Chemistry & Biology, Vol. 11, 1651–1658, December, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.09.011

High-Affinity Small Molecule Inhibitors of T Cell Costimulation: Compounds for Immunotherapy

Philip Huxley,[1] Deborah H. Sutton, Phillip Debnam, Ian R. Matthews, Joanna E. Brewer, Jennifer Rose,[2] Matthew Trickett,[3] Daniel D. Williams, Torben B. Andersen, and Brendan J. Classon*
Avidex Limited
57c Milton Park
Abingdon, OX14 4RX
United Kingdom

Summary

Costimulatory molecules are important regulators of T cell activation and thus favored targets for therapeutic manipulation of immune responses. One of the key costimulatory receptors is CD80, which binds the T cell ligands, CD28, and CTLA-4. We describe a set of small compounds that bind with high specificity and low nanomolar affinity to CD80. The compounds have relatively slow off-rates and block both CD28 and CTLA-4 binding, implying that they occlude the shared ligand binding site. The compounds inhibit proinflammatory cytokine release in T cell assays with submicromolar potency, and as such, they represent promising leads for the development of novel therapeutics for immune-mediated inflammatory disease. Our results also suggest that other predominantly β proteins, such as those that dominate the cell surface, may also be accessible as potentially therapeutic targets.

Introduction

Optimal activation of T cells requires the interaction of the T cell receptor (TcR) with major-histocompatibility complex (MHC)-peptide antigens and the engagement of a costimulatory receptor by its respective ligand. The most important and well-characterized T cell costimulatory ligands are CD28 and CTLA-4 (CD152), which engage CD80 and CD86 receptors on antigen-presenting cells (APCs). Ligation of CD28 enhances T cell activation [1, 2], resulting in enhanced stability of cytokine mRNA [3], increased glucose metabolism [4], and upregulation of antiapoptotic genes [5]. In contrast, engagement of CTLA-4 delivers a negative signal to the T cell, resulting in attenuation of kinase activation [6] and overall inhibition of activation [7]. T cell costimulation is regulated by altered expression profiles of both costimulatory receptors and their ligands over the course of the immune response. For example, CD28 is constitutively expressed on naive cells, whereas CTLA-4 expression is upregulated on activated T cells [8]. Similarly, while CD86 is constitutively expressed on monocytes, B cells, and dendritic cells, CD80 is only expressed at significant levels on these cells following activation [9–14]. Therefore, it is generally believed that CD86 is the major costimulatory receptor in primary immune responses, whereas CD80 is thought to act as the dominant costimulatory receptor in established immune responses [15]. Although CD28 and CTLA-4 are each capable of binding both CD80 and CD86, there are data that suggest CD80 is the preferred receptor for CTLA-4 [16, 17]. The net inhibitory effect of this interaction is thought to play a role in maintaining immune tolerance [18], a possibility consistent with the observation that anti-CTLA-4 blocking antibodies enhance antitumor T cell responses in vivo [19]. Conversely, the elevated expression of CD80 on activated APCs and activated T cells [20] suggests a role for CD80 in ongoing immune responses, particularly at distal sites of inflammation in autoimmune disease [21, 22].

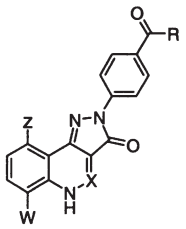
An important consequence of targeting T cell costimulation as a means of immunosuppression is that only those cells that have already received an antigen-specific signal via their TcR are expected to be susceptible to inhibition [15]. Some degree of antigen-specific inhibition is therefore anticipated, which may enhance the therapeutic potential of costimulation blockade. The effectiveness of costimulatory blockade as a means of immunosuppression was first demonstrated over a decade ago, when it was shown that CTLA-4Ig inhibits graft rejection [23] and induces long-term tolerance in mice [24]. Subsequently, CTLA-4Ig has been shown to be an effective antagonist of costimulation in rodent models of autoimmune disease, including collagen-induced arthritis [25, 26] and the experimental allergic encephalomyelitis (EAE) model of multiple sclerosis (MS) [27, 28]. CTLA-4Ig is thought to block CD28 signaling by preventing its engagement by CD80 and CD86, although a direct immunomodulatory effect of CTLA-4Ig on dendritic cells has recently been proposed [29]. Selective blockade of CD80 with monoclonal antibodies (mAbs) in the mouse EAE model has also resulted in decreased severity of disease [30–33]. For example, blocking CD80 with monoclonal antibodies during the initiation phase of EAE decreased severity of disease [30, 31]. Furthermore, it was shown that the selective blockade of costimulation using anti-CD80 F(ab) fragments during EAE remission could prevent antigenic epitope spreading and disease relapse [32, 33]. However, since CD80 is capable of engaging both stimulatory (CD28) and inhibitory (CD152) ligands [16], it is likely that timing and duration of CD80 blockade in the clinical setting will be important in achieving the desired therapeutic outcome.

Encouraging results in animal models have led to clinical trials with CTLA-4Ig in rheumatoid arthritis [34], and a recent phase III trial has since confirmed this promise [35]. A significant drawback of protein-based therapies, however, is their high treatment cost and the requirement for parenteral administration. By comparison, small compound inhibitors offer an attractive alternative, provided that target specificity, potency, and

*Correspondence: brendan.classon@avidex.com
[1]Present address: Galapagos Genomics NV, Industriepark Mechelen Noord, Generaal De Wittelaan L11 A3, B-2800 Mechelen, Belgium.
[2]Present address: Oxagen Ltd., 91 Milton Park, Abingdon, OX14 4RY, United Kingdom.
[3]Present address: Cancer Research Technology, Dominion House, 59 Bartholomew Close, London, EC1A 7BE, United Kingdom.

oral bioavailability can be achieved. Several attempts to identify small compound inhibitors of the CD80/CD28 interaction have been reported. In the first of these, a microbial metabolite isolated from a natural product library was shown to inhibit the CD28/CD80 interaction with an IC_{50} of 0.3 μM in a biochemical assay, but a relatively high concentration of the compound (20 μM) was required to inhibit IL-2 production in a cellular assay [36]. In a second study, a series of condensed aromatic peptide inhibitors with increased cellular activity were identified [37, 38]; however, nonspecific binding of these compounds to other proteins [39] suggested that they were poor lead candidates. Another study identified a third class of compounds that inhibit the CD80/CD28 interaction, but their lack of potency in cellular adhesion assays and their relatively weak inhibition of the intracellular CD28/PI3-kinase interaction were disappointing [40, 41].

We report herein a series of small compounds (average MW 424 ± 75) that bind specifically to human CD80 with low nanomolar affinity in standard binding assays. The compounds antagonize CD28 and CTLA-4 binding to CD80 and inhibit the release of the proinflammatory cytokines IL-2, IFNγ, and TNFα at submicromolar doses in T cell costimulation assays. Our data suggest that these agents represent promising leads for the development of novel small molecule autoimmune therapies.



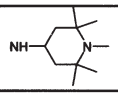
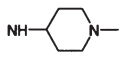
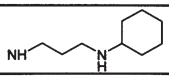
Compound	W	Z	X	R
1	F	H	CH	OH
2	F	H	CH	$NHCH_2CH_2CH_2NMe_2$
3	H	H	N	
4	F	H	N	
5	F	H	N	
6	F	F	N	

Figure 1. Chemical Structures of the Small Molecule Human CD80 Inhibitors

Results

Identification and Synthesis of Small Molecule Inhibitors

Our interest in CD80 inhibitors was prompted by ActiveBiotech AB, who identified novel small compounds [42] that blocked the CD28/CD80 interaction in a scintillation proximity assay [43]. The inhibitory properties of selected compounds were confirmed at Avidex Ltd. in a CD80/CD28 time-resolved fluorescence resonance energy transfer (TR-FRET) assay (see Experimental Procedures), which was also used to screen compound analogs produced in lead optimization programs. The structures of a lead pyrazoloquinoline (compound 1) and a more potent analog (compound 2) together with four representative examples of a second (pyrazolocinnoline) series of compounds (compounds 3–6) are shown in Figure 1. The degree to which each compound inhibited the CD80/CD28 interaction in the TR-FRET assay was determined and expressed as an EC_{50} value. The data presented in Table 1 show that we were able to identify inhibitors with potency as low as 1.3 nM. Inhibition surface plasmon resonance (SPR) experiments were also undertaken to confirm the early hits identified by TR-FRET. In the SPR experiments, biotinylated CD80 was immobilized onto the sensor surface, and a series of samples containing a fixed amount of CD28-Fc and an increasing concentration of lead compound were injected over the surface. A concentration-dependent inhibition of CD28-Fc binding to CD80 was observed for each compound and two representative examples (compounds 2 and 5; Figure 2A). The EC_{50} values of compounds 2 and 5 for inhibition of CD28/CD80 binding in SPR (see Figure 2 legend) correlated well with those determined by TR-FRET (Table 1). Compounds 2 and 5 also inhibited CTLA-4-Fc binding to CD80 (Figure 2B), but with 3-fold and 1.7-fold higher EC_{50} values, respectively (see Figure 2 legend), consistent with the higher affinity and bivalency of the CTLA-4/CD80 interaction compared to CD28/CD80 [16].

Table 1. Biochemical and Cellular Inhibitory Properties of CD80 Compounds

	TR-FRET	SPR			Jurkat Cell
Compound	EC_{50} (nM)	K_D (nM)	k_d (s^{-1})	k_a ($M^{-1}s^{-1}$)	IC_{50} (μM)
1	630	125	0.12	988,400	ND
2	66	11.3	0.023	2,237,250	13.5 (n = 2)
3	4.7	5.0	0.0089	1,708,000	1.6 ± 0.77 (n = 3)
4	3.0	3.1	0.0036	1,146,000	0.84 ± 0.19 (n = 4)
5	1.3	1.3	0.0024	1,873,000	0.77 ± 0.33 (n = 3)
6	2.7	2.4	0.0025	1,053,000	0.75 ± 0.14 (n = 3)

ND, not determined.

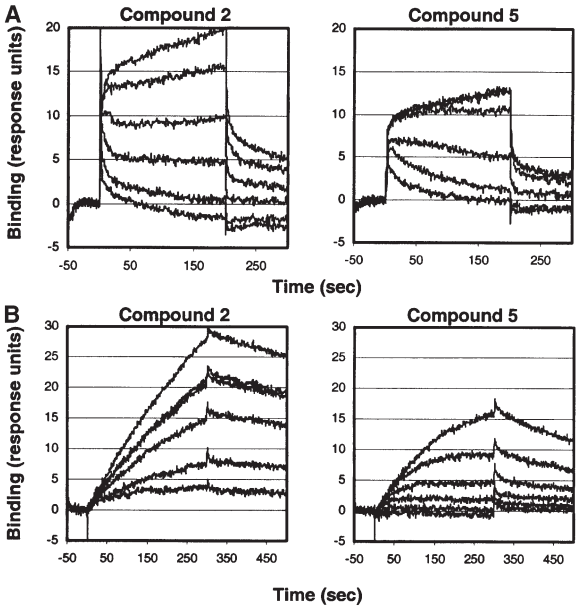


Figure 2. Inhibition of the CD28/CD80 Binding on the Biacore

Soluble biotinylated CD80 protein was immobilized on a streptavidin-conjugated CM5 chip prepared using amine coupling chemistry. Soluble-CD28-Fc (A) or CTLA-4Fc (B) at 270 nM and 5.9 pM, respectively, were mixed with increasing doses of compound 2 or compound 5 and injected onto the immobilized sCD80 surface. All compound dilution series were 3-fold dilutions (6 points) from 1000 μM, except inhibition of the CD28-CD80 interaction by compound 5 ([B], right panel), where 5-fold dilutions (6 points) from 100 μM were used. The EC_{50} values were calculated as follows: for CD28-CD80 inhibition by compound 2, 45 nM, and compound 5, 3 nM; and for CTLA4-CD80 inhibition by compound 2, 142 nM, and compound 5, 5 nM.

Small Molecule Inhibitors Are Uniquely Specific for Human CD80

The properties of the compounds were further investigated by direct SPR binding experiments, which also enabled precise identification of target protein as CD80. Selected fusion proteins were immobilized on a streptavidin-coated sensor surface, and titrated amounts of compound were applied to the surface. The data of Figure 3 show that compound 2 binds specifically to human CD80 but not the related protein, human CD86 (Figure 3B), nor human CD28-Fc (data not shown), nor do the compounds bind mouse, rat, or canine CD80, although binding to rhesus monkey CD80 was observed (data not shown). The binding affinity and kinetic rate constants for a selection of CD80 compounds are summarized in Table 1. Significantly, all the inhibitors exhibit significantly slower off-rates than the natural ligand (CD28), and a representative example (compound 4) is shown in the SPR sensorgram depicted in Figure 4.

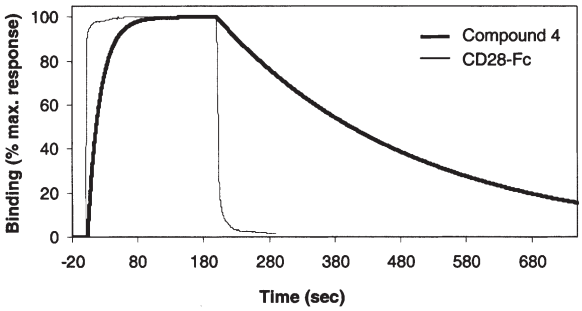


Figure 4. Binding of Compound 4 and CD28-Fc to Immobilized CD80 Analyzed by SPR

Compound and protein binding (normalized as a percentage of maximum binding for ease of comparison) is shown over time. The dissociation phase of the curve reveals rapid dissociation of the natural ligand (CD28; k_d, $\geq$ 1.6 s^{-1} [54]) compared to the small compound.

CD80-Specific Small Molecules Block T Cell Costimulation in Cell-Based Assays

The activity of the CD80-specific compounds in T cell costimulation assays was investigated. Since CD80 and CD86 are coexpressed on Raji cells (Figure 5A, inset), their relative contributions to costimulation-dependent IL-2 release were investigated in blocking experiments (Figure 5A). Partial inhibition of IL-2 secretion (approximately 25%) was observed in the presence of an anti-CD86 mAb (Figure 5A, shaded bars), whereas a CD80 mAb resulted in more pronounced inhibition, reaching a plateau of 95% at a mAb concentration of 0.03 μg/ml (Figure 5A, solid bars). This result was consistent with a previous report showing functional dominance of CD80 over CD86 on Raji cells in proliferation assays [44]. In all experiments, IL-2 secretion by Jurkat

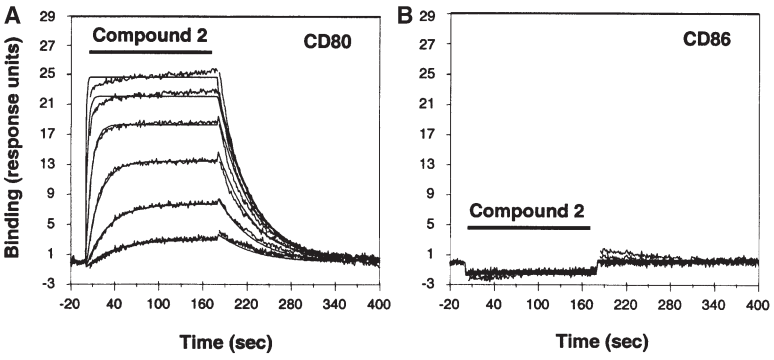


Figure 3. Binding of Compound 2 to Human CD80

Biotinylated human CD80 (A) and CD86 (B) were labeled on to spot 1 and spot 2, respectively, of a CM5 chip which had been prepared with streptavidin using standard amine coupling chemistry. The two proteins were bound to the chip to approximately 3000 RU. A range of compound concentrations (1000 nM and six 3-fold dilutions thereof) were injected sequentially, with solvent correction performed using a standard series of DMSO concentrations. A 200 s injection was used, followed by a dissociation phase of 400 s. The solid black line overlayed on each sensorgram trace represents a global fit assuming a 1:1 interaction between the compound and protein.

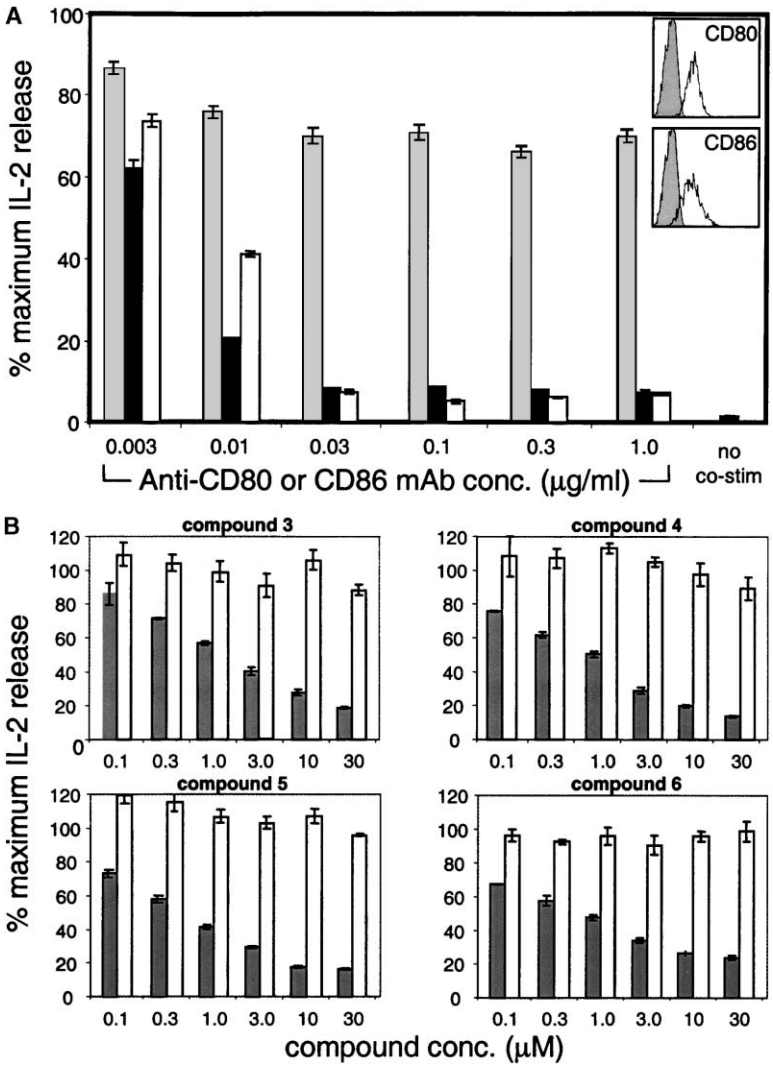


Figure 5. A CD80-Dependent Jurkat T Cell Costimulation Assay

(A) Jurkat T cells were stimulated with anti-CD3 mAb in the presence of Raji B cells, which coexpress CD80 and CD86 (inset). Anti-CD80 mAb (solid bars) or anti-CD86 mAb (shaded bars) or both mAbs (open bars) were added to the cultures at various concentrations. IL-2 release is expressed as a percentage of maximum release obtained in the absence of blocking mAbs. "No-costim" control indicates the level of IL-2 secreted by Jurkat T cells and CD3mAb in the absence of Raji cells.

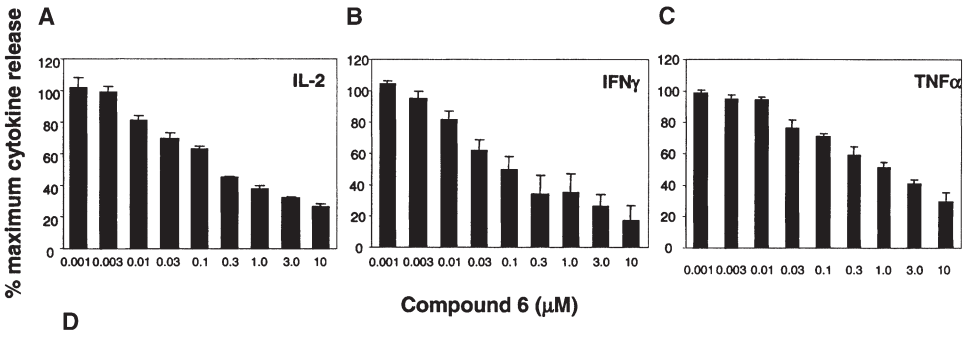
(B) CD80-specific small compounds 3–6 inhibit Jurkat T cell costimulation. Inhibition of IL-2 release by compounds (shaded bars) in the Jurkat assay (A) is expressed as percentage of maximum release obtained in the presence of DMSO alone. Compounds failed to inhibit the anti-CD3/anti-CD28 mAb (CD80-independent) control assay (open bars).

cells was insignificant in the absence of added Raji cells (see Figure 5A, "no costim" control). Using this assay, dose-dependent inhibition of IL-2 release was seen for all four compounds (Figure 5B), with IC_{50} values ranging from 0.75–0.84 μM (Table 1). An earlier lead compound (compound 2) with a lower biochemical EC_{50} (66 nM) exhibited significantly lower potency (13.5 μM) in the cell assay than lead optimized compounds 3–6. None of the compounds showed significant inhibition of IL-2 release in the control assay, when anti-CD28 antibody was added to overcome the effect of compound-mediated CD80 blockade (Figure 5B, open bars).

Finally, compounds were tested in assays using purified peripheral blood $CD4^+$ primary T cells in place of Jurkat cells. CD3 mAb and Raji cells were used to provide the primary stimulus and costimulation as before, and supernatants were assayed for IL-2, TNFα, and IFNγ at 24 and 48 hr. The data of Figure 6A show that compound 6 inhibited IL-2 secretion in the $CD4^+$ T cell assay with approximately similar potency to that observed with the Jurkat cell assay (IC_{50} = 0.75 μM versus 0.57 μM). In the same assay, compound 6 also inhibited TNFα and IFNγ secretion with IC_{50} values of 0.97 μM and 0.15 μM, respectively. For IL-2 and IFNγ, compound potency was reduced approximately 2-fold when comparing the 24 hr and 48 hr time points, whereas compound potency with respect to TNFα inhibition was unchanged over this time period (Figure 6D).

Discussion

There is a strong biological rationale for developing small molecule CD80 antagonists. The challenge facing medicinal chemists hoping to exploit this opportunity lies in the difficulties of identifying inhibitors of protein-protein interactions [45–47]. A low success rate is usually ascribed to the fact that small orally bioavailable inhibitors bind with low affinity to the (generally) flat surfaces found at the interface between interacting proteins. The compounds reported herein appear to be exceptions to this rule, given that they inhibit the CD80/CD28 interaction with IC_{50} values as low as 1–3 nM. The binding site on CD80 for CTLA-4 (and presumably also CD28) is a shallow depression that comprises



D

IC_{50} (μM) (mean ± S.D., n=3)		
	24 hr	48 hr
IL-2	0.57±0.31	1.2±0.62
IFNγ	0.15±0.031	0.26±0.20
TNFα	0.97±0.32	1.0±0.59

Figure 6. Inhibition of Cytokine Release in a $CD4^+$ T Cell Costimulation Assay

Purified T cells were incubated with anti-CD3 mAb and Raji cells in the presence of titrated amounts of compound 6. After 24 and 48 hr (data not shown), supernatants were assayed for IL-2, IFNγ, and TNFα (A–C). Cellular IC_{50} values calculated with ExcelFit using the data obtained from three experiments (employing two different blood donors) are shown in (D).

around 600 $Å^2$ of the solvent-accessible surface [48, 49]. It is possible that the inhibitors we describe bind at or near this site and effectively block ligand binding, although it is difficult to envisage how sufficient binding energy could be generated in such a shallow pocket. We therefore favor the view that binding follows a more complex mechanism, such as the adaptive ligand binding seen with a nonpeptidyl small molecule antagonist of IL-2 [50]. This issue will be resolved very easily in planned mutational analyses and/or cocrystallization studies.

CD80 contains two tandemly arranged immunoglobulin (Ig)-like domains with the active site located in the membrane distal V domain [49]. Ig-like domains consist almost exclusively of β sheet structure and are by far the most abundant protein domain observed in cell surface molecules. Since there are relatively few examples of small compounds known to interact with all β domains, the compounds described herein are of significant interest. One such example, SB 247464, binds the N-terminal half of the extracellular domain of the GM-CSF receptor [51], which consists of an Ig-like domain, a cytokine receptor domain, and two fibronectin type III domains, all of which are rich in β sheets. However, SB 247464 does not compete with the native ligand and instead acts as a cytokine mimetic, probably by inducing receptor crosslinking [52]. It will be of considerable interest to determine whether other leukocyte receptor/ligand pairs, many of which represent excellent immunotherapeutic targets, are also amenable to blockade with small compound inhibitors in the manner of CD80. Among potentially therapeutic, CD80-related molecules is the costimulatory molecule LICOS, which is implicated in sustaining T cell activation and progression of autoimmune disease [53].

To be clinically useful, small compound inhibitors of cell surface protein-protein interactions need, by definition, to inhibit binding events at the junction between interacting cells. Cell surface proteins are constrained within two dimensions, and as such, binding is favored between cognate receptor-ligand pairs when closely approximated. The inhibitory properties of the small compounds described herein are consistent with the multivalent nature of the CD28/CD80 interaction on cells. For example, whereas compounds 2–6 described herein showed potent inhibition of CD28/CD80 binding in direct binding assays, their corresponding IC_{50} values in cellular assays are between two and three orders of magnitude higher (Table 1). This reflects the avidity enhancement of cell surface protein-protein interactions not manifest when their soluble counterparts interact in solution. Additional support for an avidity-driven mechanism for CD28/CD80 binding is provided by a recent study in which CD80-conjugated beads were shown to bind CD28-transfected cells with an apparent half-maximal binding value ($B_{max}/2$) of 2–5 nM [43]. This is approximately three orders of magnitude lower than the K_D of the interacting monomers (4 μM) [54]. Compound efficacy in cellular assays will also be influenced by other, possibly more favorable factors, however, such as the threshold below which there are insufficient numbers of CD28/CD80 interactions to effect appropriate cellular responses. Significantly, it has been shown in the NOD mouse model of autoimmune diabetes that as little as a 50% reduction in the level of endogenous CD80 cell surface expression due to gene-dosage effects has a significant impact on the course of disease [55].

Previously, Erbe et al. described CD80-specific compounds that exhibited 4–17 nM potency in direct binding assays, but these compounds were unable to inhibit adhesion between CD28- and CD80-transfected CHO

cells [41] and had only weak activity (20%–70% inhibition at 50 μM) in a CD28-PI3-kinase association assay (using Jurkat cells and CD80-transfected CHO cells) [40]. For comparison, we synthesized compound 6q [40] and showed that it had an EC_{50} value of 177 nM in our TR-FRET assay, compared with an IC_{50} of 4nM in the original ELISA assay [41]. This discrepancy alone may explain the relative lack of potency of compound 6q in cellular assays. However, the level of CD80 surface expression on transfected CHO cells may also be significant. Based on previous studies [56] and our own observations (data not shown), we noted relatively high cell surface expression of CD80 on transfected fibroblasts. By comparison, the Raji cells used for the assays herein express lower levels of CD80 and coexpress CD86 (Figure 5A, inset). Thus, Raji cells more faithfully represent the $CD80^+/CD86^+$ phenotype of an activated APC [10, 12, 14] and are therefore a more physiologically relevant source of costimulation than transfected fibroblasts. In terms of the responder cells, it is significant that primary $CD4^+$ T cells proved susceptible to inhibition with the CD80 compounds at submicromolar doses (Figure 6D), since results obtained with primary cells are clearly more likely to predict in vivo efficacy.

Significance

Targeting T cell costimulation remains one of the more promising avenues for modulating undesired immune responses in autoimmune disease and transplantation. The CD80 costimulatory pathway is thoroughly validated as a therapeutic target for the amelioration of disease in a variety of indications, including multiple sclerosis, rheumatoid arthritis, and graft rejection. The most effective currently available biologic therapies suffer from the coincident problems of administration and dosing, leading to high treatment costs. The CD80-specific small compound antagonists described herein offer a realistic starting point for the development of alternative, nonprotein-based orally available therapies for autoimmune disease. Protein-protein interactions, such as those involving CD80 and CD28, are generally held to be problematic as targets for small, drug-like compounds. Our results suggest that proteins involved in these interactions, in particular, those comprised of β-rich domains that dominate the cell surface, may also be accessible as therapeutic targets using small molecules.

Experimental Procedures

Chemical Synthesis

Synthesis methods for compounds 1 and 2 have been previously described in patents WO03004495 [42] and WO2004048378 [57], respectively. Compounds 3–6 can be readily prepared according to published methods in patent WO2004081011 [58]. In brief, the 4-chloro-cinnoline-3-carboxylic acid methyl ester and 4-hydrazino-benzoic acid are condensed together to form 4-(3-oxo-1,3-dihydro-pyrazolocinnolin-2-yl)-benzoic acid. This product can then be readily converted to the acid chloride with oxalyl chloride in dichloromethane. Reaction with the appropriate amine affords the desired product. The relevant patents describing synthesis methods [42, 57, 58] may be accessed free of charge at http://ep.espacenet.com.

Recombinant Protein Production

Human soluble CD80 (sCD80) and human and mouse soluble CD28 (sCD28) proteins were available as Fab and Fc fusions from Active Biotech AB (Lund, Sweden) [43]. Human, rhesus monkey, mouse, rat, and canine CD80 constructs with C-terminal BirA-tags were derived by PCR using oligonucleotide primers to amplify cDNA sequences from either commercially available libraries or cDNA generated by the reverse-transcriptase method using peripheral blood mRNA or assembled using overlapping synthetic oligonucleotides according to published sequences. cDNAs encoding rat and canine CD28 were made in similar fashion and expressed as Fc-fusion proteins. All constructs were subcloned into the pEE14.4 vector (Lonza Biologics, Slough, UK) for expression in CHO cells, and recombinant proteins were purified by lentil lectin affinity chromatography as described previously [59]. Soluble CD80 proteins were biotinylated using the BirA enzyme in the presence of 1.5 mM D-biotin, 7.6 mM $MgCl_2$, and 5 mM ATP and then separated from reaction products by size exclusion chromatography. Details of the construct design and characterization will be described elsewhere.

TR-FRET Assay

The interaction between sCD80-Fab fusion protein and sCD28-Fc fusion protein in solution was detected by anti-Fc and anti-kappa secondary antibodies and by a europium-conjugated tertiary antibody and streptavidin-allophycocyanin conjugate. On formation of the complex, europium and allophycocyanin are brought into close proximity and a signal is generated. Nonspecific interaction was measured by substituting a mouse immunoglobulin Fab fragment for the sCD80-Fab fusion protein. The assay was carried out in black 384-well plates in a final volume of 30 μl in 150 mM NaCl, 50 mM Tris (pH 7.8) (HCl), 0.1% (w/v) bovine serum albumin. Compounds were added to the above reagents in a concentration series ranging from 100 μM to 1.7 nM, and the reaction was incubated for 4 hr at room temperature. Dual measurements were made using a Victor 1420 Multilabel Counter (Wallac). First measurement, excitation 340 nm, emission 665 nM, delay 50 μs, window time 200 μs; second measurement, excitation 340 nm, emission 615 nm, delay 50 μs, window time 200 μs. Counts were automatically corrected for fluorescence crossover, quenching, and background. Inhibition of the CD80/CD28 interaction was detected by diminution of emitted fluorescence at 665 nm. The assay was robust and capable of generating reliable SAR, with a signal to background ratio of 2.5:1, giving an average Z factor per 384-well plate of 0.75.

SPR Assays

All assays were performed on Biacore S51 (Biacore AB, St Albans, Hertfordshire, UK) at an analysis temperature of 25°C. Biotin-labeled recombinant proteins were immobilized by high-affinity capture to a series S sensor chip (CM5) surface that was first coated with streptavidin. Immobilization levels were 3000–3500 RU using CD80 on spot 2 and CD86 as a negative control on spot 1. Running buffer was 10 mM HEPES (pH 7.4), 150 mM NaCl containing 0.005% surfactant P-20 and supplemented with 5% (v/v) DMSO where required. Compound characterization assays (Biacore S51 software wizard) were run using a flow rate of 30 μl/min with three start-up cycles and eight-point solvent correction cycle run after every second dilution series. Sample cycles consisted of a 60–200 s injection phase followed by a 400–1000 s dissociation phase and a final wash with 50% DMSO.

Flow Cytometry

Cell surface expression of costimulatory receptors was analyzed by fluorescence-activated cell sorter (FACS) analysis. Cells (2×10^5) were incubated with 100 μl appropriately diluted CD80 (clone MEM-233, Serotec) or CD86 (clone BU63, Serotec) mAb which had been directly conjugated with FITC. Cells were washed and then analyzed using a FACSVantage (BD Biosciences) equipped with a 488 nM laser.

Cellular Assays

Compounds were prescreened at a concentration of 30 μM on Jurkat and Raji cells (ATCC, LGC, Teddington, Middlesex, UK) using the Alamar Blue redox indicator dye assay to exclude toxic candi-

dates prior to screening in functional assays [60]. A costimulation-dependent IL-2 release assay was developed by dispensing human Raji (Burkitt's lymphoma) cells at a density of 2×10^5 cells per well in RPMI-1640 medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine (RPMI medium) in a 96-well round bottom microtitre plate. Compounds (dissolved in 100% DMSO) were added to make up a volume of 200 μl/well at the desired final concentration. In control experiments, antagonist mAbs to CD80 (clone L307.4, BD-Pharmingen) and CD86 (clone IT2.2, BD Pharmingen) were added in place of compounds. After 20 min preincubation of Raji cells and blocking agent at 37°C, Jurkat T cells were added to a final density of 2×10^5 cells per well. Monoclonal antibody to CD3 (OKT3, ATCC) was added to the cultures at a final concentration of 0.06 μg/ml. Cells were cultured at 37°C for 5 hr, after which the plates were centrifuged and the supernatants harvested for IL-2 ELISA assay (DIACLONE Research, Besancon, France) according to the manufacturer's instructions. Maximum IL-2 levels at 5 hr post-stimulation usually ranged from 0.3 ng/ml–1.5 ng/ml, and supernatants were appropriately diluted (usually 1:3) prior to assay. An anti-CD3/anti-CD28 control assay was developed by adding the antibody CD28.2 (BD-Pharmingen, San Jose, CA) to the Jurkat assay at a final concentration of 0.3 μg/ml. After 5 hr, supernatants were harvested and assayed for IL-2 as described above. Primary human $CD4^+$ T cells were purified from buffy coats (National Blood Transfusion Service, Bristol, UK) using a CD4 T cell negative isolation kit (Miltenyi, Gladbach, Germany). $CD4^+$ T cells were cocultured with Raji cells as described for the Jurkat assay, except that the CD3 mAb UCHT-1 (BD-Biosciences) was used at 0.125 μg/ml. Supernatants were harvested and analyzed for IL-2, IFNγ, and TNFα by ELISA (DIACLONE).

Acknowledgments

We are grateful to Dorthe da Graca Thrige and colleagues at Active Biotech AB and Simon Davis for helpful advice and discussions. We acknowledge Evotec OAI and Chemovation for help with synthetic and molecular compound design.

Received: June 4, 2004
Revised: September 3, 2004
Accepted: September 29, 2004
Published: December 17, 2004

References

1. Michel, F., Attal-Bonnefoy, G., Mangino, G., Mise-Omata, S., and Acuto, O. (2001). CD28 as a molecular amplifier extending TCR ligation and signaling capabilities. Immunity *15*, 935–945.
2. Harding, F.A., McArthur, J.G., Gross, J.A., Raulet, D.H., and Allison, J.P. (1992). CD28-mediated signalling costimulates murine T cells and prevents induction of anergy in T-cell clones. Nature *356*, 607–609.
3. Linsley, P.S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N.K., and Ledbetter, J.A. (1991). Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. J. Exp. Med. *173*, 721–730.
4. Frauwirth, K.A., Riley, J.L., Harris, M.H., Parry, R.V., Rathmell, J.C., Plas, D.R., Elstrom, R.L., June, C.H., and Thompson, C.B. (2002). The CD28 signaling pathway regulates glucose metabolism. Immunity *16*, 769–777.
5. Boise, L.H., Minn, A.J., Noel, P.J., June, C.H., Accavitti, M.A., Lindsten, T., and Thompson, C.B. (1995). CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. Immunity *3*, 87–98.
6. Lee, K.M., Chuang, E., Griffin, M., Khattri, R., Hong, D.K., Zhang, W., Straus, D., Samelson, L.E., Thompson, C.B., and Bluestone, J.A. (1998). Molecular basis of T cell inactivation by CTLA-4. Science *282*, 2263–2266.
7. Krummel, M.F., and Allison, J.P. (1996). CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. J. Exp. Med. *183*, 2533–2540.
8. Linsley, P.S., Greene, J.L., Tan, P., Bradshaw, J., Ledbetter, J.A., Anasetti, C., and Damle, N.K. (1992). Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. J. Exp. Med. *176*, 1595–1604.
9. Nabavi, N., Freeman, G.J., Gault, A., Godfrey, D., Nadler, L.M., and Glimcher, L.H. (1992). Signalling through the MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression. Nature *360*, 266–268.
10. Vandenberghe, P., Delabie, J., de Boer, M., De Wolf-Peeters, C., and Ceuppens, J.L. (1993). In situ expression of B7/BB1 on antigen-presenting cells and activated B cells: an immunohistochemical study. Int. Immunol. *5*, 317–321.
11. Schmittel, A., Scheibenbogen, C., and Keilholz, U. (1995). Lipopolysaccharide effectively up-regulates B7–1 (CD80) expression and costimulatory function of human monocytes. Scand. J. Immunol. *42*, 701–704.
12. Creery, W.D., Diaz-Mitoma, F., Filion, L., and Kumar, A. (1996). Differential modulation of B7–1 and B7–2 isoform expression on human monocytes by cytokines which influence the development of T helper cell phenotype. Eur. J. Immunol. *26*, 1273–1277.
13. Fleischer, J., Soeth, E., Reiling, N., Grage-Griebenow, E., Flad, H.D., and Ernst, M. (1996). Differential expression and function of CD80 (B7–1) and CD86 (B7–2) on human peripheral blood monocytes. Immunology *89*, 592–598.
14. Freedman, A.S., Freeman, G.J., Rhynhart, K., and Nadler, L.M. (1991). Selective induction of B7/BB-1 on interferon-gamma stimulated monocytes: a potential mechanism for amplification of T cell activation through the CD28 pathway. Cell. Immunol. *137*, 429–437.
15. Salomon, B., and Bluestone, J.A. (2001). Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu. Rev. Immunol. *19*, 225–252.
16. Collins, A.V., Brodie, D.W., Gilbert, R.J., Iaboni, A., Manso-Sancho, R., Walse, B., Stuart, D.I., van der Merwe, P.A., and Davis, S.J. (2002). The interaction properties of costimulatory molecules revisited. Immunity *17*, 201–210.
17. LaBelle, J.L., Hanke, C.A., Blazar, B.R., and Truitt, R.L. (2002). Negative effect of CTLA-4 on induction of T-cell immunity in vivo to B7–1+, but not B7–2+, murine myelogenous leukemia. Blood *99*, 2146–2153.
18. Sansom, D.M., Manzotti, C.N., and Zheng, Y. (2003). What's the difference between CD80 and CD86? Trends Immunol. *24*, 314–319.
19. Leach, D.R., Krummel, M.F., and Allison, J.P. (1996). Enhancement of antitumor immunity by CTLA-4 blockade. Science *271*, 1734–1736.
20. Azuma, M., Yssel, H., Phillips, J.H., Spits, H., and Lanier, L.L. (1993). Functional expression of B7/BB1 on activated T lymphocytes. J. Exp. Med. *177*, 845–850.
21. Windhagen, A., Newcombe, J., Dangond, F., Strand, C., Woodroofe, M.N., Cuzner, M.L., and Hafler, D.A. (1995). Expression of costimulatory molecules B7–1 (CD80), B7–2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. J. Exp. Med. *182*, 1985–1996.
22. Karandikar, N.J., Vanderlugt, C.L., Eagar, T., Tan, L., Bluestone, J.A., and Miller, S.D. (1998). Tissue-specific up-regulation of B7–1 expression and function during the course of murine relapsing experimental autoimmune encephalomyelitis. J. Immunol. *161*, 192–199.
23. Lenschow, D.J., Zeng, Y., Thistlethwaite, J.R., Montag, A., Brady, W., Gibson, M.G., Linsley, P.S., and Bluestone, J.A. (1992). Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. Science *257*, 789–792.
24. Lenschow, D.J., and Bluestone, J.A. (1993). T cell costimulation and in vivo tolerance. Curr. Opin. Immunol. *5*, 747–752.
25. Webb, L.M., Walmsley, M.J., and Feldmann, M. (1996). Prevention and amelioration of collagen-induced arthritis by blockade of the CD28 costimulatory pathway: requirement for both B7–1 and B7–2. Eur. J. Immunol. *26*, 2320–2328.
26. Tellander, A.C., Pettersson, U., Runstrom, A., Andersson, M., and Michaelsson, E. (2001). Interference with CD28, CD80, CD86 or CD152 in collagen-induced arthritis. Limited role of IFN-gamma in anti-B7-mediated suppression of disease. J. Autoimmun. *17*, 39–50.

27. Perrin, P.J., Scott, D., Quigley, L., Albert, P.S., Feder, O., Gray, G.S., Abe, R., June, C.H., and Racke, M.K. (1995). Role of B7:CD28/CTLA-4 in the induction of chronic relapsing experimental allergic encephalomyelitis. J. Immunol. *154*, 1481–1490.
28. Anderson, D.E., Sharpe, A.H., and Hafler, D.A. (1999). The B7–CD28/CTLA-4 costimulatory pathways in autoimmune disease of the central nervous system. Curr. Opin. Immunol. *11*, 677–683.
29. Grohmann, U., Orabona, C., Fallarino, F., Vacca, C., Calcinaro, F., Falorni, A., Candeloro, P., Belladonna, M.L., Bianchi, R., Fioretti, M.C., et al. (2002). CTLA-4-Ig regulates tryptophan catabolism in vivo. Nat. Immunol. *3*, 1097–1101.
30. Racke, M.K., Scott, D.E., Quigley, L., Gray, G.S., Abe, R., June, C.H., and Perrin, P.J. (1995). Distinct roles for B7–1 (CD-80) and B7–2 (CD-86) in the initiation of experimental allergic encephalomyelitis. J. Clin. Invest. *96*, 2195–2203.
31. Kuchroo, V.K., Das, M.P., Brown, J.A., Ranger, A.M., Zamvil, S.S., Sobel, R.A., Weiner, H.L., Nabavi, N., and Glimcher, L.H. (1995). B7–1 and B7–2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. Cell *80*, 707–718.
32. Miller, S.D., Vanderlugt, C.L., Lenschow, D.J., Pope, J.G., Karandikar, N.J., Dal Canto, M.C., and Bluestone, J.A. (1995). Blockade of CD28/B7–1 interaction prevents epitope spreading and clinical relapses of murine EAE. Immunity *3*, 739–745.
33. Vanderlugt, C.L., Neville, K.L., Nikcevich, K.M., Eagar, T.N., Bluestone, J.A., and Miller, S.D. (2000). Pathologic role and temporal appearance of newly emerging autoepitopes in relapsing experimental autoimmune encephalomyelitis. J. Immunol. *164*, 670–678.
34. Moreland, L.W., Alten, R., Van den Bosch, F., Appelboom, T., Leon, M., Emery, P., Cohen, S., Luggen, M., Shergy, W., Nuamah, I., et al. (2002). Costimulatory blockade in patients with rheumatoid arthritis: a pilot, dose-finding, double-blind, placebo-controlled clinical trial evaluating CTLA-4Ig and LEA29Y eighty-five days after the first infusion. Arthritis Rheum. *46*, 1470–1479.
35. Kremer, J.M., Westhovens, R., Leon, M., Di Giorgio, E., Alten, R., Steinfeld, S., Russell, A., Dougados, M., Emery, P., Nuamah, I.F., et al. (2003). Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. N. Engl. J. Med. *349*, 1907–1915.
36. Hida, T., Hattori, M., Kurokawa, T., and Nakanishi, A. September 17, 1997. Takeda Chemical Industries Ltd., Japan. (Oxo-) xanthene derivatives, their preparation and their use as immunomodulators. European Patent Appl. EP0795554.
37. Jenh, C.H., Zhang, M., Wiekowski, M., Tan, J.C., Fan, X.D., Hegde, V., Patel, M., Bryant, R., Narula, S.K., Zavodny, P.J., et al. (1998). Development of a CD28 receptor binding-based screen and identification of a biologically active inhibitor. Anal. Biochem. *256*, 47–55.
38. Fine, J.S., Macosko, H.D., Justice, L., Chou, C.C., Jenh, C.H., Narula, S.K., and Zavodny, P.J. (1999). An inhibitor of CD28–CD80 interactions impairs CD28-mediated costimulation of human CD4 T cells. Cell. Immunol. *191*, 49–59.
39. Hegde, V.R., Puar, M.S., Dai, P., Patel, M., Gullo, V.P., Chan, T.M., Silver, J., Pramanik, B.N., and Jenh, C.H. (2003). Condensed aromatic peptide family of microbial metabolites, inhibitors of CD28–CD80 interactions. Bioorg. Med. Chem. Lett. *13*, 573–575.
40. Green, N.J., Xiang, J., Chen, J., Chen, L., Davies, A.M., Erbe, D., Tam, S., and Tobin, J.F. (2003). Structure-activity studies of a series of dipyrazolo[3,4-b:3′,4′-d]pyridin-3-ones binding to the immune regulatory protein B7.1. Bioorg. Med. Chem. *11*, 2991–3013.
41. Erbe, D.V., Wang, S., Xing, Y., and Tobin, J.F. (2002). Small molecule ligands define a binding site on the immune regulatory protein B7.1. J. Biol. Chem. *277*, 7363–7368.
42. Bjork, P.A., Fex, T., Pettersson, L.O.G., Sorensen, P., and da Graca Thrige, D. Jan 16, 2003. Active Biotech AB, Sweden. Novel immunomodulating compounds. PCT Int. Appl. WO03004495.
43. Sorensen, P., Kussmann, M., Rosen, A., Bennett, K.L., da Graca Thrige, D., Uvebrant, K., Walse, B., Roepstorff, P., and Bjork, P. (2004). Identification of protein-protein interfaces implicated in CD80–CD28 costimulatory signaling. J. Immunol. *172*, 6803–6809.
44. Olsson, C., Michaelsson, E., Parra, E., Pettersson, U., Lando, P.A., and Dohlsten, M. (1998). Biased dependency of CD80 versus CD86 in the induction of transcription factors regulating the human IL-2 promoter. Int. Immunol. *10*, 499–506.
45. Toogood, P.L. (2002). Inhibition of protein-protein association by small molecules: approaches and progress. J. Med. Chem. *45*, 1543–1558.
46. Cochran, A.G. (2000). Antagonists of protein-protein interactions. Chem. Biol. *7*, R85–R94.
47. Arkin, M.R., and Wells, J.A. (2004). Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. Nat. Rev. Drug Discov. *3*, 301–317.
48. Stamper, C.C., Zhang, Y., Tobin, J.F., Erbe, D.V., Ikemizu, S., Davis, S.J., Stahl, M.L., Seehra, J., Somers, W.S., and Mosyak, L. (2001). Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses. Nature *410*, 608–611.
49. Ikemizu, S., Gilbert, R.J., Fennelly, J.A., Collins, A.V., Harlos, K., Jones, E.Y., Stuart, D.I., and Davis, S.J. (2000). Structure and dimerization of a soluble form of B7-1. Immunity *12*, 51–60.
50. Arkin, M.R., Randal, M., DeLano, W.L., Hyde, J., Luong, T.N., Oslob, J.D., Raphael, D.R., Taylor, L., Wang, J., McDowell, R.S., et al. (2003). Binding of small molecules to an adaptive protein-protein interface. Proc. Natl. Acad. Sci. USA *100*, 1603–1608.
51. Tian, S.S., Lamb, P., King, A.G., Miller, S.G., Kessler, L., Luengo, J.I., Averill, L., Johnson, R.K., Gleason, J.G., Pelus, L.M., et al. (1998). A small, nonpeptidyl mimic of granulocyte-colony-stimulating factor. Science *281*, 257–259.
52. Doyle, M.L., Tian, S.S., Miller, S.G., Kessler, L., Baker, A.E., Brigham-Burke, M.R., Dillon, S.B., Duffy, K.J., Keenan, R.M., Lehr, R., et al. (2003). Selective binding and oligomerization of the murine granulocyte colony-stimulating factor receptor by a low molecular weight, nonpeptidyl ligand. J. Biol. Chem. *278*, 9426–9434.
53. Iwai, H., Kozono, Y., Hirose, S., Akiba, H., Yagita, H., Okumura, K., Kohsaka, H., Miyasaka, N., and Azuma, M. (2002). Amelioration of collagen-induced arthritis by blockade of inducible costimulator-B7 homologous protein costimulation. J. Immunol. *169*, 4332–4339.
54. van der Merwe, P.A., Bodian, D.L., Daenke, S., Linsley, P., and Davis, S.J. (1997). CD80 (B7–1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. J. Exp. Med. *185*, 393–403.
55. Salomon, B., Lenschow, D.J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., and Bluestone, J.A. (2000). B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity *12*, 431–440.
56. Manzotti, C.N., Tipping, H., Perry, L.C., Mead, K.I., Blair, P.J., Zheng, Y., and Sansom, D.M. (2002). Inhibition of human T cell proliferation by CTLA-4 utilizes CD80 and requires CD25+ regulatory T cells. Eur. J. Immunol. *32*, 2888–2896.
57. Matthews, I.R., Coulter, T.S., Ghiron, C., Brennan, C.J., Uddin, M.K., Pettersson, L.O.G., da Graca Thrige, D., and Huxley, P. June 10, 2004. Active Biotech AB, Sweden. Pyrazoloquinolines with immunomodulating activity. PCT Int. Appl. WO2004048378.
58. Matthews, I.R. September 23, 2004. Avidex Ltd., United Kingdom. Immunomodulating heterocyclic compounds. PCT Int. Appl. WO2004081011.
59. Pellicci, D.G., Kortt, A.A., Sparrow, L.G., Hudson, P.J., Sorensen, H.V., Davis, S.J., and Classon, B.J. (2000). Expression and purification of antigenically active soluble derivatives of the heterodimeric and homodimeric forms of the mouse CD8 lymphocyte membrane glycoprotein. J. Immunol. Methods *246*, 149–163.
60. O'Brien, J., Wilson, I., Orton, T., and Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. *267*, 5421–5426.