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The determination and correlation of molecular and cellular equilibrium K_d and kinetic k_{off} values for small molecule allosteric antagonists of LFA-1

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ABSTRACT

Molecular K_d and k_{off} parameters are often used to define the molecular potency of drugs. These constants, however, are determined on purified target proteins, and their relationship to *in vivo* binding phenomena is poorly understood. Herein, we report two novel assays to determine the off-rates of allosteric antagonists from lymphocyte function-associated antigen 1 (LFA-1). The SPR assay involves using the non-blocking mAb TS2/4 to immobilize full-length LFA-1 on a hydrophilic chip surface, and the soluble, native ligand sICAM-1 to probe the fraction of free LFA-1. To determine the fraction of free LFA-1 on cell surfaces, a flow cytometry assay was developed utilizing the fluorophore-labeled Fab R3.1. The R3.1 antibody has been previously demonstrated to block the ability of both ICAM-1 and antagonists to bind to purified and cell-surface LFA-1. The molecular and *ex vivo* cellular parameters were determined for a set of nine structurally-related LFA-1 allosteric antagonists. The relationships between the parameters determined in the ELISA (K_d), SPR (k_{off}), and flow cytometry (k_{off}) assays were shown to be linear with slopes approximately equal to 1, and a correlation analysis showed that the three assay datasets were equivalent at the $\alpha = 0.05$ level. These results were unexpected, as the ELISA and SPR assays involve high affinity LFA-1, and the flow cytometry assays involve cell surface LFA-1 in whole-blood, in which a distribution of affinity states would be expected. Nevertheless, the results presented herein show that the K_d and k_{off}'s determined in molecular assays can be used as predictors of LFA-1 receptor occupancy in *ex vivo* assays.

1. Introduction

The integrin lymphocyte function-associated antigen-1 (LFA-1) is a cell-surface receptor which plays a major role in leukocyte trafficking and activation [1,2]. Through its interactions with intercellular adhesion molecules (ICAM-1, -2, and -3), LFA-1 promotes leukocyte arrest and transmigration, antigen presentation, and T cell-mediated cytolysis [3]. Antibodies directed against LFA-1 have been shown to be efficacious for the treatment of psoriasis in humans [4–10]. Consequently, many pharmaceutical companies have focused on the development of small molecule inhibitors to antagonize the functionality of

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LFA-1 [11]. The molecular mechanisms of LFA-1 functionality have been studied in detail, and have been reviewed by Shimaoka et al. [17], and Giblin and Lemieux [11].

The interaction between a soluble form of ICAM-1 (sICAM-1) and full-length LFA-1, or it's isolated I domain, has been characterized at the molecular level using ELISA (enzyme-linked immunosorbent assay) [12–14] and SPR (surface plasmon resonance) [15–18]. These and other studies have shown that LFA-1 exists in at least three conformations with different binding affinities for ICAM-1, ranging from ~100 nM to ~1 mM [14–16,18–21]. SPR studies have determined the k_{on} and k_{off} rate constants for ICAM-1 to immobilized, full-length LFA-1 to be $2.2 \times 10^5\ M^{-1}\ s^{-1}$ and $3 \times 10^{-2}\ s^{-1}$ ($K_d = 140$ nM), respectively, and that the high affinity conformation of LFA-1 is induced and stabilized by the metal cations $Mn^{2+} > Mg^{2+} \gg Ca^{2+}$ [15].

Small-molecule antagonists of LFA-1 have been reported to inhibit the binding of full-length LFA-1 to surface-immobilized sICAM-1 [22]. Structural studies [23,24,22] showed that these antagonist bind to an allosteric site in the I domain, distal to the LFA-1-ICAM-1 binding interface, and have been described as αI allosteric antagonists. This class of allosteric antagonists prevents the conformational changes in the I domain required for high affinity ICAM binding, as evidenced by the lack of binding by mAb24 [24], an antibody that recognizes an epitope exposed in the extended, active LFA-1 conformation. Since the mAbs (monoclonal antibodies) R3.1 and R7.1 have demonstrated the ability to block the binding of both ICAM-1 [25,26] and the aforementioned allosteric antagonists [23,22] to LFA-1, they can be used as tools to monitor antagonist binding.

Herein, we report the development of two novel assays for the determination of molecular and cellular antagonist off-rates from full-length LFA-1. The first assay uses Fab R3.1 to probe the fraction of antagonist-free, cell-surface LFA-1 using flow cytometry; the second assay uses sICAM-1 to probe the fraction of antagonist-free, surface-immobilized LFA-1 by SPR. Both assays were carried out in the presence and absence of a set of 9 LFA-1 allosteric antagonists (based on a hydantoin structure and their bicyclic derivatives [27]) as a function of time. The kinetic and equilibrium constants were regressed and correlated to determine if molecular observations could be used to predict receptor occupancy *in vivo*, using an *ex vivo* whole blood assay as a model system.

2. Materials and methods

2.1. Materials

TS2/4.1.1 was the kind gift from Dr. Timothy Springer; TS2/4.1.1 and anti-CD11a R3.1 monoclonal antibodies and Fab fragments were produced and purified by the protein resources group at BIPI. R3.1 Fab fragments were directly labeled with rPE (Molecular Probes, Eugene, OR) using a protein conjugation kit (Molecular Probes). The 1:1 conjugate was purified using 300 mm Bio-Rad Bio-Sil and Bio-Silect size exclusion columns run in tandem, respectively (BioRad, Hercules, CA). Equivalent fractions from multiple runs were pooled, and studied using analytical ultracentrifugation (Beckman, Fullerton, CA) for molecular weights and purity of the pools. TS2/4.1.1 Fab fragments were directly labeled using the FluoReporter Oregon Green 488 (OG-488) protein labeling kit (Molecular Probes). The LFA-1 antagonists used in this study were synthesized by BIPI. LFA-1 was purified as previously described [28].

2.2. LFA-1 molecular K_d assay (ELISA)

LFA-1 binding to ICAM-1 was monitored as previously reported [22]. Briefly, sICAM-1 was plated in a 96-well format for 30 min at room temperature. Plates were then blocked with BSA at 37 °C for 30 min. Blocking solution was removed from the wells, and test compounds were titrated in diluting buffer (PBS, 2 mM $MgCl_2$, 0.1 mM PMSF). Fifty μL of each antagonist concentration was added to the wells, followed by the addition of ~25 ng of LFA-1 in 50 μL, and allowed to incubate at 37 °C for 1 h. Wells were then washed with 200 μL of PBS. Addition and incubation of 100 μL of a rabbit anti-LFA-1 polyclonal (CD11a) for 30 min at 37 °C was used to detect the fraction of the surface-immobilized sICAM-1 bound by LFA-1. Wells were washed, and visualized by the addition of HRP conjugated to goat anti-rabbit Ig (Zymed, South San Francisco, CA). This reagent was allowed to incubate for 20 min at 37 °C, wells were washed as above, and 200 μL of the ABTS substrate (Zymed) for the HRP was added to each well to develop a quantitative colorimetric signal, which is linearly proportional to the amount of LFA-1 bound to sICAM-1. sICAM-1 (70 μg/mL) was used as a positive control for inhibition of the LFA-1/ICAM-1 interaction. A dose-response curve was obtained for all test compounds (Figs. 1 and 2).

2.3. LFA-1 Molecular trap assay (SPR)

A previously described assay for the analysis of sICAM-1 binding to immobilized LFA-1 on a biosensor chip [15] was used to determine the antagonist off-rates in this study. The LFA-1 immobilization was a two-step process. Purified TS2/4.1.1, a non-blocking monoclonal antibody that recognizes the CD11a chain of LFA-1, was immobilized onto a BIAcore™ CM5 Research Grade Sensor Chip using standard amine coupling chemistry [29]. In the second step, LFA-1 was captured by

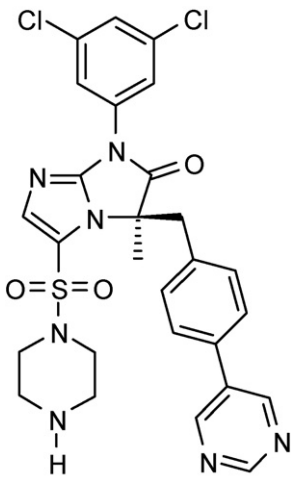

Fig. 1 – Structure of the representative analog, Compound G (see Table 1).

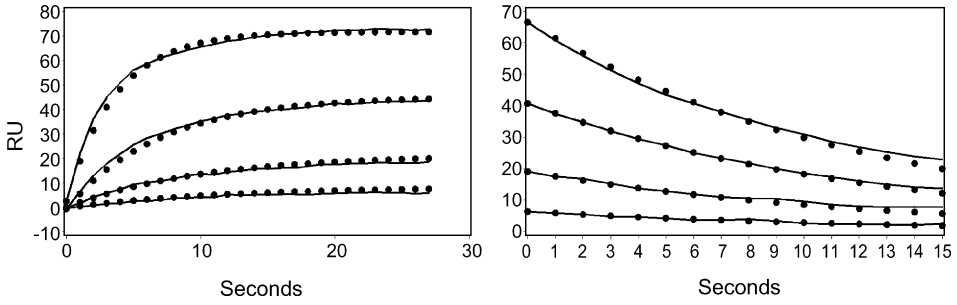

Fig. 2 – Kinetic assay for sICAM-1 binding to full-length LFA-1. Association data (Left Panel) and dissociation data (Right Panel) were globally fitted for k_{on} and k_{off}, giving 1.72(0.03) × 10^5 M^{-1} s^{-1} and 8.01(0.11) × 10^{-2} s^{-1}, respectively, with an R_{sq} of 0.997, where the numbers in parentheses are the StdErr's. The concentrations of sICAM-1 were 62.5, 125, 500, and 1000 nM, and the calculated K_d for the interaction is 4.66 × 10^{-7} M.

immobilized TS2/4.1.1 at a 5 μL/minute flow rate. The amount of LFA-1 immobilized ranged between 500 and a 1000 RU's. Assay buffer consisted of phosphate buffered saline pH 7.4 (Gibco BRL, Grand Island, NY), supplemented with 2 mM $MgCl_2$ and 100 μM $CaCl_2$. The molecular sICAM-1 on and off-rates were determined using a previously described global analysis [30]. The functional activity of the immobilized LFA-1 was evaluated before every antagonist off-rate experiment by injecting 5 μM sICAM-1 over the surface at a flow rate of 15–30 μL/minute to define the maximum response. A sICAM-1 binding signal of 50 RU's or more was set as the criteria for a working surface. All antagonists were diluted to final concentrations in assay buffer containing 1% DMSO. Two experimental assays were designed to measure off-rates for weak and potent antagonists. An initial injection of sICAM-1 ($10 \times K_d^{sICAM^{-1}}$) was performed to determine the maximum response for the experiment in both assays. For the faster antagonist off-rates (See Fig. 3), a COINJECT protocol was used. The first injection consisted of antagonist at a concentration $10 \times K_d^{antagonist}$ for 5 min, followed by a 300 s injection of sICAM-1 at $10 \times K_d^{ICAM}$. The sICAM-1 injection was performed at flow rates of 50–100 μL/min. For the slower antagonist off-rates (See Fig. 4), antagonist at concentrations ($10 \times K_d^{antagonist}$) were injected over the LFA-1 surface for 5 min at a flow rate of 15 μL/min. The antagonist loading time used was sufficient to achieve greater than 50% antagonist occupancy of the surface-immobilized LFA-1. A brief needle wash step was inserted to prevent antagonist carryover. sICAM-1 at 5–10 μM was then injected over the LFA-1 surface every 3–5 min until R_{max} was achieved. To further address antagonist carryover, experiments were conducted on a single chip containing an empty flow-cell and a flow-cell with immobilized LFA-1. Antagonist(s) was injected over the blank flow cell for 5 min at 15 μL/min. Since the BIAcore 3000 micro fluidics directs buffer flow over the flow cells in a serpentine format, the blank surface was downstream of the LFA-1 surface to prevent flow-cell to flow-cell contamination with antagonist. Then, several sICAM-1 injections were performed over the immobilized LFA-1 flow-cell. If carryover was a problem, the sICAM-1 binding responses would be diminished. When antagonist concentrations were kept below 1 μM, carryover was negligible. All data were fit using Eq. (5) (Computational Analyses, see below).

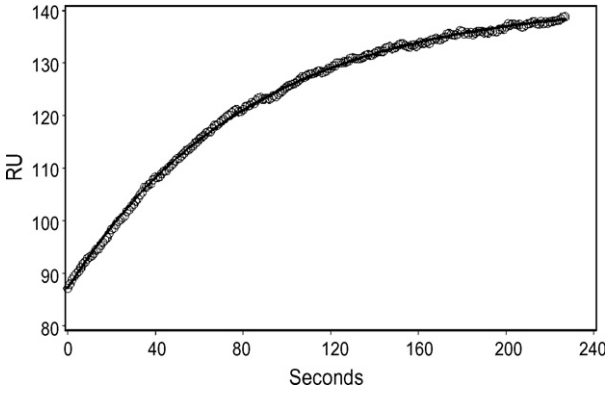

Fig. 3 – SPR Method 1 for determining the off-rates of weak antagonists of LFA-1 exhibiting antagonist half-lives less than 5 min. As Compound A (see Table 1) dissociates, sICAM-1 binds to free LFA-1. These data were fitted to Eq. (5), giving an estimate for k_{off} of 1.21(0.05) × 10^{-2} s^{-1} ($t_{1/2}$ = 0.96 min) and an R_{max} of 1.41(0.01) × 10^2 RU with an R_{sq} of 0.999 (numbers in parentheses are the StdErr's).

2.4. *LFA-1 whole blood receptor-occupancy assay (flow cytometry)*

LFA-1 antagonist off-rates in whole blood were monitored using the LFA-1 Fab R3.1 to probe the fraction of free cellular LFA-1 in the absence and presence of allosteric antagonists. Two mL of whole blood were treated with compounds added to a final concentration of 2 μM. Tubes containing compound-treated blood were incubated for 30 min at 37 °C, 5% CO_2, while mixing on a nutator inside a tissue culture incubator. After incubation, cells were washed 2× by the addition of 1 mL of untreated autologous serum, followed by centrifugation at 1000 rpm (4 °C, 5 min), and supernatant discarded. Untreated, autologous serum was added to reconstitute the cells to a volume of approximately 2 mL. This process of washing introduced about a 5 min time period in which no Fab is available to bind LFA-1 as antagonist dissociates. Treated blood (1 mL) was then mixed with fluorescently labeled Fabs, either R3.1 (blocking anti-CD11a-I-domain) or Fab TS2/4.1.1 (non-blocking, anti-CD11a) at a concentration 5 times higher

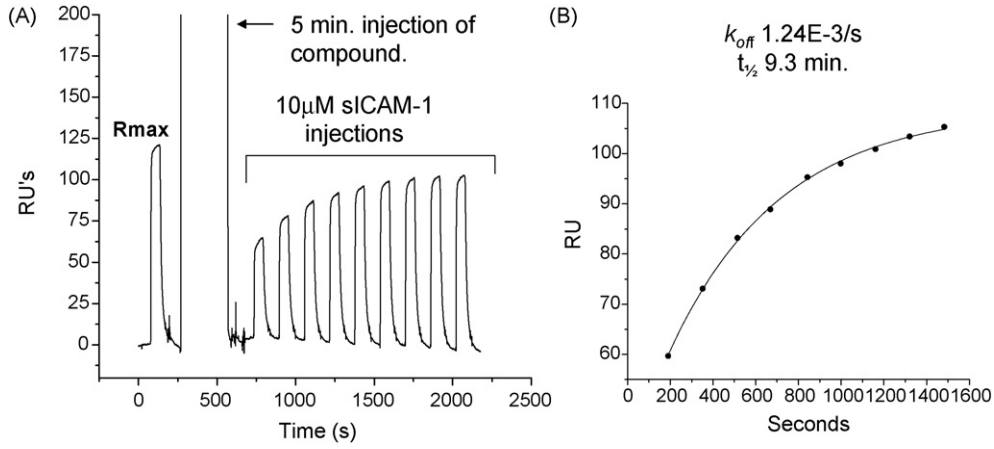

Fig. 4 – An indirect method for determining the k_{off} for a potent antagonist bound to immobilized LFA-1. A, SPR measurements (50 μM ICAM injections Recovery of sICAM-1 binding post exposure of LFA-1 to compound. B, sICAM-1 recovery data fit to Eq. (5). The k_{off} was estimated to be 1.2×10^{-3} s^{-1}.

than the observed saturating concentrations. 50 μL aliquots of whole blood were removed at pre-defined time points, washed by addition of 250 μL FACS buffer, centrifuged at 1000 rpm (4 °C, 5 min), and supernatant discarded. Cells were kept in suspension as described above to insure dynamic binding phenomena between the Fab and antagonist with cell surface LFA-1. Samples were then lysed by the addition of 50 μL of Optilyse B solution (BD Immunotech, France), incubated for 10 min at room temperature, fixed with paraformaldehyde, followed by the addition of 500 μL of distilled H_2O for flow cytometry analysis. Fixed samples were analyzed by flow cytometry using a BD FACSAN. The wash buffer consisted of 100 mM sodium phosphate, 150 mM sodium chloride, 0.1% sodium azide with the pH adjusted to 7.2. Buffer supplemented with 2% BSA, was used to dilute antibody reagents.

Preliminary experiments showed slow on-rates for association of the Fab R3.1 (see Fig. 5). In this case, Scheme 3 can not be reduced to a simple antagonist dissociation problem, as was possible for the SPR assay (Computational Analyses, vide infra). Examination of Eq. (10) shows the collinear dependency between k_1 and k_2'. The association of Fab R3.1 in the absence of antagonist was run as a control for each dataset, and the fitted estimate for apparent k_2' (see Eq. (11)) was hard-coded in Eq. (10) for the analysis of the antagonist off-rates, with a lag time set to 5 min. Since expression of LFA-1 does not vary significantly for 6 h (data not shown), the distribution of LFA-1 molecules/cell remains constant. Thus, the data taken in the absence of antagonist serve as the internal reference for the analysis of antagonist off-rates.

2.5. *Computational analyses*

All data were analyzed using the SAS statistical software system (version 9.1, SAS Institute Incorporated, Cary, NC) on a HP NC6000. ASCII data files containing either fluorescence or SPR measurements were converted into SAS datasets. Using the SAS MODEL procedure, data analyses were performed by ordinary nonlinear least-squares regression techniques to the model using the Marquardt-Levenberg minimization method (SAS-ETS *Users Guide*, Version 9).

2.6. *I. kinetic models for the analysis of SPR data*

The dissociation of antagonist in the presence of saturating concentrations of sICAM-1 is described in Scheme 1.

LFA = chip-immobilized LFA-1, ICAM = sICAM-1, Inh = antagonist, k_1 is the off-rate of the antagonist, and k_2 is the on-rate of ICAM. In this assay, the LFA-1 chip surface is charged with antagonist, and subsequent injection(s) of saturating concentrations of sICAM-1 are used to probe the fraction of free LFA-1 sites as antagonist dissociates. Since the concentrations of sICAM-1 are saturating, the rate-limiting step is the dissociation of Inh, i.e., $k_2[ICAM]_T \gg k_1$, and Scheme 1 reduces to Scheme 2.

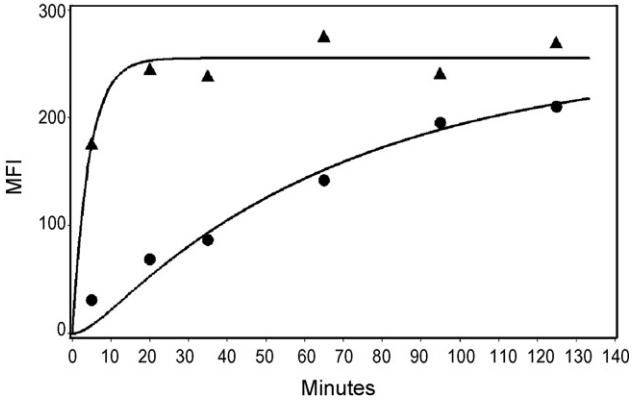

Fig. 5 – Association of R3.1 in the absence (triangles) and presence (dots) of Compound C (see Table 1). The estimates for the on-rate (k_2', see Eq. (11)) of the Fab was 3.88 (0.75) $\times 10^3$ M^{-1} s^{-1}, with an R_{sq} of 0.771. The off-rate of Compound C (k_1, see Eq. (12)) was determined to be 2.43(2.00) $\times 10^{-4}$ s^{-1} ($t_{1/2}$ = 47.5 min) with R_{sq} of 0.961 (the numbers in parentheses are the StdErr's). The experimental time-lag was 5 min.

$$\mathrm{LFA-Inh} \xrightarrow[\text{Chip Surface}]{k_1} \mathrm{LFA} \xrightarrow[\text{Chip Surface}]{k_2\cdot\mathrm{ICAM}} \mathrm{LFA-ICAM}$$

Scheme 1

$$\mathrm{LFA-Inh} \xrightarrow[\text{Chip Surface}]{k_1} \mathrm{LFA}$$

Scheme 2

The mass balance for the reaction is then:

$$[\mathrm{LFA}]_T = [\mathrm{LFA}] + [\mathrm{LFA-Inh}] \quad (1)$$

where T refers to the total concentration. The differential rate equation for the dissociation of Inh is:

$$\frac{d[\mathrm{LFA-Inh}]}{dt} = -k_1[\mathrm{LFA-Inh}] \quad (2)$$

and integration gives:

$$[\mathrm{LFA-Inh}]_t = [\mathrm{LFA-Inh}]_0 \times e^{-k_1 t} \quad (3)$$

Substitution Eq. (1) for t (time) = 0 and time = t into Eq. (3) and rearrangement gives:

$$[\mathrm{LFA}]_t = [\mathrm{LFA}]_T - ([\mathrm{LFA}]_T - [\mathrm{LFA}]_0) \times e^{-k_1 t} \quad (4)$$

$[\mathrm{LFA}]_t$ is measured in resonance units (RU). $[\mathrm{LFA}]_0$ defines the ICAM-1 SPR response at time $t = 0$ (first measurement), and $[\mathrm{LFA}]_T$ is the response amplitude ($\mathrm{RU_{max}} - \mathrm{RU_{min}}$). Rewriting Eq. (4) gives the expression for fitting SPR data.

$$\mathrm{RU}_t = (\mathrm{RU_{max}} - \mathrm{RU_{min}}) - (\mathrm{RU_{max}} - \mathrm{RU_{min}} - \mathrm{RU}_0) \times e^{-k_1 t} \quad (5)$$

2.7. II. Kinetic models for the analysis of flow cytometry data

The dissociation of antagonist in the presence of saturating concentrations of labeled R3.1 Fab is depicted in Scheme 3.

LFA = cell surface LFA-1, Inh = antagonist, Fab* = a high-affinity, labeled, blocking R3.1 Fab, k_1 = the dissociation rate constant for Inh, and k_2 is the association rate constant for Fab*. Note the similarity with Scheme 1. The difference in Scheme 3 is that the phenomena occurs at a cell surface, and involves a labeled probe (Fab*) for the detection of free, cell surface LFA-1. Another difference is that the assumption of $k_2[\mathrm{ICAM}]_T > k_1$ can not be made, and Scheme 3 can not be reduced as with Scheme 1. The mass balance for total [LFA] is given by:

$$[\mathrm{LFA}]_T = [\mathrm{LFA}] + [\mathrm{LFA-Fab^*}] + [\mathrm{LFA-Inh}] \quad (6)$$

$$\mathrm{LFA-Inh} \xrightarrow[\text{Cell Surface}]{k_1} \mathrm{LFA} \xrightarrow[\text{Cell Surface}]{k_2\cdot[\mathrm{Fab^*}]} \mathrm{LFA\_Fab^*}$$

Scheme 3

Assuming negligible dissociation of the high affinity Fab*, the differential rate equation for the appearance of LFA-Fab* is:

$$\frac{d[\mathrm{LFA-Fab^*}]}{dt} = k_2'[\mathrm{LFA}] \quad (7)$$

k_2' is the first-order rate constant for the association for Fab*. Substituting Eq. (3) for [LFA – Inh] into the Eq. (6) gives:

$$[\mathrm{LFA}]_T = [\mathrm{LFA}] + [\mathrm{LFA-Fab^*}] + [\mathrm{LFA-Inh}]_0 \times e^{-k_1 t} \quad (8)$$

Substitution of [LFA] from Eq. (8) into Eq. (7), and rearrangement gives:

$$\frac{d[\mathrm{LFA-Fab^*}]}{dt} + k_2'[\mathrm{LFA-Fab^*}] = k_2'([\mathrm{LFA}]_T - [\mathrm{LFA-Inh}]_0 \times e^{-k_1 1}) \quad (9)$$

Integration of this first-order differential equation gives the concentration of the bound probe at time t:

$$[\mathrm{LFA-Fab^*}]_t = [\mathrm{LFA}]_T(1 - e^{k_2' t}) + \frac{k_2'}{k_2' - k_1}[\mathrm{LFA-Inh}]_0(e^{k_2' t} - e^{-k_1 t}) \quad (10)$$

Free LFA-1 is measured by determining $[\mathrm{LFA-Fab^*}]_t$, the mean fluorescence intensity (MFI) at time t. $[\mathrm{LFA}]_T$ is the amplitude of the signal ($\mathrm{MFI_{max}} - \mathrm{MFI_{min}}$). Since k_1 and k_2' are collinear in this expression, one of the parameters must be known. k_2' is the only parameter that can be determined independently. In the absence of antagonist, Eq. (10) reduces to:

$$\mathrm{MFI}_t = (1 - e^{-k_2' t})(\mathrm{MFI_{max}} - \mathrm{MFI_{min}}) + \mathrm{MFI_{min}} \quad (11)$$

When $t = 0$ (the first observation), $\mathrm{MFI}_t = \mathrm{MFI_{min}}$. Knowing k_2', the antagonist off-rates k_1 can now be determined. In these experiments, the cells have been saturated with antagonist prior to the addition of Fab*. Assuming $[\mathrm{LFA-Inh}]_0 = [\mathrm{LFA}]_T$, Eq. (10) can be rewritten to give the fitting expression:

$$\mathrm{MFI}_t = \left[(1 - e^{-k_2' t}) + \frac{k_2'}{k_2' - k_1}(e^{-k_2' t} - e^{-k_1 t})\right](\mathrm{MFI_{max}} - \mathrm{MFI_{min}}) + \mathrm{MFI_{min}} \quad (12)$$

3. Results

In a previous SPR study, LFA-1 was immobilized on a hydrophobic HPA surface, and the on and off-rates of sICAM were measured [15]. Since most drug molecules are hydrophobic, nonspecific binding to the hydrophobic HPA chip surface precluded using this surface in the present study. The methodology used herein involved the immobilization of TS2/4.1.1, an LFA-1 specific, non-blocking mAb, on a hydrophilic CM5 surface. The immobilized mAb was shown to capture LFA-1 with high affinity, as little or no LFA-1 was observed to leach over a series of experiments; in addition, this surface was shown to be robust in that the maximum sICAM signal did not decrease within any experiment. Fig. 2 shows the global

fitting analysis for the association and dissociation of sICAM-1 binding, in which the k_{on} and k_{off} parameters were estimated to be $1.7 \times 10^5\ M^{-1}\ s^{-1}$ and $8.0 \times 10^{-2}\ s^{-1}$, respectively. The calculated K_d of 471 nM is approximately three-fold weaker than the previously published value of 140 nM using the HPA chip [14,15]. The difference was manifested by an increase in the sICAM-1 off-rate, probably due to small LFA-1 conformational changes that occur upon capture by surface-immobilized mAb TS2/4.1.1.

Preliminary SPR studies showed that antagonist off-rates ranged from very fast to moderately long times. For antagonists exhibiting fast k_{off}'s, immobilized LFA-1 was first saturated with antagonist, followed by a continuous flow of saturating concentrations of sICAM-1 in the absence of antagonist. A typical experiment for monitoring the shorter dissociation rates is shown in Fig. 3. As antagonist dissociates, sICAM-1 rapidly binds and traps the antagonist from rebinding ($k_2[\text{sICAM}]_T \gg k_1$), and Scheme 1 reduces to a first-order kinetic problem as shown in Scheme 2 (see Eq. (5)). For antagonists exhibiting slower k_{off}'s, LFA-1 is saturated with antagonist as before, but followed by pulses of excess sICAM-1 in the absence of antagonist at pre-selected time points. The only difference between the two assays is the time period of the measurements. The antagonist concentration was kept approximately 10-fold higher than its K_d determined by ELISA, and proved to be sufficient to saturate at least 50–80% of the immobilized LFA-1 in all cases. Absorption and leaching of potent antagonists from system components can be problematic, as carry over of the more potent antagonists can affect the measurement of antagonists with faster off-rates. The methods presented herein were developed to minimize these effects (see Section 2). A typical kinetic experiment for monitoring the longer dissociation rates is shown in Fig. 4. The observed k_{off} values (Eq. (5)) ranged from 1 to 30 min (see Table 1).

The flow cytometry-based assay developed herein extends a previously reported receptor-occupancy, equilibrium assay [31] to determine antagonist off-rates in an ex vivo format that closely resembles the physiological environment in which LFA-1 normally functions. The assay protocol was to add antagonist to whole blood at saturating conditions, wash, add R3.1 Fab, fix, stain, and analyze by flow cytometry. The on-rate for the control, non-blocking mAb TS2/4 Fab was not altered by the presence of antagonists, verifying that the binding of Fab R3.1 is specific to and blocks the antagonist binding site in the I domain (data not shown). Kinetic data for the association of Fab R3.1 in the presence and absence of Compound C is shown in Fig. 5. In this case, Scheme 4 can not be reduced to a simple first-order dissociation equation, as was possible for the SPR assay. The computational solution for this problem shows a collinear dependency between k_1 and k_2' (see Eq. (10)). Since the apparent on-rate of the Fab R3.1 (k_2') is the only parameter that can be determined independently using Eq. (11), each antagonist experiment included a dataset for the determination of k_2' using Eq. (12). The on-rate of the R3.1 Fab in the absence of antagonist was determined to be $6.10(1.48) \times 10^5\ M^{-1}\ s^{-1}$ over all experiments, where the value in parentheses is the standard deviation. This on-rate is typical for large MW proteins binding at surfaces. In the presence of antagonist, the association rate of R3.1 Fab is dramatically reduced (see Fig. 5) due to the slow dissociation of the antagonist ($t_{1/2} = 47.5$ min). Off-rates for all of the compounds were evaluated multiple times in different experiments and are given in Table 1.

Linear regression relationships for the assays reported herein are shown in Fig. 6, (see Table 2). Interestingly, the parameters determined in the three assays all show linear relationships with each other, with slopes of approximately one. The relationship between k_{off} determined by SPR and FACS gave a slope = 0.95, and an intercept = 0, which is quite remarkable given that one assay is molecular and the other is cellular (whole blood). Regression analysis involving K_d had slopes close to one, with an intercept of 4.91(0.40); when $K_d = 1$ M, the antagonist $k_{off} \approx 1 \times 10^5\ s^{-1}$. Since $K_d = k_{off}/k_{on}$, the antagonist k_{on} for this set of experiments is approximately $1 \times 10^5\ M^{-1}\ s^{-1}$. Small MW antagonists typically show much faster times approaching the diffusion limit, and the low k_{on} is probably due to the viscosity of whole blood. While linear regressions show that the slopes between all of the parameters are approximately 1, it does not provide information on the similarity between the datasets. For this purpose, a correlation analysis was performed, and the results are given in Table 2. It can be seen that all of the correlation coefficients are above their critical values for n number of observations, indicating that all 3 datasets are equivalent at a confidence level of $\alpha = 0.05$.

Table 1 – Antagonist equilibrium and kinetic rate constants determined for a set of LFA-1 allosteric antagonists

Compound name	K_d_ELISA (M)	n (#)	S.D. (%)	k_{off}_SPR (s^{-1})	n (#)	S.D. (%)	$t_{1/2}$ (min)	k_{off}_FACS (s^{-1})	n (#)	S.D. (%)	$t_{1/2}$ (min)
A	1.61×10^{-07}	9	43	1.40×10^{-02}	5	33	0.8	[a]	3	[a]	[a]
B	2.10×10^{-09}	4	43	5.70×10^{-04}	3	12	20.3	3.62×10^{-04}	1	0	31.9
C	1.86×10^{-09}	4	94	3.90×10^{-04}	4	13	29.6	2.43×10^{-04}	4	6	47.5
D	3.58×10^{-09}	5	80	1.20×10^{-03}	3	23	9.6	1.23×10^{-03}	3	9	9.4
E	8.87×10^{-09}	3	29	1.70×10^{-03}	3	18	6.8	2.51×10^{-03}	4	32	4.6
F	9.00×10^{-09}	1	0	1.70×10^{-03}	3	12	6.8	1.34×10^{-03}	1	0	8.6
G	4.33×10^{-09}	5	26	1.20×10^{-03}	4	28	9.6	9.70×10^{-04}	5	22	11.9
H	2.00×10^{-09}	5	8	4.90×10^{-04}	4	19	23.6	7.03×10^{-04}	5	34	16.4
I	5.10×10^{-09}	4	44	1.90×10^{-03}	4	26	6.1	7.50×10^{-04}	2	20	15.4

n = number of determinations, and $t_{1/2} = \ln(2)/k_{off}$.
[a] The dissociation of antagonist was too fast to be measured by this method.

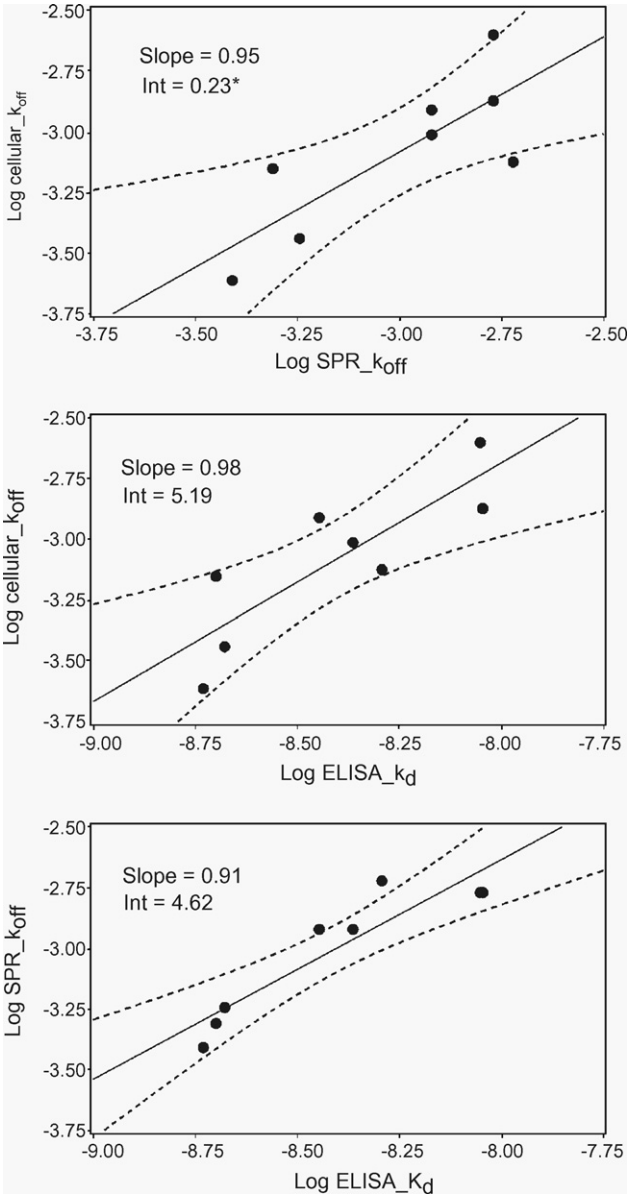

Fig. 6 – Correlations between antagonist equilibrium and kinetic parameters determined by flow cytometry, SPR, and ELISA (see Table 1; note that Compound A is not included because the antagonist off-rate was too fast to be determined by the flow cytometry assay). The linear regression estimates are given in Table 2.

4. Discussion

LFA-1 is a high MW, cell surface adhesion molecule that binds ICAMs and other integrin-related ligands. In its low affinity state, the α7 helix of the LFA-1 heterodimer is in a bent conformation, in which the housed I domain is positioned close to the cell membrane; in its high affinity state, the α7 helix is extended to present the I domain to the extra-cellular ICAM binding pocket [17]. The high affinity conformation of LFA-1 can be induced by ligand binding to the low affinity state of LFA-1, and by external stimuli that result in signal transduction and intracellular activities. Distinct LFA-1 affinity states have been reported [14–16,18–21], highlighting the structural changes that occur in the regulation of LFA-1 function. The small molecule antagonists of LFA-1 studied herein bind to the I domain near the α7 helix, but distally to the ICAM binding site. When the allosteric antagonist is bound, the α7 helix is prevented from assuming its high affinity, extended conformation, thus attenuating the high affinity, receptor-ligand interaction in an allosteric manner. Given the complexity of LFA-1 structural and cell biology, the focus of this study was to ask whether or not antagonist off-rates obtained with purified LFA-1 (always high affinity) would mimic those obtained from cell surface-expressed LFA-1 in whole blood (presumably a distribution of affinity states). The small MW antagonists of LFA-1 studied herein are hydantoin-based compounds (see Fig. 1 for an example). Dissociation constants for these allosteric antagonists were determined using an established ELISA assay, involving purified, high affinity LFA-1 and plate-immobilized sICAM-1 [22]. A set of nine LFA-1 antagonists were chosen for kinetic studies using SPR and flow cytometry.

Allosteric antagonism involves conformational changes within the LFA-1 binding site. In case-1, antagonist may only cause only a moderate structural distortion at the ICAM-1 binding site, leading to only a moderate change in ICAM-1 affinity. In this case, the ICAM-1 can still bind, and saturate the receptor in the presence of saturating concentrations of antagonist. In case-2, antagonist may induce a conformation change that causes a complete loss of ICAM-1 affinity for the LFA-1 binding site. In this case, allosteric and competitive binding models are indistinguishable. We have observed cases (data not shown) in SPR where the ICAM-1 binding to surface LFA-1 could not be driven to zero, only to about 10-20%, in support of case-1 above. For the antagonists studied herein, however, ICAM-1 binding could be driven to zero. In contrast, the receptor occupancy assay does not involve ICAM-1, rather Fab R3.1. It is not clear from literature or in-house studies

Table 2 – Linear regression and correlations between molecular and cellular inhibition constants*

Correlation*	n	Slope	StdErr	P-value	Intercept	StdErr	P-value	R_{sq}	Pearson r	Critical value***
Cellular* SPR	8	1.03	0.02	<0.0001	**	**	**	1	0.66	0.64
Cellular* ELISA	8	0.98	0.26	0.01	5.19	2.15	0.05	0.67	0.82	0.6
SPR* ELISA	9	0.91	0.16	0	4.62	1.31	0.01	0.82	0.84	0.6

* The log of the K_d and $t_{1/2}$ data from Table 1 (excluding Compound A) were fit to a linear regression.
** The slope for this analysis was 0.95, but the 0.80 P-value on the intercept (−0.23) was not statistically significant. Consequently, these data were re-fit no intercept.
*** The critical values for significance of the Pearson r values at the $\alpha = 0.05$ level.

whether R3.1 binding leads to exclusive or non-exclusive antagonist binding. The model being used herein assumes that R3.1 serves to probe the fraction of free LFA in a manner (case-2) which is indistinguishable from competitive binding (see Section 2).

The relationships between the parameters determined in the ELISA (K_d), SPR (k_{off}), and flow cytometry (k_{off}) assays were shown to be linear with slopes approximately equal to 1, and a correlation analysis showed that the three assay datasets were equivalent at the $\alpha = 0.05$ level. These results were unexpected, as the ELISA and SPR assays involve high affinity LFA-1, and the flow cytometry assays involve cell surface LFA-1 in whole-blood, in which a distribution of affinity states would be expected. Only in an ideal case would these very different molecular and cellular results be expected to be equivalent and related to each other with a slope of 1 (see Fig. 6 and Table 2). It should be pointed out that that purified, high-affinity, full-length LFA-1 was used in the ELISA and SPR assays, so these results may not be that unexpected. In contrast, however, given the biology and the multiple affinity states reported for cell surface LFA-1, a distribution of affinities would be expected to have an attenuating effect on the k_{off}'s and k_{on}'s determined using high-affinity LFA-1, but this was not observed. It may be possible that the manipulation of whole blood in the receptor occupancy assay activates and shifts the expected distribution to the LFA-1 high-affinity state; e.g. introduction of divalent cations (Mg^{2+}, Mn^{2+}) into the whole blood preparation via assay buffers/media or transient exposure of LFA-1-bearing cells to sICAM-1 present in the blood or membrane bound ICAM-1 via homotypic cell-cell interactions during the concentration of cells during incubation or centrifugation. These conditions have been demonstrated to induce cell-surface LFA-1 to adopt a high affinity conformation [reviewed in Stewart and Hogg; 32].

The efficacy of a receptor antagonist depends upon the residence time of the antagonist, which is a function of the antagonist off-rate, i.e., $t_{1/2}$. The object of this study was to determine if antagonist off-rates determined in molecular assays could be used to predict receptor-occupancy in whole blood. Since the on-rates for a set of structurally-related antagonists is usually a consistent value, the K_d's determined by ELISA are usually driven by the antagonist off-rate. Nevertheless, this is not always true, and SPR, which provides a direct measurement of k_{off}, may be the more appropriate measurement for predictions of receptor occupancy, particularly when antagonists with different scaffolds are being compared. While the whole blood assay requires considerable resources, and would be impractical to employ at the drug discovery level, these kinds of data will provide valuable insights into the relationships between physical binding parameters measured in a molecular assay and those in a whole blood assay. The results presented herein for LFA-1 show that the K_d's and k_{off}'s determined in molecular assays can be used a predictor of receptor occupancy in *ex vivo*. It remains to be shown, however, if these results will translate to the *in vivo* setting.

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