Inhibition of destructive autoimmune arthritis in FcγRIIa transgenic mice by small chemical entities

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The interaction of immune complexes with the human Fc receptor, FcγRIIa, initiates the release of inflammatory mediators and is implicated in the pathogenesis of human autoimmune diseases, including rheumatoid arthritis and systemic lupus erythematosus, so this FcR is a potential target for therapy. We have used the three-dimensional structure of an FcγRIIa dimer to design small molecule inhibitors, modeled on a distinct groove and pocket created by receptor dimerization, adjacent to the ligand-binding sites. These small chemical entities (SCEs) blocked immune complex-induced platelet activation and aggregation and tumor necrosis factor secretion from macrophages in a human cell line and transgenic mouse macrophages. The SCE appeared specific for FcγRIIa, as they inhibited only immune complex-induced responses and had no effect on responses to stimuli unrelated to FcR, for example platelet stimulation with arachidonic acid. In vivo testing of the SCE in FcγRIIa transgenic mice showed that they inhibited the development and stopped the progression of collagen-induced arthritis (CIA). The SCEs were more potent than methotrexate and anti-CD3 in sustained suppression of CIA. Thus, in vitro and in vivo activity of these SCE FcγRIIa receptor antagonists demonstrated their potential as anti-inflammatory agents for autoimmune diseases involving immune complexes.

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block the interaction between FcγRIIa and IgG in immune complexes. This paper describes the *in vitro* and *in vivo* activity of several of these FcγRIIa receptor antagonists and investigates their potential as anti-inflammatory agents.

**RESULTS**

**Drug design strategy**

We have previously used X-ray crystallography to determine the 2.0-Å resolution structure of the H134 allelic form of FcγRIIa. A dimer of receptor monomers was formed around the unique two-fold crystallographic axis of the orthorhombic P2₁2₁2 crystals (Figure 1a). The dimer interface contained a large solvent-filled groove that partially overlapped, and was flanked by, the IgG-binding sites of the two FcγRIIa monomers (Figures 1b and c). Thus, molecules designed to bind across this groove (that is, target site) may inhibit the binding of immune complexes and affect the initiation of downstream signaling events that lead to inflammation.

A series of compounds were designed to take advantage of three prominent features of the target site. First, the phenyl rings of F132 lying flat against walls of the groove and at either end of the groove, separated by a distance of approximately 10 Å (1 nm), were used to design SCE containing planar ring systems, capable of participating in π–π stacking interactions with F132. These hydrophobic planar rings were joined by linkers that could be easily manipulated to increase diversity and vary flexibility while maintaining an optimal distance between hydrophobic moieties.

The second prominent attribute of the target site used was the position of the side chains of K120, located between the two F132 residues and overhanging the central part of the groove. Thus, SCEs featured groups capable of participating in electrostatic interactions with the K120 side chains. These were: (i) acidic moieties such as carboxylic acids for charge neutralization (salt bridge formation) or (ii) polar groups capable of hydrogen bonding with the positively charged amino groups.

Third, the floor of the target site was formed by hydrophobic and uncharged polar groups provided by the side chains of the residues T122, F124, N157, L162 and S164 and these determined placement of the SCE within the groove. Examples of the positions of three of the active the compounds (VIB153, 384 and 113) within the groove are shown (Figures 1d–f, respectively). Using this detailed knowledge of the three-dimensional structure and chemical attributes of the target site, more than 100 compounds were synthesized as sodium salts and

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**Figure 1** Target site used for design of small chemical entity (SCE). Solvent-accessible surface views of the FcγRIIa dimer showing the target site used for design of SCE. (a) Side view with the IgG-binding sites at the top of the FcγRIIa dimer; (b) view looking down onto the IgG-binding site and the target site and (c) close-up view of the groove-shaped target site. Key target residues are F132 (yellow), H134 (green) and K120 (red). Other residues lining the groove are: T122, F124, T155, N157, L162 and S164 (dark grey). A prominent IgG-binding site residue is highlighted (Y160), although F132 and K120 also directly participate in binding IgG. (d) Docking of VIB153, (e) docking of VIB384 and (f) docking of VIB113.
screened for inhibition of binding of FcγRIIa to immune complexes. As the SCEs were designed to fit within the groove of the FcγRIIa dimer,22 and the His/Arg polymorphic side chain is located outside the groove (Figure 1a), this polymorphism is unlikely to influence the binding of SCE. Herein, we present results for the six most active compounds that were tested in both in vitro and in vivo assays for inhibition of FcγRIIa-dependent activity (Table 1).

**Strategy for screening SCE in vitro**

To select compounds for in vivo testing, SCEs were initially subjected to an in vitro screening to determine the efficiency and specificity of action. Thus, SCEs were selected after meeting the following criteria: (i) inhibition of FcγRIIa-dependent platelet activation, (ii) inhibition of platelet aggregation and (iii) failure to inhibit arachidonic acid-induced aggregation of platelets. SCEs selected by platelet assays were also tested for inhibition of heat-aggregated gamma-globulin (HAGG)-induced TNF secretion.

### Table 1 Compounds showing activity in vitro

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Structure/chemical name</th>
<th>Inhibition of TNF-α (IC50 mM)</th>
<th>Inhibition of HAGG-mediated activation (IC50 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIB113</td>
<td>1,2-Bis(m-carboxyphenyl)ethane</td>
<td>ND</td>
<td>1.2</td>
</tr>
<tr>
<td>VIB152</td>
<td>3-{(m-carboxyphenyl)methoxy}benzoic acid</td>
<td>ND</td>
<td>0.3</td>
</tr>
<tr>
<td>VIB153</td>
<td>3-{3-(3-Carboxyphenyl)-3-oxo-propenyl}benzoic acid</td>
<td>0.31</td>
<td>0.57</td>
</tr>
<tr>
<td>VIB294</td>
<td>3-(2-Oxo-2-phenylethyl)sulfanyl]benzoic acid</td>
<td>&gt;1.47</td>
<td>0.8</td>
</tr>
<tr>
<td>VIB384</td>
<td>4-(2-Oxo-2-phenylethyl)sulfanyl]benzoic acid</td>
<td>0.34</td>
<td>0.18</td>
</tr>
<tr>
<td>VIB197</td>
<td>6-{Carboxyloxymino}caproic acid</td>
<td>&gt;1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>BRI6801</td>
<td>4-amino-1-{3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-pyrimidin-2-one</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: HAGG, heat-aggregated gamma-globulin; ND, not documented; TNF, tumor necrosis factor.
IC50 results for the fluorescence-activated cell sorting (FACS) tests. All His/His or Arg/His phenotype (data not shown). Table 1 shows the difference in activity when SCEs were tested on platelets of Arg/Arg, were collected from different donors each week. There was no activation pathways, including ADP and arachidonic acid. Platelets aggregation and for specificity by comparing their effects on other for their ability to inhibit immune complex-induced (HAGG) platelet aggregation in a dose-dependent manner and specificity was demonstrated by the lack of inhibition of aggregation mediated by ADP. Platelets aggregated normally after subsequent addition of arachidonic acid.

SCE inhibition of TNF-α secretion

Although TNF-α secretion is not specific for arthritis induction, TNF is a potent, clinically relevant inflammatory mediator in the development of RA, and immune complexes are known to induce TNF secretion. The four most active SCEs, selected by platelet assays, were tested for their capacity to inhibit immune complex-dependent TNF-α secretion from human and transgenic mouse macrophages. Using a differentiated human monocyte cell line, U937 that expresses the R134 allelic form of the FcR, three compounds, VIB153, 384 and 294 showed dose-dependent inhibition of TNF-α secretion (Figure 3a), with activity similar to the anti-FcRRIIa antibody IV.3 (71–78% inhibition at the highest dose). VIB197, although inhibiting immune complex-dependent platelet aggregation, was a poor inhibitor of TNF-α secretion (Figure 3a). Table 1 also shows the IC50 for the compounds in the TNF-α tests with U937 cells. Further analysis of VIB153 and 384 (375 μg ml⁻¹) using HAGG stimulated peritoneal macrophages from R134-expressing huFcRIIa transgenic mice, compared with wild-type C57BL/6 mice, showed that both SCEs inhibited TNF-α (Figure 3b; 70% inhibition, P<0.05 compared with untreated cells). Less inhibition (51%) was seen with C57BL/6 macrophages (P<0.05). Thus, SCE had a general anti-inflammatory effect in vitro, possibly acting through other FcRs on both strains of mice. These cross-reactions were not seen in vivo (see below).

Effects of SCE in vivo

Effects of SCE on blood cells and body weight. In the huFcRIIa transgenic mice treated with SCE or control substances, body weight and full blood counts were monitored at 3, 24 and 48 h, and 4 and 6 days. All mice remained well, with no significant weight loss (Figure 3c). In mice treated with VIB113, platelet and white cell counts decreased significantly, to levels comparable with methotrexate (P<0.05 compared with phosphate-buffered saline (PBS) controls; Figure 3c). No significant changes were seen at other time points, lower doses of VIB113 or with multiple doses of 7.5 mg of VIB197 or 153 (data not shown).

Anti-inflammatory activity of SCE in collagen-induced arthritis. Compounds that specifically inhibited immune complex-mediated activation and aggregation of platelets were tested in vivo for activity in the collagen-induced arthritis (CIA) model. Mice were treated with collagen II in complete Freund’s adjuvant (CFA) 21 days prior to treatment with SCE, receiving their first dose of SCE on the same day as a second dose of collagen II/CFA. Although transgenic mice (RR134-expressing huFcRIIa) were highly susceptible to CIA induction and in some cases developed disease after one injection of collagen/CFA (see Figures 4b and d, and 5c and d), all mice received two injections of collagen/CFA and were randomly allocated by cage to treatment groups. No difference was seen in disease incidence between males and females.

Transgenic mice receiving VIB153 (7.5 mg per mouse, 275 mg kg⁻¹ for a 30 g mouse) on days 21, 24, 27 and 30 showed profound suppression of arthritis development, with a mean arthritis index of...
1.2 (maximum possible 12) on day 35, and no further progression of disease at day 49 (Figure 4a). No disease was seen in non-susceptible C57BL/6 mice. To evaluate how changes in the dose of VIB153 may affect arthritis development, huFcγRIIA mice were tested with the following treatment regimens: (a) 7.5 mg on day 21 followed by 14 daily doses of 1 mg; (b) 1 mg (33.3 mg kg\(^{-1}\)) on days 21, 24, 27 and 30 being the most effective treatment. Treated with VIB153 showed decreased severity of arthritis (Figure 4a), with 7.5 mg on days 21, 24, 27 and 30 being the most effective treatment.

To test the in vivo specificity of VIB153 for huFcγRIIA, VIB153 was tested for inhibition of CIA in non-transgenic DBA/1 mice. This strain is the ‘gold standard’ CIA-susceptible strain but does not carry the FcγRIIA transgene. VIB153 had no significant effect (P>0.05 compared with untreated DBA/1 controls) on the development of CIA in DBA/1 mice (Figure 4b). The lack of effect on CIA in non-transgenic DBA/1 mice argues that the inhibitory activity is predominantly directed at FcγRIIA and not other pathways that may be involved in the development of CIA.

Compounds VIB152, 113 and 197 were tested at 7.5 mg per mouse, given intraperitoneally (i.p.) on days 21, 24, 27 and 30 and compared with VIB153 at this dose (Figure 4c). Mean arthritis indices at day 35 were, 6.4 for PBS, 8.4 for VIB152, 4.5 for VIB197 and 1.3 for VIB113, compared with an index of 2.5 for VIB153 at this time point. VIB113 was as effective as VIB153 for inhibition of CIA (P<0.05) at days 35 and 49. The SCE, in order of effectiveness, were: VIB153 = 113 > 197 > 152. VIB152 was not significantly different from PBS controls (P>0.05 at days 35 and 49).

As VIB294 was very effective in vitro (Figures 2 and 3), particularly at inhibiting TNF-α secretion from the U937 macrophage cell line (Figure 4a), it was compared in vivo with VIB153 (1 mg × 14 doses; Figure 4d). There was no antiarthritic effect with VIB294, whereas VIB153 at this dose again showed significant inhibition of disease (P<0.05 compared with PBS at 35 days). Although reasons for the in vivo compared with in vitro differences are not clear, they are likely to be due to differences in pharmacological properties, as modification of the VIB294 structure, by changing the position of the carboxyl group, created VIB384 with similar activity to VIB294 (Table 1; Figures 3a and b), but improved in vivo activity. In CIA, VIB384 at all time points was as effective as VIB153 at the 7.5 mg dose. Moreover, VIB384 was effective in delaying disease progression when treatment was commenced at day 28 in mice with mild disease (index=2). In these mice, treatment with 7.5 mg on days 28, 31, 34 and 37 significantly delayed disease, with arthritis indices lower than controls (P<0.05) at days 28, 31 and 35 (Figure 5a). Treatment of mice with more developed disease (scores >4) was not effective with any of the compounds (data not shown).

In a dose–response study, mice were injected on days 21, 24, 27 and 30 with varying doses of VIB384 (7.5, 2.5 or 1.25 mg) or PBS (Figure 5b). The anti-inflammatory effects were seen to be dose dependent. The 1.25-mg dose group had indices similar to the PBS controls (P>0.05), whereas mice injected with the 2.5 mg dose developed moderate arthritis, compared with very mild arthritis in the 7.5 mg dose group (P<0.05 compared with PBS at 49 days). Clearly, VIB384 was as effective as VIB153 at inhibiting CIA in mice,
and in vivo testing confirmed the activity of two (VIB153 and 384) of the six compounds selected by in vitro assays.

Comparison of VIB153 and VIB384 with non-SCE treatments of CIA. Methotrexate and immunosuppressive anti-CD3 mAb are known to be effective in treating CIA in DBA/1 mice. We have shown earlier that fragments of anti-FcRIIa antibodies can inhibit the development of CIA in the huFcRIIa mice. To compare the efficacy of VIB153 and 384 with these treatments of CIA, groups of mice were treated with PBS, VIB153 or VIB384 (both at 7.5 mg day\(^{-1}\)) on days 21, 24, 27 and 30; the published dose and schedule of methotrexate for the six compounds selected by

**DISCUSSION**

FcγRIIa plays a pivotal role in immune complex-mediated autoimmune inflammation and our recent work has demonstrated that antibody fragments specific for FcγRIIa can effectively inhibit inflammatory responses in the huFcγRIIa transgenic mice. As FcγRIIa is expressed on many human leukocytes, modulation of FcγRIIa function could have wide-reaching effects on immune activation. The SCEs described herein were designed to inhibit activation through FcγRIIa, and can effectively treat CIA in the huFcγRIIa transgenic mice, further confirming that this receptor is a valid target for new therapies for diseases such as RA and systemic lupus erythematosus and demonstrating the effectiveness of drug design based on molecular structures defined by X-ray crystallography.

The data presented here clearly demonstrate that compounds designed to bind in the groove formed by dimerization of FcγRIIa can selectively interfere with cellular responses following immune complex binding to the FcγRIIa receptor, as demonstrated by their specificity in the platelet activation and aggregation assays. Platelets were considered ideal as target ‘cells’ as they express only FcγRIIa, thus avoiding confounding results due to IgG interaction with other FcR. Agonist pathways for platelet responses are well defined, allowing evaluation of specificity and action of SCE on non-FcγRIIa pathways (for example, arachidonic acid, ADP or thrombin). Compounds that inhibited IgG-dependent platelet activation and aggregation pathways

**Figure 4** Comparison of activity of small chemical entities (SCEs) in collagen-induced arthritis (CIA). (a) The huFcγRIIa or C57BL/6 mice immunized with collagen II (days 0 and 21) were treated either with: 7.5 mg of VIB153 on days 21, 24, 27 and 30 (huFcγRIIa n=18); 7.5 mg on day 21, then 1 mg day\(^{-1}\) on days 22–35 (7.5 mg, huFcγRIIa n=14); 1 mg day\(^{-1}\) on days 21–35 (huFcγRIIa n=7) or with 0.33 mg day\(^{-1}\) on days 21–35 (huFcγRIIa n=44). Controls were treated with phosphate-buffered saline (PBS), 0.5 ml day\(^{-1}\) i.p. days 21–35 (huFcγRIIa n=24; C57BL/6, n=12).

(b) Specificity of action of VIB153: treatment of CIA-susceptible non-transgenic DBA/1 mice with VIB153, 7.5 mg per mouse i.p. on days 21, 24, 27 and 30, following induction of CIA (n=15). The arthritis index at days 35 and 49 (P<0.05) was not significantly different when compared with PBS treatment (n=12). The huFcγRIIa mice given VIB153, 7.5 mg per mouse (n=12) on days 21, 24, 27 and 30 showed significantly reduced disease compared with PBS (n=12) controls (P<0.05 at day 49).

(c) VIB153 (n=11) compared with VIB152 (n=5), 197 (n=11) and 113 (n=11) at doses of 7.5 mg per mouse i.p. on days 21, 24, 27 and 30 in the huFcγRIIa mice, with PBS controls. (d) VIB153 (n=7) compared with VIB294 (n=6) in the huFcγRIIa mice at 1 mg day\(^{-1}\) i.p. on days 21–35. PBS (n=9) controls (0.5 ml i.p. on days 21–35).
were selected and tested for TNF-α inhibition in vitro and in vivo testing in the CIA model, using the huFcγRIIA transgenic mice.18

The effectiveness of the in vitro screening techniques was demonstrated, as four of the six compounds selected were active in vivo, causing significant inhibition of CIA. Comparative activity in the assays was as follows:
(a) platelet activation (FACS): VIB153=384>294>197>
152>113>>BRi6801;
(b) for TNF-α inhibition: VIB294>153=384>197;
(c) CIA inhibition: VIB153=384=113>
197 (152 and 294 inactive).

Figure 5 Comparison of activity and specificity of VIB153 and VIB384.
(a) The huFcγRIIA mice were given 7.5 mg per mouse, i.p. on days 21, 24, 27 and 30 in VIB153 (n=16), VIB384 (n=12) and phosphate-buffered saline (PBS) (n=18) groups (both small chemical entities (SCEs) were P<0.05 compared with PBS at days 35 and 49). Also, mice with index=2 on day 28 were treated with VIB384 (n=5) on days 28, 31, 34, 37 (P<0.05 compared with PBS at days 35 and 42). (b) Dose response of VIB384, from 7.5 mg (n=16), 2.5 mg (n=7) or 1.25 mg (n=5) per mouse in 0.5 ml of PBS, given i.p. on days 21, 24, 27 and 30 after collagen-induced arthritis (CIA) induction in the huFcγRIIA mice. PBS (0.5 ml i.p. on days 21, 24, 27 and 30, n=19). (c) Comparison of VIB384 and methotrexate: huFcγRIIA mice given VIB384, 7.5 mg per mouse i.p., days 21, 24, 27 and 30 (n=7), or methotrexate, 30 μg per mouse i.p., days 21–31 (n=11). These treatments were equally effective at day 35 (P<0.05 compared with PBS controls), but by day 49 the index for methotrexate-treated mice had risen (P<0.05 compared with PBS group), whereas a low mean index was maintained in the VIB153- and 384-treated groups (P<0.05 compared with PBS, n=11 at day 49). (d) Comparison of PBS (n=6) with anti-FcγRIIA antibody fragment, 8.7 F(ab′)2 therapy (0.1 mg i.p. days 21, 23, 27 and 30, n=7) with anti-CD3/KT3 (0.5 mg i.p. days 20, 22, 23 and 25, n=6) and VIB153, 7.5 mg per mouse i.p., days 21, 24, 27 and 30 (n=7). These treatments were effective during treatment at day 28, but 8.7 F(ab′)2 and anti-CD3/KT3 showed diminishing inhibition once treatment ceased (>day 35).

Figure 6 (a) Photograph of the paw of a huFcγRIIA transgenic mouse with severe arthritis (score 4), 35 days after collagen-induced arthritis (CIA) induction and (b) paw of a VIB153-treated (7.5 mg per mouse, i.p. on days 21, 24, 27 and 30) huFcγRIIA transgenic age-matched mouse at 35 days. (c) Representative section (hematoxylin and eosin-stained) showing the histopathologic features of destructive arthritis in a huFcγRIIA transgenic mouse 35 days after CIA induction with mononuclear cell infiltration and advanced pannus. (d) Histopathologic features of a knee joint from a huFcγRIIA transgenic mouse 36 days after initial collagen injection and treatment with VIB153 (7.5 mg per mouse, i.p. on days 21, 24, 27 and 30) (hematoxylin and eosin-stained; original magnification ×40).
TNF-α is a major component of the inflammatory cascade and FcγRIIa has been identified by others as a target for modulation of the expression of TNF-α.29 Although inhibition of TNF-α production in vitro cannot be extrapolated to predict inhibition of TNF-α in vivo, antibodies that block TNF-α have been very effective in treating RA,30 so TNF-α inhibition was a valuable in vitro screening test to select compounds for in vivo assessment. This approach was validated by showing that SCEs, notably VIB153 and 384, were active in vitro in this assay and also active in vivo. Different effects of the compounds in vivo could be a consequence of their respective ADME properties. Interaction with other receptors, such as FcγRIII, may play a minor role in the inhibition of TNF-α and, although this was not seen with CIA. Induction of CIA in DBA/1 mice is FcγRIII dependent.31

CIA in mice is routinely used as a model of immune complex-dependent autoimmunity, and arthritis development is dependent on binding of immune complex to FcR.2,23 and deletion or blockade of FcR inhibits the development of CIA.18,31 In vivo, VIB113, 153 and 384 were potent inhibitors of CIA. Both VIB153 and 384 showed better long-term suppression of CIA than methotrexate, anti-T-cell antibody and FcγRIIa-specific anti-FcR fragments. Given to mice with mild disease (score 2 on day 28) VIB384 significantly delayed disease progression, with arthritis indices lower than controls until day 42 (Figure 5a). However, none of the SCEs was able to control well-established disease (scores >4, data not shown). VIB384 was more effective than methotrexate and anti-CD3, agents that are used primarily in cell depletion therapies in mice,26,27 with disease returning in these animals when therapy ceased and depleted cells were replaced. FcγRIIa-specific anti-FcR fragments, although demonstrating that FcγRIIa was a valid target for CIA therapy, achieved similar short-term inhibition of disease. The 8.7 F(ab′)2 binds to the FcγRIIIa immune complex-binding site (see Figure 1), inhibiting receptor interaction with immune complexes.15 The SCEs may act quite differently, as they were designed to fit within the dimeric ‘groove’, below the immune complex-binding sites. Recent studies of dimer configurations suggest that SCEs may lock the dimer into an inactive form.1,36 We have not been able to block the binding of antibody to receptor with the SCE in in vitro studies using transfected cell lines and human platelets. The antibody-binding site is outside the dimer groove and the drugs were designed to bind in the groove, therefore binding sites should not overlap. We cannot rule out the possibility of some allosteric effects due to the binding of SCEs in the groove that may prevent activation of cells by immune complexes. Affinity constants for binding of SCEs to receptor have not yet been measured, because the target receptor is cell based and labeled ligands are not available. The pharmacokinetic parameters are also yet to be determined. The SCEs are small (molecular weight 250–300) and would be cleared rapidly in vivo, and this is reflected by the higher doses needed for activity.

Rheumatoid arthritis is an autoimmune disorder of unknown etiology, characterized by erosive synovitis, often accompanied with extra articular involvement, such as renal, cardiac, pulmonary and vascular inflammation.37 Treatment for RA include a variety of therapies aimed to relieve pain and swelling of the joints, to slow disease progression and to stop cartilage and bone destruction.38 However, many current drugs, although used long term, do not prevent disease progression.39–42 Novel anti-inflammatory drugs that inhibit FcγRIIa function could block disease development early, before activation of the inflammatory cascade. Although FcγRIIa antagonists could reduce host resistance to infection, this is unlikely given that humans dispose of immune complexes through C1q binding and subsequent elimination by the liver through capture by CR1 on erythrocytes.43 Blocking FcγRIIa should not interfere with these pathways. Increased expression of inhibitory FcγRIIB44–46 or blockade of FcγRIIa47,48 has been investigated as therapeutic targets for drugs and some have been validated in mouse models48,49 and in human diseases such as systemic lupus erythematosus and RA.46,47 In summary, anti-FcR agents could be less immunosuppressive and act downstream of immune complex formation, blocking FcR-mediated cellular activation. Our earlier studies of immune complex disease in the huFcγRIIa mice validate this receptor’s role in autoimmune inflammation,12,13,18 and the data presented herein show that treatments designed to block FcγRIIa function can inhibit the development of immune complex-mediated disease.

METHODS

Design and synthesis of SCE

The design, synthesis and characterization of the SCE was based on the three-dimensional structure of FcγRIIa, identified as a crystallographic dimer and detailed elsewhere.27 The compounds (listed in Table 1) were docked into the FcR dimer using the MINIMIZE Dock subroutine in Sybyl 7.2 (Tripos Associates, St Louis, MO, USA) assuming a rigid host site and using Gasteiger–Huckel charges. The ligand (as the dicarboxylate) was then allowed to relax within the confines of the active site (using the Tripos forcefield, default convergent conditions). COMPARE ENERGY Dock was then used to ascertain that the resulting docked structure was both electrostatically and sterically favorable. Selected compounds were synthesized with >95% purity and tested in vitro and in vivo as aqueous solutions of sodium salts as detailed earlier.49

HAGG preparation

HAGG was prepared as described earlier49 from Sandoglobulin (Sandoz, Novartis Pharmaceuticals Co., East Hanover, NJ, USA). Briefly, Sandoglobulin (8 mg/ml−1 in PBS) was heated for 30 min at 63°C and centrifuged at 10,000 g, 4°C, 10 min, and the supernatant was incubated on ice for 30 min with 1% (w/v) polyethylene glycol 6000 (PEG 6000; Sigma Chemical Co., St Louis, MO, USA) in PBS. The precipitated complexes were centrifuged (10,000 g, 4°C, 10 min), the supernatant was discarded and complexes were dissolved in PBS at 5 mg ml−1 and stored at −20°C.

SCE in HAGG-mediated platelet activation

The platelet-rich plasma from whole blood (centrifuged at 1000 r.p.m. for 10 min) was washed in Tyrodes/Heps buffer and aliquoted at 5×107 cells per 200 μl. Compounds (50 μl of 5 mg ml−1) or PBS was added, plus 1 μM final concentration of EDTA pH 8.0 to prevent aggregation (0.9 μl of 0.5% EDTA) and incubated for 30 min. Activation agents (200 μl) were added (HAGG 400 μg ml−1 or arachidonic acid 2.5 μg ml−1) and incubated for 30 min. Samples were fixed (400 μl of 4% paraformaldehyde in PBS) and incubated for 30 min before washing with PBS/0.5% bovine serum albumin (2000 r.p.m. for 5 min). Platelets were resuspended in 100 μl of conjugated antibody diluted in PBS/bovine serum albumin, for example, fluorescein isothiocyanate-anti-human IgG, from Serotec, Göttingen, Germany, www.serotec.com.au) and phycocerythrin-anti-gpll CD41, isotype controls (IgG1-fluorescein isothiocyanate-anti-human IgG1-phycocerythrin, from Silenus, Victoria, Australia, www.chemicon.com/techsupp/Silenus.asp) and platelets were detected by FACScan. Theplatelet FACScan assay was carried out for each compound using three separate platelet donors and average percentage (%) inhibition ± standard deviation (s.d.) was calculated.

Platelet aggregation assay

Platelet suspensions (400 μl at 200–300×106 cells l−1) in a two-channel aggregometer (Chronolog, Havertown, PA, USA) were stimulated with 50 μl agonist containing 10 μg HAGG or the control agonist at concentrations as follows: 50 μM ADP; 1.6 μM arachidonic acid in 0.2 mM Tris pH 7.4 (Sigma Chemical Co.). For stimulation by ADP or arachidonic acid, 10 μl of plasma was added. Platelet aggregation was monitored for 15 min.51,52 To evaluate inhibitory activity, SCEs were dissolved in PBS (pH 7.5) and 50 μl (300 μg ml−1) of SCE suspension was added to the platelets 5 min before agonist administration.
TNF-α release from human cells simulated with HAGG

The human monocyte-like cell line U937 was differentiated in phenol 12-myrystate 13-acetate (Sigma Chemical Co.) at 20 ng ml⁻¹ in RPMI, for 24 h at 37 °C Non-adherent cells and phenol 12-myrystate 13-acetate were removed by washing with PBS. Adherent cells were harvested, plated into 24-flat well tissue culture plates (4×10⁶ cells per 500 μl well) and SCES were added to 375, 187, 93 or 47 μg ml⁻¹ final concentration. In other wells, anti-FcγRIa mAb (IV3) was added at 12.5, 6.25, 3.125 or 1.6 μg ml⁻¹ final concentration. Control cultures received an equal volume of PBS. Cultures were incubated for 2 h at 37 °C, stimulated with HAGG (250–500 μg ml⁻¹ in RPMI) and then incubated for 24 h at 37 °C. Supernatants were assayed by ELISA for TNF-α on plates coated with mouse-anti-human TNF-α mAb (Pharmingen, San Diego, CA, USA, www.pharmingen.com) at 2.5 μg ml⁻¹ (see below).

HAGG stimulation of mouse TNF-α and detection by ELISA

To assess TNF-α release from peritoneal macrophages, huFcγRIIA mice were injected intraperitoneally (i.p.) with 4% thiglycollate and macrophages were lavaged from the peritoneum 4 days later. Adherent peritoneal macrophages were isolated (1×10⁶ cells ml⁻¹) and incubated with 100 μg HAGG, with or without anti-FcγRIIA antibody or SCE as detailed above, at 37 °C for 24 h. Plates (polyvinyl chloride 96 well, Costar-Corning from DKSH, Hallam, VIC, Australia, www.DKSH.com.au) were coated with hamster anti-mouse/rat TNF-α (BD Biosciences, Franklin Lakes, NJ, USA) at 2.5 μg ml⁻¹, supernatant was added (10 μl for 1 h at 37 °C). Plates were washed 3× with PBS/Tween-20 and detected with secondary biotin anti-mouse TNF-α (BD Biosciences) and tertiary streptavidin-HRP (Amersham Lifescience, Buckinghamshire, UK) and absorbance was read at 405 nm by Fluostar optima (BMG LABTECH, Offen- burg, Germany).

SCE effects in vivo on mouse body weight and whole blood counts

SCES were injected i.p. into 8- to 12-week-old huFcγRIIA mice. Body weight and full blood counts, measured by Coulter Counter (Coulter Electronics Ltd, Dunstable, Beds, UK), were monitored at 3, 24 and 48 h, and 4 and 6 days. Results were compared with those of mice receiving PBS or met Hobhezine (30 μg per mouse i.p.).

Induction and treatment of CIA

B6.SJL/huFcγRIIA (H-2b) transgenic mice are homozygous for the R34 allele of FcγRIIa.15 No difference in disease incidence was seen in male and female mice.16 CS7BL/6 (H-2b) male and female and DBA/1 (H-2b) male mice at 8–15 weeks of age were used. Collagen type II (Sigma Chemical Co.), 2 μg ml⁻¹ in 10 μl acetic acid, was emulsified in an equal volume of CFA (Difco Laboratories, Detroit, MI, USA) and 100 μl was injected intradermally into the base of the tail. The same dose of collagen II in CFA was administered 21 days later15 and mice were examined daily for arthritis, scored for each limb: 0=mild inflammation of the paw, 2=severe inflammation of the paw, two or more digits affected, 3

in vitro tests, mean ± s.d. for triplicate samples was compared by analysis of variance. All statistical analyses were done using Microsoft Excel analysis tools (www.microsoft.com). A probability of P<0.05 was regarded as significant.

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