



## Identification of novel agonists of the integrin CD11b/CD18

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### ABSTRACT

We report the identification of novel small molecule agonists of integrin CD11b/CD18, which increased, in a dose-dependent manner, the adhesion of the integrin CD11b/CD18 expressing cells to two physiologically relevant ligands: Fibrinogen and iC3b. Compound **6** showed an ex vivo EC<sub>50</sub> of 10.5 μM and in vitro selectivity for binding to the recombinant αA-domain of CD11b/CD18. In silico docking experiments suggest that the compounds recognized a hydrophobic cleft in the ligand-binding αA-domain, implying an allosteric mechanism of modulation of integrin affinity by this novel compound.

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The α/β integrin heterodimers mediate important cellular functions including cell adhesion, migration and signaling.<sup>1</sup> β2 integrins, with a common β-subunit (β2, CD18) but distinct α-subunits (CD11a, CD11b, CD11c and CD11d), are critical leukocytic receptors that are important in inflammation and immunity.<sup>2</sup> The integrin CD11b/CD18 (also known as Mac-1, CR3 and αMβ2) is the predominant β2 integrin in neutrophils, macrophages and monocytes and mediates pro-inflammatory functions in these cells.<sup>3–5</sup> CD11b/CD18 recognizes the complement fragment iC3b, Fibrinogen, and ICAM-1 as ligands, among various others. CD11b/CD18 has been implicated in many inflammatory and autoimmune diseases, such as ischemia-reperfusion injury (including acute renal failure and atherosclerosis), tissue damage, stroke, neointimal thickening in response to vascular injury and the resolution of inflammatory processes.<sup>2,6–9</sup> Thus, there is a considerable potential for agents that modulate the function of CD11b/CD18 as therapeutic agents for the treatment of such inflammatory conditions.

Progress towards identifying small molecules that selectively target integrin CD11b/CD18 has been slow, with only a few

reported discoveries,<sup>10,11</sup> primarily due to the lack of good high-throughput screening (HTS) assays. We recently described a simple, no-wash cell-adhesion based HTS assay in the 384-well plate format that is ideal for the discovery of small molecules against the integrin CD11b/CD18.<sup>12</sup> Our cell-based assay does not require expensive reagents, is very quick, and provides a quantitative and consistent readout. In this preliminary report, we describe the discovery of several novel small molecule agonists of integrin CD11b/CD18 (Fig. 1).

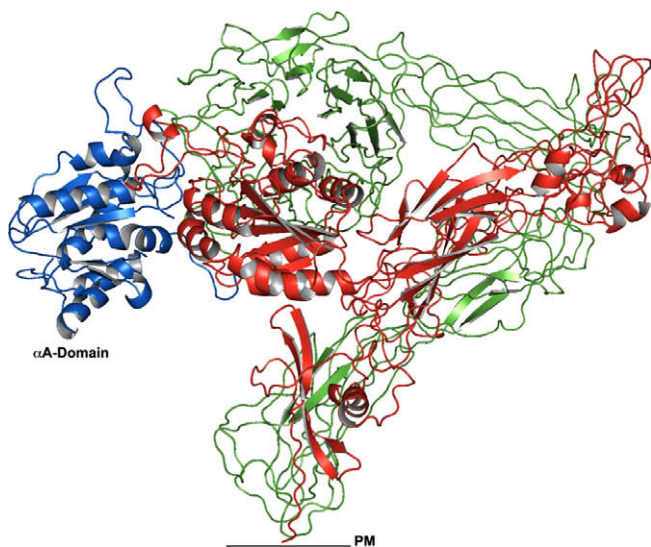
We screened a commercially available library of >13,500 small molecules to identify novel agonists of integrin CD11b/CD18 using our previously described assay,<sup>12</sup> which relies on the ability of small molecules to increase adhesion of mammalian K562 cells stably transfected with the wild type integrin CD11b/CD18 (K562 CD11b/CD18) to Fibrinogen, a physiologic ligand of integrin CD11b/CD18. K562 CD11b/CD18 cells showed virtually no binding to immobilized Fibrinogen (Fg) when incubated in the assay buffer (1 mM each of physiologic ions Ca<sup>2+</sup> and Mg<sup>2+</sup> in Tris buffered saline (TBS<sup>++</sup>)) alone. However, 208 compounds selectively increased adhesion of K562 CD11b/CD18 cells and were identified as hits from this primary screen (hit-rate of ~1.5%).

Next, we ‘cherry-picked’ the top 87 compounds that produced the highest level of cell-adhesion for validation using a secondary assay—small molecule induced binding of K562 CD11b/CD18 cells

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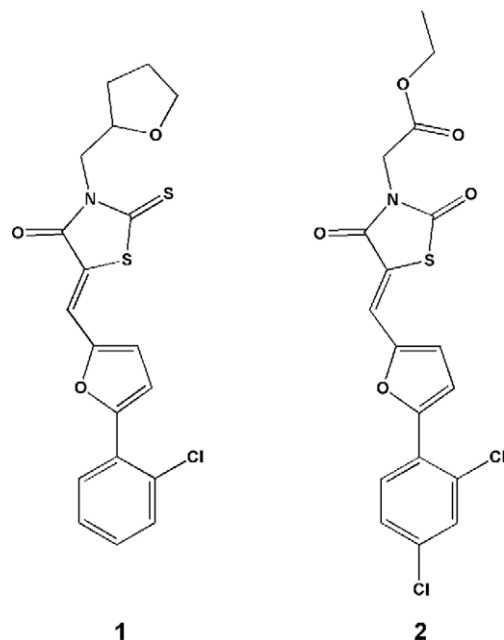
**Figure 1.** A ribbon diagram of a model of integrin CD11b/CD18 based on the crystal structure of  $\alpha V\beta 3$  ectodomain.<sup>13</sup> The  $\alpha$ -chain (CD11b) is shown in green, with the ligand binding  $\alpha A$ -domain in blue. The  $\beta$ -chain (CD18) is shown in red. PM = Plasma Membrane.

to two different integrin CD11b/CD18 ligands (Fg and iC3b). 46 compounds showed increased K562 CD11b/CD18 cell adhesion in the secondary assays in a dose dependent fashion and thus were confirmed as agonist hits (hit confirmation rate of ~53%). Majority of the agonists showed the  $EC_{50}$  (effective concentration for 50% increase in cell adhesion) values in the 10–30  $\mu M$  range.

Surprisingly, we found that a large subset of the hits contained a central five-membered 2,4-di-oxo-thiazolidine motif.<sup>12</sup> A subsequent primary screen with a chemical library of >92,000 compounds also identified a number of similar 2,4-di-oxo-thiazolidine motif containing compounds as hits.<sup>14</sup> Furthermore, we found that this family of hit compounds are structurally similar to compounds recently reported as CD11b/CD18 agonists in an independent screen using purified CD11b A-domain ( $\alpha A$ -domain),<sup>11</sup> helping us quickly narrow down the target binding site for this class of molecules to the CD11b A-domain. We confirmed targeting of the  $\alpha A$ -domain by the 2,4-di-oxo-thiazolidine motif containing compounds using binding assays with purified recombinant  $\alpha A$ -domain, where these compounds increased binding of  $\alpha A$ -domain to immobilized Fg.<sup>15</sup> Additionally, binding was selective as cells not expressing CD11b/CD18 did not show any appreciable binding and the binding of CD11b/CD18 expressing cells could be blocked with known blocking monoclonal antibodies (mAbs) 44a<sup>3</sup> (anti-CD11b) and IB4<sup>16,17</sup> (anti-CD18).

Next, we determined the selectivity of compounds for integrin CD11b/CD18 over highly homologous integrin CD11a/CD18 (also known as LFA-1). Since both compounds **1** and **2** (Fig. 2) produced high levels of K562 CD11b/CD18 cell-adhesion in secondary assays, we obtained pure **1** and **2** in the powder form from commercial vendors for these assays. We also generated K562 cells stably transfected with wild type integrin CD11a/CD18 (K562 CD11a/CD18). Next, we measured the ability of **1** and **2** to increase cell adhesion to immobilized ICAM-1, a physiologic ligand of integrin CD11a/CD18. Although **1** was not highly soluble in aqueous buffers, **2** showed twofold higher selectivity for integrin CD11b/CD18 over CD11a/CD18, with  $EC_{50}$  values of  $13.6 \pm 5 \mu M$  with K562 CD11b/CD18 cells. This is in contrast with the previously described compounds,<sup>11</sup> which showed equal binding to both integrins in our assays.

In order to determine the exact binding mode of **2** in the  $\alpha A$ -domain, we explored the structure–activity relationship (SAR) of



**Figure 2.** Chemical structure of two identified hits containing a central 5-(furan-2-ylmethylene)-2,4-di-oxo-thiazolidine motif from the primary screen for agonists of integrin CD11b/CD18.

various substitutions on the core 5-(furan-2-ylmethylene)-2,4-di-oxo-thiazolidine motif that was common between compounds **1**, **2** and a number of other primary hits. We obtained a limited series of compounds with various  $R_1$  and  $R_2$  substitutions (Table 1) from commercial sources and determined their effect on K562 CD11b/CD18 cell adhesion to its natural ligand Fg. The  $EC_{50}$  values of these derivatives are listed in Table 1.

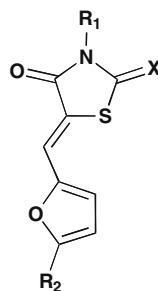
In general, substitutions at the C-5 position of the furan ring ( $R_2$  substituents) had the largest effect on agonist potency. Non-aromatic (**3–4**) or non-conjugated (**5**) substituents that disrupted the  $\pi$ -conjugation with the planar furanyl ring were strongly dis-favored. Planar aromatic rings were preferred and non-substituted phenyl ring (**6**) was most preferred over aliphatic or polar heteroatoms at *ortho* or the *para* positions of the phenyl ring (**7–11**). For the  $R_1$  substituents at the N-3 position of the thiazolidine ring, shortening the length of the substituted ester (from ethyl to methyl) (**12–13**), and shortening the aliphatic chain length (**14–15**) was highly dis-favored. Similarly, substitution of the aliphatic chain with a phenyl ring was dis-favored (**16**). Long-chain, bulky residues were also dis-favored at  $R_1$  (**17–20**). However, compound **21** containing methylene substituted small aromatic ring bound to a level similar to **6**. Conversely, a co-substitution of benzyl at  $R_1$  with a highly electron-withdrawing and bulky *para*-substituted aromatic at  $R_2$  was highly dis-favored (**22**).

Compound **6** also showed selective binding to the purified recombinant  $\alpha A$ -domain by increasing its binding to immobilized Fg<sup>15</sup> as well as a high selectivity for integrin CD11b/CD18 ( $EC_{50} = 10.5 \pm 5 \mu M$ ) over CD11a/CD18 (no appreciable binding). Thus, we believe that we have discovered a novel and unique integrin CD11b/CD18 selective agonist.

To further evaluate the compounds we also calculated various physicochemical descriptors using Schrodinger QikProp program. Our best compounds (**1**, **2**, **6**, **7**, **8**) have good predicted Caco-2 cell permeability and human oral absorption. Among them **6** has a slightly better *clog P* and better predicted solubility. Because it is more active than **2** and smaller, it also has the highest ligand efficiency ( $BEI = 14$ ).<sup>18</sup>

**Table 1**

Results of the cell adhesion assay with a series of compounds containing the central 5-(furan-2ylmethylene)-2,4-di-oxo-thiazolidine motif



	R <sub>1</sub>	R <sub>2</sub>	EC <sub>50</sub>
2			13.6 ± 5
3			NB
4			23.2 ± 20
5			NB
6			10.5 ± 5
7			40.4 ± 5
8			18.6 ± 7
9			>50
10			40.9 ± 9
11			NB

**Table 1 (continued)**

	R <sub>1</sub>	R <sub>2</sub>	EC <sub>50</sub>
12			NB
13			NB
14			NB
15			NB
16			>50
17			NB
18			35.6 ± 22
19			>50
20			22.3 ± 14
21			18 ± 6
22			NB

EC<sub>50</sub> values (μM) are shown. X = O, except for **21**, where it is S. NB = no increase in binding of K562 CD11b/CD18 cells to ligand Fg.

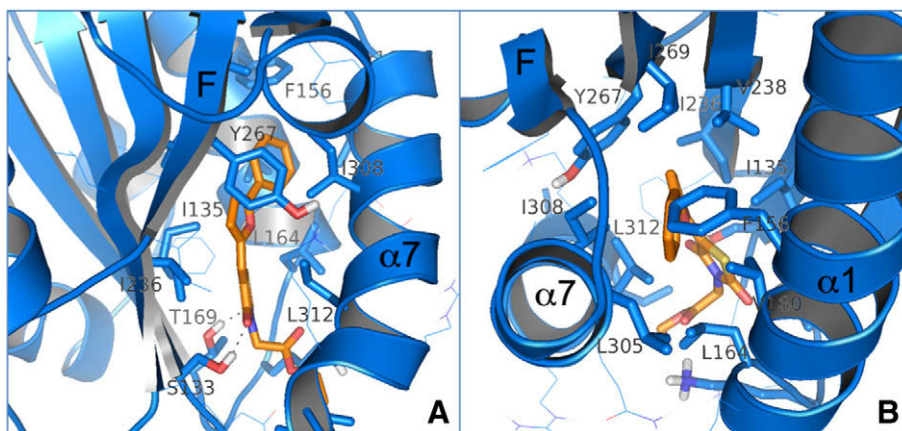
Next, in order to gain insights into potential binding pockets for these small molecules in the  $\alpha$ A-domain, we conducted in-silico docking experiments. The high-resolution three-dimensional structure of CD11b A-domain in both its closed (inactive) and open (active, ligand-competent) conformations is available from PDB.<sup>19–21</sup> However, the  $\alpha$ 7 helix in  $\alpha$ A (that creates part of a hydrophobic pocket known as Socket for Isoleucine (SILEN) in CD11b<sup>21</sup> or IDAS in CD11a<sup>22</sup> and that shows the highest conformational change upon  $\alpha$ A-activation<sup>19,23,24</sup>) is shorter by three residues in the three-dimensional structures of the open form<sup>20,21</sup> as compared to that of the closed form of  $\alpha$ A.<sup>19,20</sup> As the newly discovered agonists are predicted to bind in this region and stabilize this conformation of  $\alpha$ A,<sup>11</sup> we constructed a model of the open (active, ligand-competent) conformation of the CD11b A-domain by manually extending the  $\alpha$ 7 helix in the high resolution structure of CD11b A-domain<sup>20,21</sup> by three additional residues from the structure of the closed form followed by hydrogen bond optimization and constrained (Impref) minimization as implemented in the Maestro protein preparation facility (Schrodinger Inc, Portland).

Conformational repositioning of the  $\alpha$ 7 helix upon activation, which appears to be stabilized upon agonist binding suggests that the agonists bind in the region between helix  $\alpha$ 7 and  $\alpha$ 1 and the central  $\beta$  sheet.<sup>11,19</sup> This has also been suggested by a previous report.<sup>11</sup> Therefore we utilized the above optimized structure of the  $\alpha$ A-domain in the open conformation to initiate compound docking. In the apo structure this activation sensitive  $\alpha$ 7 helix region is spatially crowded by many hydrophobic residues lining the pocket. We applied an induced fit docking procedure implemented in the Schrodinger software suite in which initial docking with a softened potential to generate an ensemble of possible poses is followed by receptor optimization and ligand re-docking.<sup>25</sup> This protocol resulted in a high scoring pose of **2** (Z configuration) in which the carbonyl oxygens of the 2,4-di-oxo-thiazolidine core are fixed by Ser133 and Thr169 and the hydrophobic 2,4-dichlorophenyl moiety is interacting in the hydrophobic pocket. In a stable 6 ns all-atom explicit solvent molecular dynamics simulation at increased temperature (using Desmond by DEShaw Research)<sup>26</sup> the  $\alpha$ 7 helix adjusts only very slightly. The induced-fit docking receptor was used to dock additional structures using Schrodinger Glide Program.<sup>27</sup> The obtained poses were then rescored using MM-GB/SA methodology<sup>28</sup> allowing receptor flexibility to obtain more accurate estimates of relative binding free energies. The resulting binding hypothesis of the best compound **6** is shown in Figure 3. As we expected, the hydrophobic phenyl furanyl moiety (the

C5-substituent on the thiazolidine ring) is burried in a hydrophobic pocket lined by residues L312, I308, L305 ( $\alpha$ 7 helix), L164, V160, F156 ( $\alpha$ 1 helix), and Y267, I269, I236, V238, I236, I135 (central beta sheet). The relative free binding energy of **6** is slightly lower compared to **2** in agreement with the experimental data. This structural model also explains why compound **13** (highly similar to **2** and **6**) is inactive, as in this binding mode the  $\alpha$ C carbon of the ethylcarboxylate moiety at N-3 position of the central thiazolidine ring of **2** is in close proximity to Ser133, Thr169, and Asp132 (less than 2.5 Å), which creates a tight fit and does not tolerate the larger methyl group at  $\alpha$ C that is present in **13** but is absent in **2** and **6**.

For the most comparable compounds **2**, **6**, **7** and **8**, the lower activity of the sterically more demanding compound can (at least partially) be attributed to increased receptor and/or ligand strain. The SAR and the binding hypothesis suggest that one hydrophobic interaction is critical. Compounds with two polar ends are inactive (**9**, **10**, **11**, **12**). The interaction in the hydrophobic pocket appears quite sensitive to sterical demand and the overall size of the molecule. For example, in case of the smaller ethyl acetate N-3 substituent, larger (**2**, **7**, **8**) as well as smaller (**6**) phenyl furanyl substituents are tolerated (although the smallest is the most active) while for structures with larger N-3 substituents (such as **17** and **20**) only the unsubstituted phenyl furanyl is active. Thus, the in silico docking studies suggest a reasonable hypothesis for the binding of these novel allosteric agonists of integrin CD11b/CD18. Additionally, our proposed model is consistent with a previous modeling study with other CD11b A-domain agonists.<sup>11</sup> However, as can be expected from an in silico model, the model presented here can not in all cases quantitatively explain the subtle differences in activity and it should be considered a hypothesis that will be further evaluated by extensive molecular dynamics and SAR studies in the future. During the various induced fit docking studies we obtained other (although lower scoring) poses. For example, one model showed the compound **6** 'flipped' along its long, vertical axis, as shown in Figure 3B. However in all cases the hydrophobic moiety interacts in the same region described and illustrated in Figure 3. Future structural studies will determine the validity of this model as well as the reasons for selectivity of agonists **2** and **6** for integrin CD11b/CD18 over highly homologous integrin CD11a/CD18.

To summarize, we have identified a series of novel substituted 5-(furan-2ylmethylene)-2,4-di-oxo-thia-zolidine motif containing agonists of integrin CD11b/CD18. Several molecules within this class showed good binding to the ligand binding  $\alpha$ A-domain of



**Figure 3.** Two views of a structural model showing proposed binding mode of the novel agonist **6** in the zoomed-in activation-sensitive  $\alpha$ 7 helix ( $\alpha$ -helix on the right in **A**) region of the CD11b A-domain (blue ribbon). **B** shows a 90° rotated view of the same pocket as shown in **A**. Compound **6** is shown as orange stick model. Interacting residues from the activation-sensitive hydrophobic region are also shown as blue sticks and are labeled.  $\alpha$ 7 helix,  $\alpha$ 1 helix and the F-strand are also labeled. Dashed lines highlight potential key interactions between **6** and  $\alpha$ A.



CD11b/CD18 and are currently subject to further evaluation. These compounds may serve as mechanistic chemical biology probes for modulating  $\beta 2$  integrin function in vivo. The proposed structural model suggests that the novel agonists increase integrin CD11b/CD18 dependent cell adhesion by binding  $\alpha A$  in the activation-sensitive F- $\alpha 7$  region and allosterically stabilizing it in the open conformation.  $\beta 2$  integrin agonists can modulate recruitment of leukocytes and lymphocytes to the sites of inflammation via a novel mechanism—by promoting cell adhesion and delaying cell de-adhesion in an integrin dependent fashion.<sup>11,12,29</sup> Thus, these newly identified integrin CD11b/CD18 agonists are also potential leads for the development of unique anti-inflammatory agents.

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