3-oxopropanoate provided the required heterocyclic intermediate that, upon treatment with $POCl_3$, provided 7-chloro-2-(3-nitrophenyl)-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-one (**5**). Addition of Boc-protected piperizine gave tert-butyl 4-(2-(3-nitrophenyl)-5-oxo-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-7-yl)piperazine-1-carboxylate (**6**) which was directly reduced to tert-butyl 4-(2-(3-aminophenyl)-5-oxo-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-7-yl)piperazine-1-carboxylate (**7**) with Raney-Ni/hydrazine. Coupling of Boc-protected glycine via EDC and global Boc deprotection provided 2-amino-N-(3-(5-oxo-7-(piperazin-1-yl)-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-2-yl)phenyl)acetamide (**8**, NCGC00183896-01).

Scheme 2

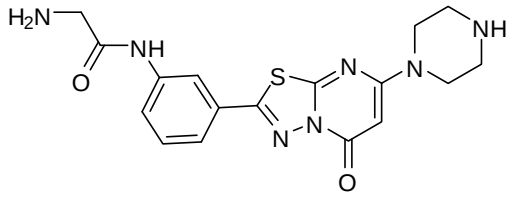


4 **5** **6** **8** **7**

3 Results

Chemical name of probe compound: 2-amino-N-(3-(5-oxo-7-(piperazin-1-yl)-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-2-yl)phenyl)acetamide

Probe chemical structure:



NCGC00183896-01
CID 44820665
ML165

Structural Verification Information of probe SID: 2-amino-N-(3-(5-oxo-7-(piperazin-1-yl)-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-2-yl)phenyl)acetamide (NCGC00183896-01) ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.31 (s, 1 H), 7.86 (d, *J*=7.43 Hz, 1 H), 7.48 - 7.58 (m, 2 H), 5.38 (s, 1 H), 3.46 (br. s., 4 H), 3.29 (s, 2 H), 2.67 - 2.76 (m, 4 H); LCMS: (electrospray +ve), *m/z* 386.1 $(MH)^+$; HPLC: t_R = 2.70 min,UV_{254} = 100%. HRMS (ESI): *m/z* calcd for $C_{17}H_{19}N_7O_2S$ $[M+H]^+$ 386.1394, found 386.1393.

The PubChem CID (SID) is 44820665 (89449681). The ML number is ML165. This probe is not commercially available.

MLS ID	NCGC ID	Type	ML
MLS002729049	NCGC00183896-04	Probe	165
MLS002729050	NCGC00183452-01	Analog	
MLS002729051	NCGC00183328-01	Analog	
MLS002729052	NCGC00183902-01	Analog	
MLS002729053	NCGC00183330-01	Analog	
MLS002729054	NCGC00184858-01	Analog	

Compound is soluble at ~10mM in water or DMSO. The compound is not fluorescent with blue excitation wavelengths (~340 nm).

Summary of known probe properties:

Calculated Property	Probe Identity
	CID_44820665 (MLS002729049)
Molecular Weight [g/mol]	385.44346
Molecular Formula	C17H19N7O2S
XLogP3-AA	-0.2
H-Bond Donor	3
H-Bond Acceptor	7
Rotatable Bond Count	4
Tautomer Count	2
Exact Mass	385.132094
MonoIsotopic Mass	385.132094
Topological Polar Surface Area	141
Heavy Atom Count	27
Formal Charge	0
Isotope Atom Count	0
Defined Atom StereoCenter Count	0
Undefined Atom StereoCenter Count	0
Defined Bond StereoCenter Count	0
Undefined Bond StereoCenter Count	0
Covalently-Bonded Unit Count	1
Complexity	714

IUPAC Name: 2-amino-N-(3-(5-oxo-7-(piperazin-1-yl)-5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-2-yl)phenyl)acetamide

Canonical SMILES:
O=C1N2C(SC(C3=CC(NC(CN)=O)=CC=C3)=N2)=NC(N4CCNCC4)=C1

3.1 Summary of Screening Results

To explore SAR, a number of analogues were synthesized and tested with the platelet adhesion and aggregation assays. Selected results are shown in Table 1 and Table 2 in Section 3.4. Alterations to the piperizine ring were made, including ring expansion and contraction, replacement with piperidine and morpholine analogues, and a variety of additions to ring carbons. All of these alterations were detrimental to the activity of this chemotype. Specific examples include 4-pryimidine (**9**) (SID_89449675; CID_44820661), 1-methylpiperazine (**10**) (SID_89449673; CID_44820641), 2,6-dimethylpiperazine (**11**) (SID_89449674; CID_44820659), and azetidin-3-amine (**12**) (SID_89449672; CID_44820646). Altering the 6-position of the 5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-one ring from hydrogen was tolerated as long as the moiety was not overly large. For instance, the hydrogen isostere fluorine (**13**) (SID_89449676; CID_44820670) was acceptable, but the relatively larger methyl group (**14**) (SID_89449677; CID_44820648) was detrimental to activity.

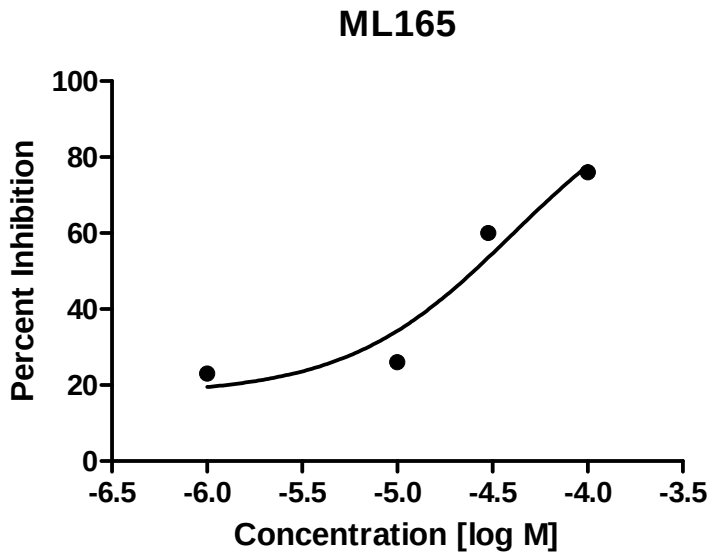
Investigation of ethyl analogues of RUC-1 (the 2-position of the 5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-one ring system) identified modifications that increased potency. Simple analogues with alkyl replacements (**15** and **16**) (SID 89449668; CID_44820655 and SID 89449669; CID_44820657, respectively) and inclusion of heteroatoms (**17**) (SID_89449671; CID_44820642) were ineffectual (Table 2). Several aromatic groups were also added to the 2-position of the 5H-[1,3,4]thiadiazolo[3,2-a]pyrimidin-5-one ring, and it was quickly determined that substitutions at the meta position of phenyl ring analogues was favored. Further, amine derivatives of varying bond lengths from an amide attachment at the meta position increased potency in both the platelet adhesion assay and the platelet aggregation assay. The most effective analogue maintained a 3-(2-aminoacetamide) moiety on the phenyl ring group (**18**,

NCGC00183896-01, SID_89449681; CID_44820665) which displayed an IC_{50} value of 1.1μM in the platelet adhesion assay and 0.16μM in the platelet aggregation assay. Expansions of this analogue included addition of aniline derivatives that maintained chirality (**19** and **20**) (SID 89449680; CID_44820654 and SID 89449679; CID_44820640, respectively), addition of an N-terminal methyl group (**21**) (SID_89449678; CID_44820667), and inclusion of ring systems (**22**) (SID_89449682; CID_44820650). None of these analogues possessed activity exceeding NCGC00183896-01 (**18**) (SID_89449681; CID_44820665).

NCGC00183896-01 (**18**) (SID_89449681; CID_44820665, ML165) represents the current best in class small molecule for inhibition of the αIIbβ3 receptor. The potent mAB's and RGD peptide mimetics that target this integrin receptor possess several liabilities, including limited use as molecular tools due to adverse physiochemical properties, and the propensity for priming the receptor into an artificial activation conformation. Based upon modeling studies, NCGC00183896-01 (**18**) (SID_89449681; CID_44820665, ML165) does not bind to the MIDAS metal ion, and should not possess this liability.

Advanced studies are in progress and include the examination of fibrinogen binding to purified αIIbβ3, displacement of fluorescent RGD peptides, X-ray crystallization and *in vivo* studies of NCGC00183896-01 (**18**) (SID_89449681; CID_44820665, ML165) in antithrombotic animal models.

3.2 Dose Response Curves for Probe

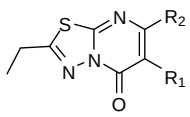


3.3 Scaffold/Moiety Chemical Liabilities

NCGC00183896-01 (MLI165) has two basic nitrogens, each of which will likely exist as cationic functional groups, limiting their membrane permeability. However, as the goal of this probe is to study the receptor, and not *in vivo* use, this liability does not hinder the overall utility of the probe.

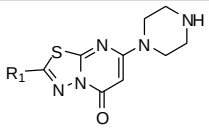
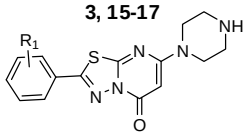
3.4 SAR Tables

Table 1. SAR of selected 5*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidin-5-ones versus the αIIbβ3 receptor

	#	SID	CID	R_1	R_2	*P.Ad.A.*[a] *% inhibition*[d]	*P.Ad.A.*[a] IC_{50}[d]	*P.Ag.A.*[a] IC_{50}[d]
 **3, 9-14**	**3** (Ruc-1)	89449667	756604	H	piperazine	35%[b]	> 20 μM	8.4 μM
	9	89449675	44820661	H	4-pyridine	25%[b]	ND	ND
	10	89449673	44820641	H	1-methylpiperazine	23%[b]	ND	> 20 μM
	11	89449674	44820659	H	2,6-dimethylpiperazine	27%[b]	ND	> 20 μM
	12	89449672	44820646	H	azetidin-3-amine	23%[b]	ND	ND
	13	89449676	44820670	F	piperazine	41%[c]	ND	8.3 μM
	14	89449677	44820648	Me	piperazine	55%[c]	ND	> 20 μM

[a] P.Ad.A. = platelet adhesion assay. P.Ag.A. = platelet aggregation assay;[b] % inhibition at 30 μM; [c] % inhibition at 100 μM [d] % inhibition and IC_{50} values were determined utilizing modifications of the platelet adhesion and aggregation assays described in Blue et al, Blood 2008, 111, 1248. ND = not determined.

Table 2. SAR of selected 5*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidin-5-ones versus the αIIbβ3 receptor

	#	SID	CID	R_2	*P.Ad.A.*[a] *% inhibition*[d]	*P.Ad.A.*[a] IC_{50}[d]	*P.Ag.A.*[a] IC_{50}[d]
 **3, 15-17**	**3** (Ruc-1)	89449667	756604	ethyl	35%[b]	> 20 μM	8.4 μM
	15	89449668	44820655	methyl	20%[b]	ND	ND
	16	89449669	44820657	tert-butyl	21%[b]	ND	ND
	17	89449671	44820642	2-(methoxymethyl)	18%[b]	ND	ND
 **18-22**	**18** (NCGC00183896-01)	89449681	44820665	3-(2-aminoacetamide)	92%[b]	1.1 μM	0.163 μM
	19	89449680	44820654	3-((S)-2-aminopropanamide)	35%[c]	8.2 μM	0.916 μM
	20	89449679	44820640	3-((R)-2-aminopropanamide)	69%[c]	> 20 μM	5.9 μM
	21	89449678	44820667	3-(2-(methylamino)acetamide)	24%[c]	ND	ND
	22	89449682	44820650	3-(piperidine-4-carboxamide)	64%[c]	> 20 μM	8.6 μM

[a] P.Ad.A. = platelet adhesion assay. P.Ag.A. = platelet aggregation assay;[b] % inhibition at 30 μM; [c] % inhibition at 100 μM [d] % inhibition and IC_{50} values were determined utilizing modifications of the platelet adhesion and aggregation assays described in Blue et al, Blood 2008, 111, 1248. ND = not determined.

3.5 Cellular Activity

All activity (adhesion and aggregation assays) listed above represent cellular assays.

3.6 Profiling Assays

This agent has been profiled versus related receptors, including murine αIIbβ3 or murine αIIb and mouse β3, and was found to be inactive.

4 Discussion

The Coller group previously demonstrated that a small molecule (RUC-1) could effectively inhibit ligand binding, platelet aggregation, and *in vivo* thrombus formation mediated by human αIIbβ3. This analysis included docking studies and crystallographic structural data, which defined the binding orientation of this agent as being solely within the αIIb domain. This is significant, as agents that bind at the β3 domain can induce an activated confirmation of the receptor. In the present study, we have built on these data by synthesizing and then analyzing the binding of NCGC00183896-01 (MLI165, RUC-2), a RUC-1 derivative that is more than one hundred-fold more potent in inhibiting platelet aggregation. NCGC00183896-01 is selective for αIIbβ3 compared to αVβ3 and does not induce the β3 LIBS epitope. In contrast to tirofiban and eptifibatide, neither RUC-1 nor NCGC00183896-01 induced recruitment of IgG in 10 of 12 patient cell lines with eptifibatide-dependent thrombocytopenia. Importantly, studies into the structural basis for the binding of NCGC00183896-01 to human αIIbβ3 have revealed (report pending) a novel mechanism of action.

4.1 Comparison to existing art and how the new probe is an improvement.

NCGC00183896-01 (CID_44820665, ML165) is more potent than RUC-1 and possesses a unique binding mode to human αIIbβ3.

4.2 Mechanism of Action Studies.

Orthosteric inhibitor.

4.3 Planned Future Studies.

X-ray crystallography is presently being explored, and advanced studies of receptor confirmation are also planned.

5 References

(1) Ginsberg, M. H.; Partridge, A.; Shattil, S. J. Integrin regulation. *Curr. Opin. Cell Biol.* **2005**, *17*, 509.

(2) Shattil, S. J.; Newman, P. J. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood* **2004**, *101*, 1606.

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