Design, Synthesis, and Analysis of a Polyethylene Glycol-Modified (PEGylated) Small Molecule Inhibitor of Integrin α4β1 with Improved Pharmaceutical Properties


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ABSTRACT

Integrin α4β1 plays an important role in inflammatory processes by regulating the migration of leukocytes into inflamed tissues. Previously, we identified BIO5192 [2(S)-[1-(3,5-dichloro-benzenesulfonyl)-pyrrolidine-2(S)-carbonyl]-amino]-4-[4-methyl-2(S)-(methyl-2-[4-(3-o-tolyliureido)-phenyl]-acetyl)-pentanoylamin]-butyric acid, a highly selective and potent (Kd of 9 pM) small molecule inhibitor of α4β1. Although BIO5192 is efficacious in various animal models of inflammatory disease, high doses and daily treatment of the compound are needed to achieve a therapeutic effect because of its relatively short serum half-life. To address this issue, polyethylene glycol modification (PEGylation) was used as an approach to improve systemic exposure. BIO5192 was PEGylated by a targeted approach in which derivatizable amino groups were incorporated into the molecule. Two sites were identified that could be modified, and from these, five PEGylated compounds were synthesized and characterized. One compound, 2a-PEG (Kd of 19 pM), was selected for in vivo studies. The pharmacokinetic and pharmacodynamic properties of 2a-PEG were dramatically improved relative to the unmodified compound. The PEGylated compound was efficacious in a rat model of experimental autoimmune encephalomyelitis at a 30-fold lower molar dose than the parent compound and required only a once-a-week dosing regimen compared with a daily treatment for BIO5192. Compound 2a-PEG was highly selective for α4β1. These studies demonstrate the feasibility of PEGylation of α4β1-targeted small molecules with retention of activity in vitro and in vivo. 2a-PEG, and related compounds, will be valuable reagents for assessing α4β1 biology and may provide a new therapeutic approach to treatment of human inflammatory diseases.

Integrins are a large family of cell surface receptors that mediate cell-cell and cell-matrix interaction. They exist as noncovalent αβ heterodimers of different combinations of α and β chains and share extensive structural homology. Integrins mediate a wide variety of physiological processes and are relevant to a wide variety of pathological conditions. The integrin α4β1 regulates normal leukocyte trafficking (Lobb and Hemler, 1994) and provides a key costimulatory signal supporting cell activation (Clark and Brugge, 1995). During inflammatory responses, α4β1 regulates leukocyte migration into the damaged tissues and has been recognized as an attractive therapeutic target. In vivo studies using blocking monoclonal antibodies (Lobb and Hemler, 1994; Issekutz et al., 1996; Enders et al., 1998; Hojo et al., 1998; Schneider et al., 1999; Ramos-Barbon et al., 2001), inhibitory peptides (Molossi et al., 1995; Abraham, 1997; van der Laan et al., 1996; Enders et al., 1998; Schneider et al., 1999; Ramos-Barbon et al., 2001), and small molecule antagonists (Lin et al., 1999; Kudlacz et al., 2002) have verified the critical role of α4β1 integrins in leukocyte-mediated inflammation and have implicated α4β1 inhibitors as potential treatments for diseases such as asthma and arthritis. Numerous EAE models that recapitulate important aspects of human multiple sclerosis

ABBREVIATIONS: EAE, experimental autoimmune encephalomyelitis; VCAM-1, vascular cell adhesion molecule-1; BIO5192, 2(S)-[1-(3,5-dichloro-benzenesulfonyl)-pyrrolidine-2(S)-carbonyl]-amino]-4-[4-methyl-2(S)-(methyl-2-[4-(3-o-tolyliureido)-phenyl]-acetyl)-pentanoylamin]-butyric acid; BIO7662, 2S-[1-benzenesulfonyl-pyrrolidine-2S-carbonyl]-amino]-4-[4-methyl-2S-(methyl-2-[4-(3-o-tolyliureido)-phenyl]-acetyl)-amino]-pentanoylamin]-butyric acid; 2a, 2S-[4R-amino-1-benzenesulfonyl-pyrrolidine-2S-carbonyl]-amino]-4-[4-methyl-2S-(methyl-2-[4-(3-o-tolyliureido)-phenyl]-acetyl)-amino]-pentanoylamin]-butyric acid; PEG, polyethylene glycol; PEGylation, PEG modification; mPEG, methoxypolyethylene glycol; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; Vss, volume of distribution at steady state; AUC, area under the curve; MBP, myelin basic protein.
are also responsive to both monoclonal antibodies and small molecule α4 inhibitors (Yednock et al., 1992; Kanwar et al., 2000b; Piraino et al., 2002; Leone et al., 2003). Recent positive phase II data using the anti-α4 antibody natalizumab in patients with multiple sclerosis have validated α4β1 as an important clinical target (Miller et al., 2003).

α4β1 mediates cell adhesion by binding to either of two protein ligands, vascular cell adhesion molecule-1 (VCAM-1) or the alternatively spliced connecting segment 1-containing fibronectin variant (Osborn et al., 1989; Wayner et al., 1989). More recently, other potential ligands have been identified (Bayless et al., 1998; Takahashi et al., 2000); however, the biological significance of these interactions is less clear. The interactions between α4β1 and its ligands are of low affinity, and binding presumably is modulated through multivalent interactions. Although expression of α4β1 and binding presumably is modulated through multivalent techniques (Wayner and Kovach, 1992; Wang et al., 1995). Many groups have used these sequences as starting points to develop small molecule inhibitors that can block the interaction between α4β1 and its ligands (Abraham, 1997; Lin et al., 1999; Kudlac et al., 2002; van der Laan et al., 2002). In studying the selectivity of different classes of α4β1 inhibitors, we identified the small molecule inhibitor BIO5192 (Leone et al., 2003; Scott et al., 2004). BIO5192 was of special interest because of its high affinity for α4β1 under all states of activation, high selectivity for α4β1, and slow dissociation rate from the bound complex. Although BIO5192 was efficacious in various animal models of inflammatory disease, its therapeutic effects required high doses of the compound and daily treatment because of its relatively short serum half-life. To address this issue, polyethylene glycol modification (PEGylation) was used as an approach to improve systemic exposure (Delgado et al., 1992, Francis et al., 1998, Pepinsky et al., 2001). Here, we extensively characterize a PEGylated version of BIO5192 that retains function following PEG modification and describe the effects of PEGylation on its pharmacokinetics, pharmacodynamics, and efficacy in blocking the disease progression in a rat model of EAE responsive to BIO7662 [2S-[1-benzenesulfonyl-pyrrolidine-2S-carbonyl]-amino-4-[4-methyl-2S-[methyl-2-[4-[3-o-tolyl-ureido]-phenyl]-acetyl]-amino-pentanoylamin]-butyric acid], a derivative of BIO5192 lacking the dichloro substitution on the benzene ring, [35S]BIO7662, and BIO8139 [4-6-(6-amino-hexanoylamino)-2S-[methyl-2-[4-[3-o-tolyl-ureido]-phenyl]-acetyl]-amino-hexanoylamino]-2(S)-[1-benzenesulfonyl-pyrrolidine-2(S)-carbonyl]-amino]-butyric acid; compound 1a, an amine-containing derivative of BIO7662 that was used for conjugation and the development of assays for assessing α4β1 function, were synthesized as previously described (Chen et al., 2001; Leone et al., 2003; Scott et al., 2004). For the generation of 1a-PEG, compound 1a (50 mM in dimethylsulfoxide) was diluted 10-fold into a freshly prepared solution containing 50 mM HEPES, pH 8.0, 150 mM NaCl, and 5 mM 20-kDa of mPEG-succinimidyl propionate (Nektar Molecular Engineering, Huntsville, AL) and incubated at room temperature for 1 h. Ethanolamine (50 mM) was added to quench unreacted polyethylene glycol, and the sample was desalted on a PD16 (Bio-Rad, Hercules, CA) desalting column to remove unreduced 1a. 1a-PEG was formulated in PBS. Compounds 2a, 2b, 2c, and 2d were generated using the same synthetic route described for 1a except that proline was replaced with amino-proline (anti) for 2a, 6-amino-hexanoyl-amino-proline (syn) for 2b, 6-amino-hexanoyl-amino-proline (anti) for 2c, or with amino-proline (syn) for 2d on the BIO7662 framework (i.e., with leucine in place of the 6-amino-hexanoyl-lysine moiety). PEGylated versions of the amino-proline-containing compounds were generated using a modified protocol as follows: 0.097 mmol of 20-kDa mPEG-succinimidyl propionate was added to 0.11 mmol of each compound in 2 ml of dimethylsulfoxide, 0.5 ml of saturated NaHCO3, and 4 ml of water and stirred overnight at room temperature. The reactions were diluted with vigorous stirring with 30 ml of ethanol and then with 100 ml of ethyl ether. The suspension was filtered, and the resulting white solid was washed three times with ethyl ether and dried under vacuum. The crude product was dissolved in hot ethanol, and the PEGylated product was precipitated out of the mixture by the addition of ethyl ether. The precipitate was collected by filtration, washed, and dried. This process was repeated one more time to provide PEGylated product as a white powder that was >99% pure when analyzed by liquid chromatography on a Superdex peptide sizing column (Amersham Biosciences Inc., Piscataway, NJ). The sizing column was run in 30% acetonitrile and 0.1% trifluoroacetic acid at a flow rate of 1 ml/min, and the effluent was monitored at 254 nm. The presence of the inhibitor in the PEGylated product was verified by NMR. Unreacted mPEG could not be removed by this method. The relative concentrations of inhibitor-modified mPEG and unreacted mPEG in the final product were quantified from the NMR analysis. Conjugation efficiencies of 50 to 70% were observed resulting in products that contained, on average, about 0.6 mol of inhibitor per mole of mPEG. For efficacy studies in EAE and for lymphocytosis studies, an inactive control of mPEG was used that was synthesized by reacting 20 kDa of mPEG-succinimidyl propionate with isobutylylamine. The structures of BIO5192, BIO7662, compound 1a, and compound 2a are shown below (Scheme 1). In selected studies, a series of 0.35, 2, 5, 20, 30, and 50 kDa of mPEG-succinimidyl propionate (Nektar Molecular Engineering) derivatives of compound 2a were synthesized and tested for function. These compounds were generated and characterized using the same protocols used for 2a-PEG.

Cell Binding and Adhesion Assays. The α4β1-expressing human Jurkat T-cell line (a gift from S. Burakoff, Dana Farber Cancer Institute, Boston, MA) and the α4β7-expressing human JY B-cell line (American Type Culture Collection, Rockville, MD) were maintained in culture at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum. K562 cell lines transfected with either the human α1-integrin chain (from Dr. Phil Gotwals, Biogen Idec, Inc., Cambridge, MA) or with the human α2-integrin chain (a gift from M. Hemler, Dana Farber Cancer Institute, Boston, MA) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1 mg/ml G418. All cells were periodically monitored for high integrin surface expression by fluorescence-activated cell sorter analysis.

The binding of the various inhibitors to α4β1 were assessed on Jurkat cells by competition using radioactive [35S]BIO7662 as a
with TBS containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$ and treated with using a Neubauer hemocytometer. The cells were further diluted and transferred to a scintillation vial containing 2.9 ml of ScintiVerse II (Fisher Scientific Co., Pittsburgh, PA). Cell-associated radioactivity was quantified by scintillation counting. All studies were performed in siliconized 1.5-ml Eppendorf tubes with a standard 1-ml sample volume. Each condition was tested in at least two independent studies. Non-specific binding of [35S]BIO7662 to cells was assessed at each cell concentration and [35S]BIO7662 concentration in TBS, but in the absence of added metal ion. Specific counts bound were calculated by subtracting non-specific counts from total counts bound. Counts bound under these conditions measure integrin that remained free to bind the [35S]BIO7662 after treatment with test compound.

The ability of test compounds to block $\alpha_{6}\beta_{1}$/collagen I and $\alpha_{6}\beta_{1}$/collagen IV interactions were evaluated in cell adhesion assays. Collagen I and collagen IV were immobilized onto a 96-well Corning Easy Wash plate (Corning catalog no. 25801). Human integrin-expressing cell lines (2 $\times$ 10$^5$/well) were labeled with a fluorescent compound, 2 $\mu$M BCECF-AM (catalog no. B1150; Molecular Probes, Eugene, OR), and treated with plus or minus test compounds. After 30 min, the plates were washed and bound cells quantified in a Cytofluor fluorescence plate reader. The assay buffer was 24 mM Tris-HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl, 2 mM glucose, and 0.1% bovine serum albumin, containing 1 mM MnCl$_2$. The specificity of binding was controlled for using integrin-specific neutralizing monovalent antibodies. The antibodies were run at 10 $\mu$g/ml on each day of the assay and were as follows: anti-$\alpha_{6}\beta_{1}$ HI1/2 antibody (Biogen Idec, Inc.), anti-$\alpha_{2}\beta_{1}$ 2G8 antibody (Biogen Idec, Inc.), and $\alpha_{6}$10.1 antibody (Biogen Idec, Inc.).

Samples were also evaluated for function using the VCAM-Ig direct binding assay. Details for the construction of the VCAM-Ig animal cell expression vector, the generation of a Chinese hamster ovary cell line expressing VCAM-Ig, conjugation of the VCAM-Ig to alkaline phosphatase, and the development of a direct binding assay for characterizing $\alpha_{6}\beta_{1}$ and $\alpha_{6}\beta_{7}$ binding to VCAM-Ig were as previously described (Lobb et al., 1995).

Assessing Pharmacokinetic Properties of 2a-PEG in Lewis Rats. Female Lewis rats (200 g, four animals for each route of administration) received BIO5192 10 mg/kg s.c. or 1 mg/kg i.v. or 2a-PEG at 1 mg/kg i.v. or s.c. Blood samples (250 $\mu$L/bled) were obtained at specific time points after administration. For BIO5192, blood samples were drawn at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 24, 32, 48, 72, and 168 h following i.v. administration and at 0.5, 1, 2, 4, 7, 24 h following s.c. administration. For 2a-PEG, blood samples were drawn at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 24, 32, 48, 72, and 168 h following i.v. administration and 1, 2, 4, 6, 8, 24, 32, 48, and 72 h following s.c. administration. Because of the large number of time points in the 2a-PEG i.v. study, two groups of animals were treated such that samples from one group were drawn at 0.08, 0.5, 2, 6, 24, 48, and 168 h and those from the other group were taken at 0.25, 1, 4, 8, 30, and 72 h (both groups were treated concurrently). Serum samples were analyzed for 2a-PEG levels using an electrochemiluminescence assay. Pharmacokinetic parameters were calculated from the mass spectrometry data by noncompartmental analysis using the WinNonLin v4.0 program. Pharmacokinetic parameters include $C_{\text{max}}$ (maximum serum concentration), $T_{1/2}$ (terminal phase half-life), and bioavailability. Area under the curve (AUC) was calculated using the trapezoidal rule. Percent bioavailability was calculated from the following equation: $(\text{AUC}_{\text{extravascular}}/\text{AUC}_{\text{oral}}) \times (\text{Dose}_{\text{oral}}/\text{Dose}_{\text{extravascular}}) \times 100$. BIO5192 blood samples were drawn and analyzed previously (Leone et al., 2003).

Levels of 2a-PEG in rat plasma were measured by electrochemiluminescence in a competition assay format with biotinylated 1a as a probe using a modified version of the assay previously described (Weinreb et al., 2002). Dilutions of test samples in 50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 1 mM MnCl$_2$, 1% rat plasma, 50 ng/ml $\alpha_{6}$1, and $0.1 \mu$g/ml ruthenium-labeled monoclonal antibody B5G10 were incubated at room temperature for 20 min. Biotin-1a (0.5 nM) was added, and samples were incubated for 60 min at room temperature. Fifty microliters of a 0.5 mg/ml solution of DYNabeads M-280 streptavidin beads (catalog no. 402-175-02; Igen, Inc., Gaithersburg, MD) that have been prepared in assay buffer were added, and samples were incubated for 30 min at room temperature with constant mixing. Origen assay buffer (150 $\mu$L) was added, and samples were analyzed in a model 1100-1000 Origen analyzer (Igen, Inc.) following the manufacturer's instructions. Concentrations of 2a-PEG were interpolated from a standard curve generated with 2a-PEG that had been diluted with plasma, reconstituted 1a with plasma, and PEG-CO-NHS, mol. wt. 3.4 kDa (Nektar Molecular Engineering) and separating the modified 1a from unmodified by size exclusion chro
matography on a Superdex peptide column (Amersham Biosciences Inc.).

Assessing Lymphocyte Counts and Subtypes Following Inhibitor Treatments. Female Lewis rats were injected with a single dose of either 2a-PEG (1 mg/kg s.c. in PBS) or BIO5192 (30 mg/kg s.c. in Tris/lactose) or with their respective vehicles at time 0. At each time point, 0.3 ml of blood from triplicate animals was drawn from the jugular vein without anesthesia and collected into Cappiject purple-top microtainer tubes containing ethylenediaminetetraacetic acid (T-MQK; Torumo Medical Corp., Somerset, NJ). Plasma samples were analyzed for lymphocyte count using an Abbott CellDyn 3500 cell analyzer. Blood samples from the 2a-PEG-treated animals were drawn at 4, 8, and 12 h and on days 1, 2, 3, 4, 5, 6, 7, 8, and 9 and for the BIO5192-treated animals, after 2, 6, 24, and 48 h.

Rat EAE Model. Female Lewis rats obtained from Harlan (Indianapolis, IN) were housed in ventilated cage racks and allowed food and water ad libitum. At approximately 9 weeks of age, animals were immunized with an emulsion of guinea pig myelin basic protein (MBP) peptide in complete Freund’s adjuvant. MBP peptide sequence of immunized with an emulsion of guinea pig myelin basic protein (MBP) and for the BIO5192-treated animals, after 2, 6, 24, and 48 h. were drawn at 4, 8, and 12 h and on days 1, 2, 3, 4, 5, 6, 7, 8, and 9 and for the BIO5192-treated animals, after 2, 6, 24, and 48 h.

Results

Designing PEGylated α4β1 Inhibitors. BIO5192 is a small molecule inhibitor with extraordinary potency and selectivity for α4β1 (Leone et al., 2003; Scott et al., 2004). Despite its high affinity for α4β1 (Kd of 9 pM), when BIO5192 was tested in vivo in EAE, a daily 30 mg/kg s.c. treatment regimen was needed to achieve efficacy (Leone et al., 2003). The high dose requirement for efficacy was largely driven by its short circulating half-life. In an attempt to improve the pharmacokinetic properties of this class of inhibitors, mPEG was attached using a targeted approach where amino groups were engineered into the inhibitor as sites for attachment. BIO7662, a derivative of BIO5192 lacking the dichloro substitution on the benzene ring, was selected as the framework for the modifications. BIO7662 is indistinguishable from BIO5192 in its integrin binding properties (see below). Figure 1 shows a schematic illustrating the positions of the attachment sites within BIO7662. Two target sites were chosen based on extensive structure–activity relationship studies we previously had performed on related α4β1 inhibitors (Lin et al., 1999; Pepinsky et al., 2002). For site 1, the leucine moiety (R1 = 2-methylpropyl) was replaced with a lysine with a 6-amino-hexanoyl linker attached (R1 = 4-(6-amino-hexanoyl)amino-n-butyl). In site 2, proline (R2 = hydrogen) was replaced with amino-proline (R2 = amino) with and without the 6-amino-hexanoyl linker attached, both in the syn and anti orientation.

The affinities of the PEGylated inhibitors for α4β1 were calculated from association and dissociation rate constants using a kinetic analysis. For these studies, Jurkat cells expressing α4β1 (75,000 copies/cell) were treated with the inhibitors and then [35S]BIO7662 was added as a probe to detect free α4β1. The levels of bound inhibitor were calculated from a measure of total [35S]BIO7662 bound in the absence of added inhibitor and counts bound following inhibitor treatment. Results from these analyses are shown in Fig. 2 and Table 1. On rates for all the samples were similar, ranging from 4.0 × 10⁶ M⁻¹ s⁻¹ for BIO7662 to 2.4 to 3.2 × 10⁶ M⁻¹ s⁻¹ for the five PEGylated compounds (Fig. 2a and Table 1). Off rates varied by 10-fold from unmodified BIO7662, which was the tightest binder, to 2d-PEG, which dissociated somewhat faster than the other PEGylated compounds (Fig. 2b). Rate constants that were calculated from the binding data are summarized in Table 1. From on- and off-rate constants, the Kd of BIO7662 for α4β1 was calculated to be 5 pM, whereas Kd values for 1a-PEG, 2a-PEG, 2b-PEG, 2c-PEG, and 2d-PEG were 50, 19, 72, 31, and 61 pM, respectively. 2a-PEG was selected as the lead candidate from this analysis because of its slightly higher affinity for α4β1.

The following studies were performed to further characterize the properties of PEGylated inhibitors. First, their selectivity for α4β1 was evaluated by assessing the binding of PEGylated inhibitors to cells expressing α4β7, α1β1, and α2β1 (Table 2). The PEGylated compounds were highly se-
selective for α4β1. Like BIO5192, the affinities of the PEGylated inhibitors were >2000-fold higher for α4β1 than for the related integrin αβ7, which shares many of the same ligands as α4β1. No binding of the inhibitors to α1β1 and α2β1 was detected at 10 μM compound, the highest dose tested (Table 2). The PEGylated inhibitors were active on human, rat, and mouse α4β1 with no apparent differences in affinity.

Second, the effect of protein binding on the affinity of the PEGylated inhibitors for α4β1 on Jurkat cells was evaluated from equilibrium binding measurements. A slight shift in IC50 values was observed for all the inhibitors in the presence of added plasma compared with values generated in the absence of plasma (Table 1). An IC50 value of 1.2 nM was measured for compound 2a-PEG in 100% plasma versus values of 2.2, 2.5, 1.5, and 2.5 nM for compounds 1a-PEG, 2b-PEG, 2c-PEG, and 2d-PEG, respectively. The apparent discrepancies in the binding constants calculated in Table 1 from the IC50 values and from the kinetic data arise because the Kd values of the hybrid inhibitors for α4β1 are lower than the concentration of α4β1 in the binding assay. Consequently, IC50 values of <1 nM reflect the concentration of α4β1 and not the affinity for α4β1 as discussed elsewhere (Pepinsky et al., 2002).

Third, the effect of PEG chain length on binding was evaluated. For these studies, a series of compounds containing 0.35, 2, 5, 20, 30, and 50 kDa of mPEG was synthesized and tested for binding to α4β1 on Jurkat cells with VCAM-Ig as a reporter (see Materials and Methods). IC50 values of <1.0, 0.8, 1.7, 3.7, 3.0, and 3.2 nM were measured for the six PEGylated compounds, respectively. Although there clearly was a dependence of PEG size on the IC50 for binding of the compounds to α4β1, the effect was small. The 20-kDa version was selected from other considerations. First, from extensive published pharmacokinetic profiling studies (see for example, Knauf et al., 1988) we can infer that compounds with PEGs of 5 kDa or smaller attached will not produce an acceptable increase in systemic exposure. Second, the attachment of a large PEG will impact a compound’s bioavailability and tissue penetration. Based on such considerations, both the 20- and 30-kDa PEGylated inhibitors were tested in vivo. The 30-kDa version was less effective at eliciting a lymphocytosis response than the corresponding product with a 20-kDa PEG attached, and no further in vivo studies on the 30-kDa version were performed (data not shown).

### Table 1

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<th>Compound</th>
<th>k_on/10^6</th>
<th>k_off/10^{-4}</th>
<th>K_D</th>
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### Table 2

Integrin selectivity data for PEGylated inhibitors

The selectivity of the PEGylated inhibitors for the four integrins indicated were tested in adhesion formats for α1β1 and α2β1 by direct binding for α4β1. Serial dilutions of each compound were tested and IC50 values calculated from the concentration dependence of the inhibition curves. Kd values for binding to α4β1 are shown, which are derived from kinetic data.

<table>
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<tr>
<th>Integrin</th>
<th>BIO7662</th>
<th>1a-PEG</th>
<th>2a-PEG</th>
<th>2b-PEG</th>
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Fig. 2. Assessing binding of PEGylated inhibitors to Jurkat cells expressing α4β1 using kinetic measurements. The binding of 1a-PEG, 2a-PEG, and 2b-PEG to Jurkat cells was evaluated using a competition assay format with [35S]BIO7662 as a probe as described previously (Chen et al., 2001). The levels of bound inhibitor were calculated from a measure of total [35S]BIO7662 bound in the absence of added inhibitor and counts bound following inhibitor treatment. a, for on-rate measurements, Jurkat cells (1 × 10⁶ cells/ml) in TBS containing 1 mM Ca²⁺ and 1 mM Mg²⁺ were treated with 1 nM test compound for varying lengths of time and then treated with 5 nM [35S]BIO7662 for 10 min. Cells were collected by centrifugation and subjected to scintillation counting. The data were fitted to an exponential equation by nonlinear regression. b, for off rates, Jurkat cells (1 × 10⁶ cells/ml) in TBS containing 1 mM Ca²⁺ and 1 mM Mg²⁺ were treated with 5 nM test compound for 1 h, pelleted, and resuspended in 5 nM [35S]BIO7662 in TBS containing 1 mM Ca²⁺ and 1 mM Mg²⁺ for varying lengths of time. Cells were pelleted at each time point and cell-associated [35S]BIO7662 measured by scintillation counting. Dissociation data are represented as a percentage of the maximum total counts bound as a function of time. The data were fitted to a single-exponential for all curves. k_on and k_off values were calculated from the curve fits. k_on is the observed rate constant; k_off is the observed binding constant divided by the concentration of BIO7662. Data shown are from a single determination of the on and off rates.
bution was only slightly reduced for the PEGylated inhibitor (168 ml/kg for BIO5192 versus 82 ml/kg for 2a-PEG). Although the effects of PEGylation on Vss are well established and the observed value of 82 ml/kg for 2a-PEG is in line with observed values for other PEGylated products, we were somewhat surprised by the relatively small Vss value for BIO5192. This may result from the binding of BIO5192 to serum albumin (Leone et al., 2003), which could have reduced its distribution into tissues.

Pharmacokinetic data following s.c. dosing for 2a-PEG and BIO5192 are shown in Fig. 3B. Again, PEGylation had a profound effect on the pharmacokinetic parameters (summarized in Table 3) as evident from the large increase in the AUC of the PEGylated compound compared with unmodified BIO5192. Following 1 mg/kg s.c. dosing for 2a-PEG, an AUC of 48.9 µg × h/ml was observed, and >100 ng/ml blood levels were maintained for over 72 h. In contrast, following a 10-fold higher dose of 10 mg/kg s.c. for BIO5192 an AUC of only 6.1 µg × h/ml was observed, and >100 ng/ml blood levels were maintained for only 12 h. The bioavailability of 2a-PEG and BIO5192 were similar, 30% and 20%, respectively.

### Comparative Pharmacodynamics of 2a-PEG in Rats

Administration of BIO5192 and 2a-PEG to rats produced an α4-integrin-dependent lymphocytosis that was sustained as long as sufficient concentrations of the inhibitors were maintained in circulation. From previous studies (Leone et al., 2003), we estimated that >90% of α4β1 receptors had to be occupied to maintain lymphocytosis and therefore a target concentration of greater than or equal to 10 ng/ml compound needs to be maintained. Figure 4 shows lymphocytosis induced in vivo by 2a-PEG treatment. A 2-fold increase in lymphocyte levels was observed. The maximal level of induction was reached at the earliest time point tested, after 4 h. Elevated lymphocyte levels were maintained for 6 days. By day 9 lymphocyte levels had returned to normal. No change in lymphocyte counts was observed following treatment of rats with 2 mg/kg PEG control. BIO5192 treatment produced

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**Table 3**

Comparison of noncompartmental pharmacokinetic parameters for BIO5192 and 2a-PEG in rats following intravenous and subcutaneous administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BIO5192</th>
<th>2a-PEG</th>
<th>BIO5192</th>
<th>2a-PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Route</td>
<td>i.v.</td>
<td>i.v.</td>
<td>s.c.</td>
<td>s.c.</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>CL (ml/kg)</td>
<td>168</td>
<td>81.6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>AUC (µg × h/ml)</td>
<td>2.3</td>
<td>161</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>% (F)</td>
<td>NA</td>
<td>20</td>
<td>30.4</td>
<td>30.4</td>
</tr>
</tbody>
</table>

F, bioavailability; CL, systemic clearance; NA, not applicable.
a lymphocytosis that lasted 1 day, and lymphocyte levels returned to normal by day 2. The biological effects of BIO5192 and 2a-PEG were clearly associated with their pharmacokinetic properties. From these data, we infer that a once-a-week treatment with 1 mg/kg 2a-PEG is sufficient to maintain efficacious doses in blood.

2a-PEG Treatment Delays Paralysis Associated with EAE. EAE was induced in female Lewis rats with myelin basic protein peptide. The time course for EAE is shown in Fig. 5. The effects of the myelin basic protein peptide treatment on the paralytic score were monitored from day 7 to day 19. The earliest onset of paralysis in the no treatment control group occurred on day 9 followed by a peak of disease between days 10 and 12 with a mean paralytic score of 3. The disease resolved to a baseline paralytic score of 0 by day 15. 2a-PEG treatment following injection on day 7 (1 mg/kg s.c.) resulted in a 2- to 3-day delay in onset of the disease. No delay in onset of the disease was observed following treatment of rats with 1 mg/kg s.c. of the PEG control. Daily (30 mg/kg s.c.) dosing with BIO5192 produced a similar 2- to 3-day shift in onset of the disease. The disease in rats from both the 2a-PEG and BIO5192 treatment groups reached a peak on day 9 followed by a peak of disease between days 10 and 12 with a mean paralytic score of 3. The disease resolved to a baseline paralytic score of 0 by day 15. 2a-PEG treatment following injection on day 7 (1 mg/kg s.c.) resulted in a 2- to 3-day delay in onset of the disease. No delay in onset of the disease was observed following treatment of rats with 1 mg/kg s.c. of the PEG control. Daily (30 mg/kg s.c.) dosing with BIO5192 produced a similar 2- to 3-day shift in onset of the disease. The disease in rats from both the 2a-PEG and BIO5192 treatment groups reached a mean paralytic score of 3.0 indicating that, with both inhibitors, there was no significant effect on the severity of the disease under the conditions tested. The statistical significance of differences in severity of disease (peak height) and day of peak disease score (peak day) were assessed using a one-way analysis of variance followed by Fisher’s protected least significant difference test. In a comparison of the day of peak disease for treated versus controls, P values of <0.0001 were obtained for BIO5192 treatment versus untreated controls and 0.0005 versus control PEG. Similarly, P values of <0.0001 were obtained for 2a-PEG treatment versus untreated controls and 0.0001 versus control PEG. No statistically significant changes were observed in peak height for either BIO5192 or 2a-PEG when compared with either untreated control or control PEG.

Discussion

We have designed, produced, and characterized a series of PEGylated small molecules that are potent and selective αβ1 inhibitors. The lead compound, 2a-PEG, is a 19 pM inhibitor. PEGylation dramatically improved the pharmacokinetic and pharmacodynamic properties of the inhibitor. Increases in serum half-life (1.1 h for unmodified inhibitor versus 17.9 h after PEGylation) and decreases in clearance (6.2 ml/h/kg after PEGylation versus 426 ml/h/kg for unmodified inhibitor) resulted in substantial improvement in the systemic exposure of the PEGylated compound. The volume of distribution was only slightly reduced as a result of PEGylation, and values approached that of the vascular space. Using lymphocytosis as a pharmacodynamic marker of efficacy, we determined that once-a-week treatment with 1 mg/kg 2a-PEG was sufficient to maintain levels capable of blocking α4β1 function. Indeed when 2a-PEG was evaluated in EAE using this dosing regimen, it was efficacious. For the unmodified inhibitor, daily 30 mg/kg BIO5192 dosing was needed to produce a similar degree of efficacy in EAE. Together, the lower dose and reduced frequency of dosing for 2a-PEG translated into a >200-fold decrease in the minimal efficacious dose of the PEGylated versus the unmodified inhibitor.

The α4β1 small molecules were PEGylated by a targeted approach using amines engineered into the molecule as the sites of attachment. Site 1 was targeted by substituting the leucine moiety with lysine (Fig. 1). A 6-aminohexanoyl linker was attached to the lysine to minimize potential steric complications that might result from modifications at the site. When 1a was tested for its affinity for α4β1, we were surprised by the 50-fold drop in affinity (250 pM) relative to 5 pM for the parent compound BIO7662, since its affinity was 50 pM after PEGylation. To reconcile this disparity, we synthesized an acylated version of 1a, compound 1b, in which the amino group was acetylated. The Kᵦ of 1b for α4β1 was 45 pM, suggesting that the positive charge on 1a impacted binding. Site 2 was targeted by substituting the proline with amino-proline (Fig. 1). Because 4-amino-proline contains two chiral centers, we initially generated and tested four compounds with and without the 6-aminohexanoyl linker in the syn and anti orientations. The amino-proline versions in the anti orientation had slightly higher affinity for α4β1 than the corresponding syn isomers, and of these, 2a-PEG was identified as the best candidate. The positive charge on the amino-proline did not appear to significantly affect binding.

The serum protein binding properties of small molecules can often be reduced by increasing the polarity/hydrophilic character of a compound. Consequently, the addition of charges in the engineered molecules and PEG moieties in the modified compounds would be expected to minimize the serum protein binding effect, which we observed in the binding studies (Table 1). In particular, the shift in IC₅₀ that resulted from serum protein binding for 2a-PEG (0.64 nM in the absence of plasma to 1.2 nM in plasma) is smaller than the shift in IC₅₀ observed for BIO7662 (0.21 nM in the absence of plasma to 1.5 nM in plasma).

Multiple sclerosis is characterized by T-cells that infiltrate into the central nervous system and brain and bind to VCAM-1 affecting demyelination of nerve cells causing the disease. Various aspects of multiple sclerosis have been recapitulated in rodent models of EAE with differing effects and efficacy profiles of α4β1 inhibition observed in rats and mice (Kanwar et al., 2000a,b; Leone et al., 2003; Theien et al., 2003). Rat models of EAE are usually monophasic, and α4β1 inhibition delays the onset of disease. Previously, we compared the role of two types of α4β1 inhibitors, the anti-rat α4

Fig. 5. Evaluation of 2a-PEG in rat EAE. Lewis rats were injected with MBP on day 0 and then monitored daily for progression of the disease. The treatment groups receiving either no treatment (△), control PEG (1 mg/kg s.c., day 7) (▲), 2a-PEG (1 mg/kg s.c., day 7) (○), or BIO5192 (30 mg/kg s.c.) administered daily during days 5 to 14 (●). Values are given as the mean ± S.D. for 13 animals.
antibody TA-2 and BIO5192 in rat EAE (Leone et al., 2003). Whereas TA-2 and BIO5192 both were efficacious, the differences in their biochemical properties revealed key features in the inhibition that led to efficacy. First, although TA-2 recognizes α4β1 and α4β7, the selectivity of BIO5192 for α4β1 indicated that engagement of α4β1 is a key event in the progression of the disease and that inhibition of α4β7 was not necessary for efficacy in this rat EAE model. The 2a-PEG data in EAE further support this point. Second, although TA-2 treatment down-regulated α4β1 expression in vivo, BIO5192 treatment had no effect on α4β1 expression, indicating that blockade of α4β1 is sufficient for inhibiting EAE. We inferred that the difference in valency (TA-2 contains two α4-binding sites and BIO5192 has only one) leads to the capping phenomenon observed with TA-2 and subsequent events leading to down-modulation. Although we have not verified this point for 2a-PEG, we can infer that it will not promote capping since, like BIO5192, it is monovalent. In fact, an Alexa-conjugated derivative of 1a was used as part of the validation, and it did not induce capping or internalization (Leone et al., 2003).

The resulting lymphocytosis that occurs when a therapeutic dose of an α4β1 inhibitor is administered is a well documented pharmacodynamic marker of α4β1 function, which is consistent with a mechanism involving the antagonism of α4β1-integrin-dependent adhesion of leucocytes that migrate to regions of inflammation. Lymphocytosis events have been reported in studies in animals and in man with α4 antibodies and with selective α4β1 small molecule inhibitors (Leone et al., 2003; Miller et al., 2003). A strong correlation between receptor occupancy, circulating lymphocyte levels, and efficacy in EAE was observed (Leone et al., 2003). In particular, receptor occupancy and lymphocytosis were directly coupled. However, since TA-2 treatment down-modulated α4β1 expression, it was impossible to rule out that this event contributed to the effects seen with TA-2. The studies with 2a-PEG allowed us to validate in the absence of down-modulation of α4β1 that continued exposure with a long-acting inhibitor would support a lymphocytosis response. Levels of 2a-PEG sufficient to block integrin function lasted 7 days, which paralleled the lymphocytosis data. The studies with 2a-PEG provide further support that lymphocytosis can be used as a surrogate marker for establishing dosing for EAE studies.

The BIO5192 family of hybrid inhibitors was generated from a structure-activity relationship analysis by fusing functional groups from two structurally distinct classes of α4β1 inhibitors. Like most of the other inhibitor series, the hybrid inhibitors are based on the LDV sequence found in the connecting segment 1 region of fibronectin. The tolyl-ureido-phenylacetyl cap, which provides selectivity (Lin et al., 1999), and benzenesulfonamide, which stabilizes the small molecule integrin interaction through its association with the metal ion binding site in the β1-A-domain (Pepinsky et al., 2002), are key components derived from the two parent series. The αβ3 crystal structure has provided the first definitive view of how integrin α- and β-chains associate to form a ligand binding pocket in the presence and absence of bound ligand (Xiong et al., 2002). Chemical cross-linking data we generated with hybrid inhibitors verify that they bind to a similar region in α4β1 (Pepinsky et al., 2002). The benzenesulfonamide provides critical contacts that stabilizes Ca2+ binding in the β1-A-domain (Chen et al., 2001) and, in turn, stabilizes the small molecule integrin binding through this interaction. Based on these observations, we infer that the small molecule is oriented in the binding pocket such that the tolylureido-phenylacetyl moiety interacts with the α4 subunit, and the aspartic acid and benzenesulfonamide interact with the β1-chain. The fact that the PEGylated compounds retain their high affinity and selectivity for α4β1 indicates that these features are maintained.

Although PEGylation has been used to improve the pharmacological properties of many therapeutic proteins, its application to small molecules has been limited. First, small molecule candidates are often inactivated by PEGylation (Greenwald et al., 1998). Our success can be explained in part by structural features of the integrin-ligand interaction. For VCAM-1, the critical residue for binding to α4β1 is Asp-40, which is present in a 5-amino acid residue loop that protrudes out from the surface of the protein (Wang et al., 1995). Because the contact is derived from such a small surface of VCAM-1, it is easy to reconcile how the PEG can be oriented away from the contact site and not interfere with binding. Since all integrins share a similar structure, a PEGylation-based strategy should be applicable to other integrin small molecule inhibitor programs. Second, the goal of most small molecule programs is to develop an oral drug candidate. Although PEGylation is unlikely to improve systemic exposure following oral delivery, the improvements in systemic exposure and the need for less frequent dosing that we achieved through PEGylation provided an attractive alternative delivery paradigm.

In summary, we have produced a PEGylated small molecule (2a-PEG) that is a potent and selective inhibitor of α4β1 function in vivo. The pharmacokinetic and pharmacodynamic properties of 2a-PEG are dramatically improved relative to the unmodified compound. 2a-PEG was efficacious in a rat model of EAE at a 30-fold lower dose than the parent compound and required only a once-a-week dosing regimen compared with daily administration for the parent. The use of potent, selective PEGylated α4β1 integrin inhibitors therapeutically for the treatment of inflammatory diseases represents an exciting new avenue for integrin drug development.

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References


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