The potent protein kinase C selective inhibitor AEB071
(Sotrastaurin) represents a new class of immunosuppressive agents
affecting early T cell activation

Jean-Pierre Evenou, Jürgen Wagner, Gerhard Zenke, Volker Brinkmann,
Kathrin Wagner, Jiri Kovarik, Karl A. Welzenbach, Gabriele Weitz-Schmidt, Christine
Guntermann, Harry Towbin, Sylvain Cottens, Sandra Kaminski, Thomas Letschka, Christina
Lutz-Nicoladoni, Thomas Gruber, Natascha Hermann-Kleiter, Nikolaus Thuille & Gottfried
Baier

Autoimmunity, Transplantation & Inflammation (J-P.E., G.Z., V.B., K.W., J.K., K.A.W., G.W-
S., C. G.), Global Discovery Chemistry (J.W.), Developmental & Molecular Pathways (H.T.),
Center for Proteomic Chemistry (S.C.), Novartis Institutes for BioMedical Research, Basel,
Switzerland; Department for Medical Genetics, Molecular and Clinical Pharmacology,
Innsbruck Medical University, Austria (S.K., T.L., C.L.-N., T.G., N.H-K., N.T., G.B.)
Running title: PKC selective inhibitor AEB071

Corresponding author: Jean-Pierre Evenou, Novartis Institute for BioMedical Research, WSJ-386.5.27, CH-4002 Basel, Switzerland; Tel: +41 (61) 324-8388, Fax: +41 (61) 324-4464

jeanpierre.evenou@novartis.com

Number of text pages: 34
Number of tables: 0
Number of figures: 7
Number of references: 37
Abstract word count: 191
Introduction word count: 576
Discussion word count: 1235

Non-standard abbreviations:
AICD, activation induced cell death; AP-1, activating protein-1; BSA, Bovine Serum albumin; CsA, cyclosporine A; DAG, diacylglycerol; FACS, fluorescence-activated cell sorter; HBSS, Hank’s balanced salt solution; I-κB, inhibitor of κB; IKK, I-κB kinase; IL-2, interleukin-2; MLR, mixed lymphocyte reaction; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor of κB; PBS, Phosphate buffered saline; PDBu, Phorbol 12,13-dibutyrate; PKC, protein kinase C; PMA, Phorbol 12-myristate 13-acetate; TBS, Tris buffered saline; TCR, T cell receptor

Recommended section assignment: Inflammation, Immunopharmacology and Asthma
Abstract

There is a pressing need for immunosuppressants with an improved safety profile. The search for novel approaches to blocking T cell activation led to the development of the selective protein kinase C (PKC) inhibitor AEB071 (INN: Sotrastaurin). In cell-free kinase assays AEB071 inhibited PKC with $K_i$ values in the sub- to low-nanomolar range. Upon T cell stimulation, AEB071 markedly inhibited in situ PKC θ catalytic activity and selectively affected both the canonical NF-κB and NFAT (but not AP-1) transactivation pathways. In primary human and mouse T cells, AEB071 treatment effectively abrogated at low nanomolar concentrations markers of early T cell activation, such as IL-2 secretion and CD25 expression. Accordingly the CD3/CD28 antibody– and alloantigen-induced T cell proliferation responses were potently inhibited by AEB071 in the absence of nonspecific anti-proliferative effects. Unlike former PKC inhibitors AEB071 did not enhance apoptosis of murine T cell blasts in a model of activation-induced cell death. Furthermore, AEB071 markedly inhibited LFA-1 mediated T cell adhesion at nanomolar concentrations. The mode of action of AEB071 is different from that of calcineurin inhibitors, and AEB071 and Cyclosporin A appear to have complementary effects on T cell signaling pathways.
Introduction

Phosphorylation of serine, threonine, and tyrosine residues is a primary mechanism for regulating protein function in eukaryotic cells. Protein kinases, the enzymes that catalyze these reactions, regulate essentially all cellular processes and have thus emerged as therapeutic targets for many human diseases. However, nearly all protein kinase inhibitors target the adenosine triphosphate (ATP) binding site. For this reason, design of inhibitors that selectively target even a subset of the approximately 570 related human protein kinase domains continues to be a daunting challenge. Nevertheless, small-molecule inhibitors of Abelson tyrosine kinase (Abl) and epidermal growth factor receptor (EGFR) have been recently developed into clinically useful anticancer drugs (reviewed by Medinger and Drevs, 2005).

The protein kinase C (PKC) family of serine/threonine kinases plays a central role in the adaptive immune system. PKC can be grouped into three categories according to the presence or absence of structural motifs that define cofactor requirements (Baier, 2003; Tan and Parker, 2003; Spitaler and Cantrell, 2004). Although PKCs were originally identified by Nishizuka and colleagues three decades ago (Takai et al., 1977), a more in-depth understanding of the unique function of each individual isotype became possible only recently through the study of individual knockout (KO) mice. Based on extensive phenotype analyses and biochemical studies, PKCα, PKCβ, and PKCθ were found to exert primarily nonredundant and PKC isotype-selective functions in T lymphocytes (Volkov et al., 2001; Long et al., 2001; Sun et al., 2000; Pfeifhofer et al., 2003; Pfeifhofer et al., 2006). As shown by confocal microscopy, PKCθ is the only isotype that is rapidly recruited to the immunological synapse upon T cell engagement (Monks et al., 1997). PKCθ plays an important role in NF-κB, NFAT, and AP-1 transactivation,
as well as in Th2 and Th17 immune responses \textit{in vivo} (reviewed by Marsland and Kopf, 2008). Furthermore, additional functions have been reported for the PKC\(\alpha\) and \(\beta\) isotypes. PKC\(\alpha\) is upregulated shortly after T cell stimulation, and PKC\(\alpha\)-deficient mice have a Th1 defect and strongly reduced interferon-\(\gamma\) (IFN\(\gamma\)) production (Pfeifhofer et al., 2006). While T cell signaling is intact in PKC\(\beta\)-deficient mice, lymphocyte function–associated antigen-1 (LFA-1)–mediated T cell locomotion is impaired (Volkov et al., 2001). However, it should be noted that most of our knowledge about physiological PKC isotype functions is based on gene ablation approaches (Baier, 2003; Tan and Parker, 2003; Spitaler and Cantrell, 2004), which have the potential to induce compensatory mechanisms.

Cell-permeant inhibitors, if selective, can greatly complement our understanding of the mechanistic role of the functions of the PKC family. The use of both enzymatic inhibition and gene ablation allows us to distinguish between the scaffold and enzymatic functions of PKC isotypes. Here, we describe the \textit{in vitro} characterization of the novel compound AEB071(INN: Sotrastaurin), a potent PKC inhibitor that is orally available and which was recently reported to significantly reduce the clinical severity of psoriasis within two weeks at well-tolerated doses (Skvara et al., 2008). Importantly, AEB071 demonstrated a high selectivity profile for classical and novel PKC family members. We report on the biochemical and pharmacological characterization of AEB071 and compare its effects on T cell signaling with the phenotypes of PKC\(\alpha\) and PKC\(\theta\) KO mice. Given that PKCs have a pivotal role in signaling pathways downstream of the T cell receptor (TCR) and the CD28 co-receptor, the pharmacological blockade of PKCs might offer an innovative rationale-based therapeutic strategy for blocking T cell activation, thus providing novel treatment options for patients suffering from T cell–dependent autoimmune pathologies.
Methods

Collection of Biological Material

Animals were housed under conventional conditions in filter-top-protected cages and cared for in accordance with Austrian and Swiss laws for animal protection and the NIH principles of Laboratory Animal Care. All experimental protocols were approved by the Austrian and Swiss veterinary authorities. Buffy coats from healthy donors with unknown HLA type were obtained from the Blood Transfusion Center (Kantonspital, Basel, Switzerland).

Enzymes and reagents

Recombinant human PKCθ was obtained from Novartis Biomolecule Production. Recombinant human PKCα, βI, δ, ε, and η were all purchased from Oxford Biomedical Research (Rochester Hills, MI). The N-terminally biotinylated tridecapeptide substrate RFARKGSLRQKNV was purchased from NeoMPS (Strasbourg, France).

Protein kinase assays

Classical and novel PKC isotypes were assayed by scintillation proximity assays (SPA) technology. Briefly, the assay was performed in 20 mM Tris-HCl buffer pH 7.4 + 0.1% BSA by incubating 1.5 μM of the peptide substrate with 10 μM [³²P]-ATP (Hartmann Analytic, Braunschweig, Germany), 10 mM Mg(NO₃)₂, 0.2 mM CaCl₂, and PKC at a protein concentration varying from 25 to 400 ng.ml⁻¹, and lipid vesicles containing 30 mol% phosphatidylserine, 5 mol% DAG, and 65 mol% phosphatidylcholine at a final lipid concentration of 0.5 μM. Incubation was performed for 60 min at room temperature (RT). The
reaction was stopped by adding 50 μl of a mixture containing 100 mM EDTA, 200 μM ATP, 0.1% Triton X-100, and 0.375 μg/well streptavidin-coated SPA beads (GE Healthcare Biosciences, Uppsala, Sweden) in PBS without Ca\(^{2+}\) and Mg\(^{2+}\). Incorporated radioactivity was measured in a MicroBeta™ Trilux counter (Perkin-Elmer, Schwerzenbach, Switzerland) for 1 min. PKCζ was assayed according to Geiges et al., 1997. *In situ* Thr-219 autophosphorylation status analysis of PKCθ was done by a phospho-site–specific antibody as described in Thuille et al., 2005.

**Analysis of T cell proliferation**

Antibody- and alloantigen-induced proliferation was measured by \(^3\)H-thymidine incorporation during the last 16 h of incubation (Sanglier et al., 1999). Naive mouse CD3\(^+\) T cells were purified from pooled spleen and lymph nodes with mouse T cell enrichment columns (R&D Systems, Minneapolis, MN). For anti-CD3 stimulations, T cells (5 x 10\(^5\)) in 200 μl of proliferation medium (RPMI supplemented with 10% FCS, 2 mM L-glutamine, and 50 U ml\(^{-1}\) penicillin/streptomycin) were added in duplicates to plates precoated with anti-CD3 antibody (clone 2C11, 10 μg ml\(^{-1}\)). Alternatively, PDBu (10 ng ml\(^{-1}\)) + Ca\(^{2+}\) ionophore ionomycin (125 ng ml\(^{-1}\)), both from Sigma-Aldrich (St. Louis, MI) was used. Where indicated, soluble anti-CD28 (1 μg ml\(^{-1}\); BD Biosciences, San Jose, CA) was added. The proliferation of lymphocytes from two genetically different mouse strains (CBA, H-2\(^k\) and BALB/c, H-2\(^d\)) was determined *in vitro* in a two-way allogeneic mixed lymphocyte reaction (MLR). Spleen cells (1 x 10\(^5\)) from each strain were incubated together for 4 days prior to harvesting. Human PBMCs were isolated on Ficoll gradients from buffy coats of donors with unknown HLA types. Individual two-way MLRs were set up by mixing cells from three different donors at a ratio of 1:1 in different combinations and
at a total cell number of 2 x 10^5 cells/well. The cells were harvested after 6 days. For the production of T cell blasts, human PBMCs were isolated on Ficoll and stimulated (7 x 10^5 cells ml^-1) for 4 days with PHA (10 μg ml^-1; Roche Diagnostics, Mannheim, Germany). PHA blasts were harvested, washed twice, and restimulated for 72 h with IL-2 (200 U ml^-1; Chiron, Emeryville, CA) at a cell density of 2 x 10^5 cells ml^-1 prior to [3H]-thymidine addition. Bone marrow cells were isolated from CBA mice and adjusted to 5 x 10^5 cells ml^-1 in complete RPMI containing WEHI- and L929-conditioned medium at appropriate dilutions. Cultures were incubated for 4 days. The proliferation of the mouse T cell line CTLL-2 (ATCC, Rockville, MD) was followed by incubating the cells (5 x 10^4 cells ml^-1) in the presence of 50 U ml^-1 mouse IL-2 (Novartis Biomolecule Production) for 24 h. Results shown are the mean ± SD of at least three independent experiments.

Analysis of cytokine production

Cytokine production in mouse CD3^+ T cells after antibody stimulation was assessed by BioPlex technology (BioRad, Hercules, CA). IL-2 & IFNγ production in CD4^+ T cells from DO11.10 transgenic mice after OVA peptide challenge was assessed by ELISA (BD Biosciences, San Jose, CA). Single cell suspensions from spleens of DO11.10 mice were prepared by homogenization. After red blood cell lysis, cells were challenged at a final concentration of 10^7 cells ml^-1 with OVA323–339 peptide (Genway Biotech, San Diego, CA) for 48 h. Human CD4^+ T cells were negatively selected from Ficoll-isolated PBMCs of healthy volunteers by magnetic cell sorting, according to the manufacturers instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). Cells (2 x 10^5 cells ml^-1) were incubated in the presence of 1 μg ml^-1 of plate-bound anti-CD3 (OKT3, Novartis Biomolecule Production) and 1 μg ml^-1 of plate-bound anti-CD28
(kind gift of L. Aarden Clone #15E8). Results shown are the mean ± SD of at least three independent experiments.

Analysis of murine cell surface activation markers by flow cytometry

Single-cell suspensions of spleen, lymph node, and thymus were prepared and incubated for 30 min on ice in staining buffer (PBS containing 2% FCS and 0.2% NaN₃) with FITC, PE, APC, or biotinylated antibody conjugates. Surface marker expression was analyzed using a FACS Calibur cytometer (BD Biosciences) with CellQuestPro software. Antibodies against murine CD3, CD4, and CD8 were obtained from Caltag Laboratories (Hamburg, Germany). Antibodies against murine CD28, CD69, CD44, and CD25 were obtained from BD PharMingen (San Jose, CA).

Western blot analysis

Immunoblotting was performed in Jurkat cells (clone E6-1) stimulated with soluble anti-CD3 (1 μg ml⁻¹) and anti-CD28 (5 μg ml⁻¹) antibodies for 10 and 30 min at 37°C prior to cell lysis in high salt buffer. Cell extract was run on a single well 12% SDS-PAGE and immunoblotted with a panel of 26 phospho-specific antibodies (Cell Signaling Technology, Danvers, MA) that cover proximal and more distal phosphorylation events downstream of the TCR and the CD28 coreceptor.
Reporter gene assays

Jurkat cells were stably transfected with luciferase reporter gene constructs containing either the IL-2 minimal promoter (Zenke et al., 2001) or promoters bearing multiple response elements for a single transcription factor (pHTS-NF-κB, pHTS-NFAT, or pHTS-AP1, Biomyx, San Diego, CA). Cells were stimulated with plate-bound anti-CD3 (3–30 ng ml\(^{-1}\)) and anti-CD28 (100–300 ng ml\(^{-1}\)) for 5 h at 37°C in RPMI + 10% FCS prior to cell lysis. Luciferase activity was measured in a Victor II microplate reader (Perkin-Elmer, Schwerzenbach, Switzerland) immediately after addition of 470 μM D-luciferin.

Gel mobility shift assays

Mouse T cells (2 x 10\(^{7}\)) were stimulated with medium alone (control) or solid-phase hamster anti-CD3 (clone 2C11, 10 μg ml\(^{-1}\)) and hamster anti-CD28 (clone 37.51, 1 μg ml\(^{-1}\)) for 16 h. Nuclear extracts were harvested from cells according to standard protocols as previously described (Pfeifhofer et al., 2003). Protein extracts (2 μg) were incubated in binding buffer with \[^{32}\text{P}\]-labeled, double-stranded oligonucleotide probes (NF-κB: 5'-GCC ATG GGG GGA TCC CCG AAG TCC-3', AP-1: 5'-CGC TTG ATG ACT CAG CCG GAA-3', and NFAT: 5'-GCC CAA AGA GGA AAA TTT GTT TCA TAC AG-3') (Nushift; Active Motif). All experiments were performed at least three times with similar outcomes.

Intracellular calcium measurements

Jurkat cells (5 x 10\(^{6}\) cells) were pretreated for 4 h with 500 nM of AEB071 and loaded for 30 min at 37°C in the dark with 5 μM fura-2 acetoxyxymylester (Molecular Probes, Eugene, OR). Dye excess was removed by washing in HBSS. Samples were prewarmed to 37°C and baseline
Ca\textsuperscript{2+} levels were determined for 100 sec on a Spex Fluorolog 2 spectrofluorometer equipped with two excitation monochrometers and a Cooper system, as described in Pfeifhofer et al., 2003. At this point, anti-CD3 antibody (OKT3, BD Biosciences, San Jose, CA) was added to a final concentration of 10 μg ml\textsuperscript{-1} and data were collected over 6.5 min. The maximal and minimal Ca\textsuperscript{2+} levels were determined by adding an excess of ionomycin and EGTA. Experiments were performed at least four times with similar outcomes.

**Adhesion assay**

All assay steps were performed at 37°C. Ninety-six-well plates were coated with 5 μg/ml human ICAM-1 mouse Cκ fusion protein (Novartis Biomolecule Production) and blocked with 3% BSA in TBS. Jurkat E6-1 and the B cell lymphoblastoid line JY were incubated with 20 μg/ml 2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF; Molecular Probes, Eugene, OR) and 4 μg/ml anti-ICAM-1 monoclonal antibody (Clone 1304, BMA Biomedicals, Augst, Switzerland) in assay buffer (RPMI 1640 containing 2.5 % fetal bovine serum and 10 mM Hepes) for 30 min. The labeled cells were washed three times with PBS and resuspended in assay buffer. 1 x 10\textsuperscript{5} cells/well were transferred to the blocked plates containing 10 ng/ml PMA and test compounds in assay buffer. Plates were centrifuged for 30 sec at 300g, incubated for 20 min and carefully washed with assay buffer. Adherent cells were quantified by measuring fluorescence using a VICTOR II microplate reader (Perkin-Elmer, Schwerzenbach, Switzerland).
Results

AEB071 is a potent and highly selective pan-PKC inhibitor in vitro

AEB071 (chemical structure and make-up are depicted in Figure 1A) was assayed in vitro on a panel of PKC isotypes that are expressed in T cells. As shown in Figure 1B, AEB071 was very effective at inhibiting classical and novel PKC isotypes with $K_i$ values in the sub- to low-nanomolar range. Dixon-plot analysis led to a $K_i$ value for PKC$\theta$ which was in the same order of magnitude as the active enzyme concentration. Considering the relevance of this isotype for T cell activation we reinvestigated the compound under mutual depletion conditions and confirmed the $K_i$ value in the 200 pM range (not shown). The compound was tested independently on atypical PKC$\zeta$ and was found to be inactive at concentrations up to 1 $\mu$M (not shown). When the compound was tested on a selected panel of kinases, the only enzyme on which AEB071 displayed an IC$_{50}$ value below 1 $\mu$M was GSK3$\beta$ (Supplemental Table 1). Upon T cell activation, a functionally critical autophosphorylation site of PKC$\theta$, that is recognized by a phospho-Thr-219–specific antibody was reported (Thuille et al., 2005; Gruber et al., 2006). Employing the phospho-status analysis of Thr-219 on PKC$\theta$, we performed an in situ catalytic activity measurement of this isotype in intact T cells. Consistent with the cell free results above, in transiently PKC$\theta$ wild-type cDNA transfected Jurkat T cells, AEB071 inhibited the phorbol ester (PDBu)-inducible autophosphorylation on Thr-219 in a concentration-dependent manner, although full inhibition was achieved only in the high nanomolar range (Figure 1C).
AEB071 effectively abrogates CD3/CD28 antibody– and alloantigen-driven T cell responses

Activation of T cells by alloantigens or by antibodies, which target the TCR/CD3 complex and the CD28 co-receptor, is well established to result in cytokine secretion and subsequent proliferation responses. In mouse CD3+ T cells, proliferation responses induced by antibody- or phorbol ester-treatment were markedly decreased by AEB071 at a concentration of 250 nM (Figure 2A). Employing the mixed lymphocyte reaction assay, proliferation responses induced by an allogeneic stimulus were inhibited by AEB071 with a 50% maximal inhibitory concentration (IC50) of around 150 nM, when mouse splenocytes were challenged (Figure 2B). Of note, AEB071 was found to be more potent on human T cells than on mouse T cells. When the allogeneic MHC stimulation assay was performed with human PBMCs, the IC50 value for AEB071 was down to 37 nM (Figure 2B). Nonetheless AEB071 is not a general inhibitor of proliferation per se as indicated by the following findings. In a model of IL-2-driven human T cell blast proliferation the compound showed a significant inhibitory activity (>50%) only at concentrations above 2 μM (Figure 2C). Accordingly, the proliferation of the IL-2–dependent mouse CTLL cell line was significantly affected by AEB071 only at concentrations above 3 μM (Figure 2C). Finally, when mouse bone marrow cells were grown in the presence of conditioned medium, proliferation was also significantly inhibited only in the low micromolar range (Figure 2C).

Consistent with the results presented in Figure 2A-C, following stimulation with anti-CD3 or anti-CD3/anti-CD28, AEB071 potently inhibited IL-2 secretion responses in mouse CD3+ T cells (data not shown). In the transgenic TCR DO11.10 mouse CD4+ T cells, following physiological stimulation with the antigenic OVA peptide, both IL-2 and IFNγ levels were
strongly inhibited by AEB071 (Figure 2D). Compared with cytokine levels in mouse T cell supernatants, IL-2 levels in stimulated human CD4+ cell supernatants were more potently inhibited by AEB071 with an IC50 value of 5.8 nM (Figure 2E), again underlining the difference in sensitivity between mouse and human cellular responses. The decrease in IL-2 secretion was primarily related to an effect on IL-2 gene expression. In Jurkat cells stably transfected with a luciferase reporter construct, which is under the transcriptional control of the IL-2 minimal promoter, the CD3/CD28-driven increase in luciferase activity was concentration-dependently inhibited by AEB071 with an IC50 of about 50 nM (Figure 2F).

TCR-induced activation of T cells is known to promote transcriptional upregulation of both IL-2 and IL-2 receptor α-chain (CD25) genes, thereby constituting the autocrine cycle of IL-2 cytokine and its high-affinity receptor. Consistent with a marked reduction in stimulation-induced IL-2 cytokine production (Figure 2), the CD3/CD28 ligation–induced surface expression intensity of CD25 (as well as the activation marker CD44) was strongly reduced in AEB071-treated CD4+ and CD8+ mouse T cells at the nanomolar range (Figure 3). Detection by FACS of the surface expression of the early T cell activation marker CD69 on human PBMCs revealed that upregulation of surface fluorescence intensity following CD3/CD28 ligation was inhibited by AEB071 in a concentration-dependent manner, with an IC50 of 61 nM. However, full inhibition was achieved only at high nanomolar concentrations (Supplemental Figure 1).

Finally, we did not observe any significant difference in the susceptibility to the in vitro apoptotic stimulus anti-CD3 antibody in AEB071-treated CD4+ and CD8+ T cell blasts (Supplemental Figure 2). This observation is in contrast to data reported for other PKC inhibitors (Geiselhart et al., 1996; Han et al., 2000; Wasem et al., 2003; and data not shown), indicating the improved target selectivity of AEB071.
AEB071 primarily suppresses the canonical NF-κB and NFAT transactivation pathways

To further elucidate the molecular basis of the impairment in antigen receptor signaling, AEB071-treated Jurkat T cells were investigated biochemically and directly compared with DMSO-treated control cells. We found that CD3 ligation–induced PLCγ1 activation was not affected, because normal Ca^{2+} mobilization responses were reproducibly observed upon AEB071 treatment (Figure 4A). By testing for inducible serine/threonine phosphorylation events in CD3/CD28-mediated signal transduction cascades, we next analyzed the endogenous phosphorylation pattern of distinct signaling molecules (Figure 4B-E). Because Jurkat cells possess constitutive Akt/PKB activity due to loss of PTEN (a phosphatase degrading PIP3, Shan et al., 2000), Akt/PKB substrates showed a high degree of phosphorylation in unstimulated Jurkat cells. Similarly, high basal phosphorylation levels were observed for other molecules, such as PKC substrate phosphorylation and p65 RelA (Figure 4B). Nevertheless, after short-term (10 min) stimulation via CD3/CD28–ligating antibodies, we observed inducible phosphorylation of the MEK/MAPK1/2 kinases and of the p70 S6 kinase and its substrate, S6 ribosomal protein (Figure 4C). Phosphorylation of I-κBα was also strongly induced upon CD3/CD28 stimulation. Importantly, AEB071 completely suppressed both MEK/MAPK1/2 and p70 S6 kinase activation and the I-κBα phosphorylation pathways (Figure 4D).

Next, Jurkat cells were stably transfected with reporter gene constructs driven by promoters bearing multiple response elements to either NF-κB, AP-1 or NFAT, three key transcription factors known to be essential to TCR/CD28-induced IL-2 promoter transactivation (Baier, 2003). As a result, AEB071 exerted a strong effect on the NF-κB reporter pathway but it had only a weak activity on the NFAT reporter pathway in these cells (Figure 5A). The latter result
might be however anomalous since, as stated before, the reported lack of PTEN and SHIP in Jurkat cells lead to constitutive activation of the PI3K pathway, resulting in aberrant control of NFAT transactivation (Reif et al., 1997). AEB071 was found essentially inactive on the AP-1 reporter pathway (data not shown). The strong effect on the NF-κB pathway was confirmed by endogenous I-κBα phosphorylation analysis in Jurkat T cells, following PMA/ionomycin stimulation (Figure 5B). In the presence of AEB071 concentration-dependent inhibition of I-κBα phosphorylation was achieved, confirming the results obtained in Figure 4D. In contrast, TNFα-induced I-κBα phosphorylation was not inhibited by AEB071 at concentrations up to 3 μM (Figure 5B).

Finally, and similar to the impaired activation-induced IL-2 cytokine secretion (Figure 2), CD3/CD28-mediated transactivation of NF-κB in primary mouse CD3+ T cells was strongly abrogated upon AEB071 treatment (Figure 5C-D), confirming the NF-κB defect observed in Jurkat cells. Additionally, and distinct from the Jurkat cell line results described above, the NFAT pathway was also found to be severely affected by AEB071 in primary mouse CD3+ T cells (Figure 5C-D). Immunoblot analysis of nuclear extracts revealed significant, albeit partial, reduction in nuclear translocation of NFATc and p50 NF-κB (but not of cFos) in activated CD3+ T cells upon AEB071 treatment (Figure 5C). Consistently, DNA-binding analysis of these nuclear extracts revealed strong inhibition of NFAT and NF-κB DNA binding in activated and AEB071-treated CD3+ T cells (Figure 5D). These results are in agreement with the reported decrease in NF-κB and NFAT activation in PKCθ−/− T cells (Sun et al., 2000; Pfeifhofer et al., 2003; Manicassamy et al., 2006a; Altman et al., 2004). In contrast, induction of AP-1 DNA binding was mostly unaffected in AEB071-treated CD3+ T cells in the same experiments (Figure
Thus, the strong NFAT transactivation inhibition that we observed in AEB071-treated CD3+ T cells appears to be independent of the AP-1 pathway.

**AEB071 and Cyclosporin A block T cell responses in a complementary manner, and AEB071 augments T cell response inhibition in PKCθ-deficient T cells**

T cell receptor ligation results in PLCγ1 activation and subsequent generation of diacylglycerol (DAG) and inositol-3 phosphate (IP3). DAG activates notably PKC and IP3 triggers the release of calcium from intracellular stores. Cyclosporin A (CsA) exerts its main effect on T cell activation by blocking the Ca2+/calmodulin-dependent activity of the phosphatase calcineurin, preventing the nuclear translocation of NFATc. When mouse CD3+ T cells were pretreated with a combination of AEB071 and CsA (both at suboptimal doses), the CD3/CD28 ligation–driven proliferation (Figure 6A) and IL-2 secretion (Figure 6B) responses were inhibited to a greater extent than with either drug alone. Our mechanistic studies of NF-κB and NFAT transcription factor transactivation and the results shown in Figure 6B suggest that AEB071 and CsA have complementary inhibitory effects on IL-2 secretion. This is consistent with the previously observed functional cooperation of calcineurin and PKCθ (Werlen et al., 1998; Ghaffari-Tabrizi et al., 1999) in IL-2 production of Jurkat T cells. This clearly indicates that AEB071 blocks T cell activation by a mechanism that is different from that of calcineurin inhibitors, and that combination therapy with such inhibitors could prove to be very effective in the treatment of T cell–mediated immune diseases.

However, we did not anticipate that when using PKCθ-deficient T cells derived from our PKCθ KO mice (Pfeifhofer et al., 2003), AEB071 would further augment CD3+ T cell inhibition processes in these KO cells. PKCθ-deficient T cells intrinsically demonstrate strongly reduced
proliferative responses and IL-2 secretion (Pfeifhofer et al., 2003; Hermann-Kleiter et al., 2006). However, complete abrogation of proliferative (not shown) and IL-2 secretion responses (Figure 6C) were achieved only upon additional AEB071 treatment in PKCθ−/− CD3+ T cells. Taken together, these results suggest that inhibition of PKCθ, although essential, is only part of the mechanism responsible for the immunosuppressive activity of AEB071 and that additional PKC isotypes are involved in critical T cell signaling pathways.

**AEB071 abrogates T cell adhesiveness**

Prompted by the recent report about the role of PKCθ in LFA-1 inside-out signaling in T cells (Letschka et al., 2008), we looked at the effect of AEB071 on phorbol ester-induced adhesion of Jurkat cells to immobilized intercellular adhesion molecule-1 (ICAM-1). AEB071 strongly inhibited the binding to ICAM-1 with an IC50 of 300 nM (Figure 7A). When AEB071 was tested on the EBV-transformed B cell lymphoblastoid line JY, strong inhibition of binding to ICAM-1 was also observed with an IC50 value of 30 nM (Figure 7B). This was in stark contrast with CsA treatment, which was inactive up to 1 μM in this assay (data not shown).
Discussion

The past decade has seen a large number of studies aiming at delineating the role of PKC in T cell responses. Early in vitro studies performed in Jurkat cells used overexpression of kinase-dead or constitutively active mutants to investigate the role of PKCθ in T cell signaling and function (reviewed by Altman et al., 2000). More recently, gene knockout approaches revealed the physiological and nonredundant functions of PKCθ and PKCα in primary CD3+ T lymphocytes in a series of ex vivo studies (Sun et al., 2000; Pfeifhofer et al., 2003; Pfeifhofer et al., 2006). PKCθ was thereby revealed as a critical intermediate in T cell receptor-induced cytokine secretion and proliferation (Sun et al., 2000; Pfeifhofer et al., 2003). PKCα was shown to be essential for IFNγ expression and Th1-dependent immune responses (Pfeifhofer et al., 2006). In vivo, PKCθ appears to be required for the development of a robust immune response controlled both by Th17 and Th2 cells (reviewed by Marsland and Kopf, 2008). These findings qualified PKC as particularly attractive targets for pharmacological intervention in T cell-mediated autoimmune diseases and transplantation.

AEB071 was characterized biochemically as a very potent, cell-permeant inhibitor of classical and novel PKC isotypes (Figure 1), and showed clear PKC selectivity when tested on a selected panel of Ser/Thr and tyrosine kinases (Supplemental Table 1). Crystallization of AEB071 with the catalytic domain of PKCα revealed a very tight fit of the ligand into the ATP-binding site, providing a rationale for the potency as well as the PKC selectivity of the compound (von Matt et al, manuscript in preparation).

In the adaptive immune system several T cell processes are involved in the immune response. T cell activation is usually followed by clonal expansion and differentiation into effector cells that
ultimately go into apoptosis, in order to insure against inadequate prolongation of the immune response. PKCθ has been reported to play a role in T cell survival by up-regulating Bcl-xL levels in CD4+ and CD8+ T lymphocytes (Barouch-Bentov et al., 2005; Manicassamy et al., 2006b; Saibil et al., 2007). The role of PKCθ in FasL-mediated apoptosis is a matter of debate. Whereas Manicassamy and Sung (2007) claimed a critical role for PKCθ in this process, our observations repeatedly showed no change in susceptibility to apoptosis of T cells from PKCα, PKCβ, and PKCθ KO animals in our activation-induced cell death (AICD) model (Pfeifhofer et al., 2006; and data not shown). Although several reports in the literature claim a role for PKC in AICD using various PKC inhibitors (Han et al., 2000; Wasem et al., 2003; Zhou et al., 1999), we were not able to confirm these results with AEB071 (Supplemental Figure 2). Together, our data suggest an additional mechanism by which PKC is dispensable for apoptosis protection. AEB071 (in contrast to previous PKC inhibitor studies, Geiselhart et al., 1996) did not affect IL-2–driven proliferation of T cell lymphoblasts (Figure 2C). Results from the previous studies were most likely due to off-target effects of the previously used less-specific compounds (Davies et al., 2000). Instead, AEB071 strongly blocked cytokine responses, a key feature of early T cell–activation processes (Figure 2). While strong blockade of the activation responses could be observed in AEB071 treated T cells at low nM concentrations, no significant survival defect was observed at 500 nM of the compound (see Figure 2C and Supplemental Figure 2). Our present study with AEB071 thus reveals fundamental similarities between AEB071 inhibition and the phenotypes of the PKCθ and PKCα KOs (Sun et al., 2000; Pfeifhofer et al., 2003; Pfeifhofer et al., 2006), because CD3/CD28 antibody– and alloantigen-induced T cell responses where similarly affected in both situations. Similar to with PKCθ-deficient CD3+ T cells, AEB071 treatment selectively abrogated the coupling of the antigen receptor signaling to NF-κB and
NFAT activation in primary T cells (Figure 5). Finally, the strong inhibitory effect of AEB071 on phorbal ester–induced adhesion of lymphocytes to ICAM-1 (Figure 7) represents another similarity between AEB071 treatment and PKCθ gene ablation (Letschka et al., 2008).

It should be noted that discrepancies have also been observed between effects derived from PKCα and/or PKCθ gene ablation versus treatment with AEB071 (Supplemental Table 2). During AEB071 treatment, intact CD3/CD28-induced AP-1 activation was reproducibly observed (Figure 5), while PKCθ-deficient CD3+ T cells have been found to be defective in AP-1 signaling (Sun et al., 2000; Pfeifhofer et al., 2003). Similarly, and again unlike the PKCθ KO, AEB071 did not affect TCR-induced calcium fluxes (Figure 4A). Such differences between knockout and pharmacological inhibition studies may be explained by the possibility that in addition to its kinase function, PKCθ exerts scaffold functions as well, which are (in sharp contrast with the loss of expression in the KO approach) not necessarily impaired upon enzymatic inhibition. Alternatively, differences may be simply explained due to lack of efficacy of AEB071 at reaching 100% inhibition of PKCθ enzymatic activity (as achieved in the PKCθ KO situation). Conversely, while no (PKCα−/−) or only partial (PKCθ−/−) defects in CD3/CD28-induced upregulation of CD25 and CD69 surface expression (as median fluorescence intensity per T cell) were observed in KO T cells (Pfeifhofer et al, 2003 and data not shown), activation-induced CD25 and CD69 surface fluorescence intensities were potently impaired by AEB071 treatment (Figure 3 and Supplemental Figure 1). Sole inhibition of the PKCθ isotype is thus not likely to be the underlying mechanism responsible for its immunosuppressive activity.

 Consistently, AEB071 strongly inhibited PDBu/ionomycin-induced proliferation and IL-2 secretion responses of mouse CD3+ T cells, while both PKCθ−/− and PKCα−/− T cells demonstrated mostly intact proliferative and IL-2 secretion responses to such mitogenic
stimulation (Pfeifhofer et al., 2003; Pfeifhofer et al., 2006; Hermann-Kleiter et al, 2006; and data not shown). Along that line, deletion of PKCθ alone was not sufficient to elicit maximum inhibition of T cell functions in vitro. Treatment of PKCθ-deficient T cells with AEB071 resulted in further reduction of IL-2 secretion in T cells, establishing that there were additional and functionally redundant PKC family members expressed in T cells in this response pathway (Figure 6C). Therefore, the mechanism most likely to be responsible for the strong immunosuppressive activity of AEB071 is the inhibition of a broader range of PKC targets beyond PKCθ, as observed here.

In summary, our findings demonstrate that the PKC-selective compound AEB071 is a specific inhibitor of early T cell activation with no general anti-proliferative activity, and with a unique mechanism of action that is different from that of CsA, resulting in complementary effects upon cell proliferation and IL-2 secretion when T cells are co-treated with these two molecules (Figure 6A-B). Our experiments revealed that AEB071 induced molecular signaling defects in the antigen receptor signal transduction pathways by counteracting both NFAT and the canonical IKK/I-κBα/NF-κB transactivation pathways and, subsequently, cytokine release, as well as CD25 and CD69 surface expression. Unlike CsA, AEB071 acts as a potent inhibitor of both early T cell activation and β2-integrin–mediated T cell adhesiveness (Figure 7). AEB071 thus may offer a significant therapeutic advantage over currently marketed drugs, providing novel treatment options for patients suffering from T cell–dependent immune pathologies. Indeed, results from a 14-day multiple-dose study in psoriasis patients demonstrated a dose-dependent improvement in the severity of psoriatic plaques, with good tolerability (Skvara et al., 2008). Furthermore, AEB071, particularly in combination with adjunct immunosuppression
agents, was found to prolong rat heterotopic heart transplant survival and cynomolgus monkey renal allograft survival (reviewed by Vincenti and Kirk, 2008).
Acknowledgments

The expert technical assistance from N. Krumböck (Innsbruck), B. Gafner, V. Hungerford, E. Andersen, C. Kristofic, C. Vedrine, C. Wioland, J. Weiss and T. Kobel (all from Basel) is greatly appreciated. Authors wish to thank Dr. T. Meyer for measuring the AEB071 effects on PKCζ, Dr. J. Trapp and his team for measuring AEB071 effects on the selected panel of kinases, and Drs. W. Schuler and R. Morris for critically reviewing the manuscript (all from Basel).
References


Footnotes

This work was supported by grants from the FWF Austrian Science Fund [SFB-021 & P19505-B05]; the European Community Seventh Framework Program under grant agreement n°HEALTH-F4-2008-201106; a collaboration agreement with Novartis Basel (GB); and by the MFI fellowship of the Medical University of Innsbruck (NT).

Address correspondence to:

Gottfried Baier, Innsbruck Medical University, Schoepfstrasse 41, A-6020 Innsbruck, Austria;
Email address: Gottfried.Baier@i-med.ac.at

Jean-Pierre Evenou, Novartis Institute for BioMedical Research, WSJ-386.5.27, CH-4002 Basel, Switzerland; Email address: jeanpierre.evenou@novartis.com
Legends for Figures

Figure 1. Inhibition by AEB071 of recombinant PKC isotypes and assessment of its effect on PKC\(\theta\) activity *in situ*. (A) Chemical structure and make-up of AEB071. (B) \(K_i\) values for human recombinant PKC isotypes. Inhibition was assessed at three different ATP concentrations and Dixon plots derived thereof. (C) AEB071 abrogates PKC\(\theta\) Thr-219 autophosphorylation in intact T cells. Jurkat cells transfected with PKC\(\theta\) wild-type expression vector were pretreated for 1 h with increasing concentrations of AEB071 or DMSO solvent control, as indicated. Subsequently, the (p)Thr-219 phospho-status was determined by employing our PKC\(\theta\) (p)Thr-219–specific antibody in immunoprecipitations (IP) from resting (-) or 100 nM PDBu–stimulated (+) T cells, followed by SDS-PAGE and immunoblotting with a PKC\(\theta\)-specific monoclonal antibody. For normalization, total PKC\(\theta\) protein levels in the cell lysates (INPUT) were used. Results shown are the mean ± SD of three independent experiments.

Figure 2. Effect of AEB071 on T cell proliferation and cytokine secretion. (A) AEB071 was tested for its effect on the proliferation of mouse CD3\(^+\) T cells following antibody or PDBu/ionomycin stimulation. Results shown are the mean ± SD of three independent experiments. In (B), the graphs represent the drug’s effect on the allogeneic responses obtained with mouse splenocytes (dotted line) or human PBMCs (solid line) in two-way MLR analysis. (C) AEB071 (at nanomolar concentrations) did not affect cytokine or growth factor–driven proliferation. IL-2–driven proliferation of PHA-induced human T cell blasts, IL-2–driven proliferation of CTLL cells, and growth factor–induced proliferation of mouse bone marrow cells are shown. Results shown are the mean ± SD of three independent experiments. (D)
AEB071 was tested for its effect on IL-2 and IFNγ secretion from DO11.10 mouse transgenic CD4+ T cells after OVA peptide stimulation. Data shown are the mean ± SD of three independent experiments. (E) IL-2 secretion in human CD4+ T cells after 48 h antibody stimulation. Results shown are the mean ± SD of three independent experiments. (F) Jurkat cells were stably transfected with a luciferase reporter construct bearing the IL-2 minimal promoter. Cells were stimulated in the presence of increasing concentrations of AEB071 with plate bound anti-CD3/anti-CD28 for 5 hrs prior to measurement.

Figure 3. Effect of AEB071 on murine CD25 and CD44 surface expression. Surface expression of activation markers was expressed as median fluorescence intensities (MFI). Flow cytometric analysis of CD4+ (A) and CD8+ (B) mouse T cells, stimulated for 16 h by CD3/CD28 ligation, were analyzed against two concentrations of AEB071 (60 min pretreatment) for the relative fluorescence signals of anti-CD25 and anti-CD44 antibodies. Of note, the total percentages of positive cells were not reduced, indicating residual surface expression above detection levels in the AEB071-treated T cells.

Figure 4. Effect of AEB071 on antibody-stimulated calcium flux and phosphorylation response patterns. (A) Jurkat T cells were loaded with Fura-2 and monitored for changes in intracellular free calcium [Ca²⁺]. CD3 stimulation was induced by soluble OKT3 and direct Ca²⁺ influx with ionomycin. A representative result out of eight independent experiments in the presence or absence of 500 nM AEB071 inhibitor is shown. (B-E) Jurkat T cells were either not stimulated
(B) or stimulated by CD3/CD28 ligation for 10 min in the absence (C) or presence (D) of 200 nM AEB071 (30 min pretreatment) prior to cell lysis and immunoblotting with phospho-site–specific antibodies, as indicated in (E). Arrows indicate major effects of AEB071 on inducible phosphorylation events.

**Figure 5.** Effects of AEB071 on AP-1, NF-κB, and NFAT transactivation in stimulated Jurkat and mouse CD3^+ T cells. (A) NF-κB and NFAT activation is shown, as measured by reporter luciferase gene assays after stimulation with plate-bound anti-CD3/anti-CD28 antibodies in the presence of increasing concentrations of AEB071. (B) PMA/ionomycin- or TNFα-driven IκBα phosphorylation was assessed in the presence of increasing concentrations of AEB071. (C) Activation-induced nuclear translocation of key transcription factors was determined by immunoblotting of nuclear extracts for cFos, p50 NF-κB, and NFATc, respectively, as indicated. (D) Gel mobility shift assays were performed using radiolabeled probes containing NF-κB, AP-1, and NFAT binding-site sequences. The specificity of p50 NF-κB, cFos, and NFATc was confirmed by supershifting the electrophoretic mobility shift with antibodies, as indicated by the arrows. Experiments were repeated at least two times with similar results.

**Figure 6.** AEB071 additively cooperates with CsA and further augments inhibition processes in PKCθ-deficient T cells. Additive inhibitory effect of AEB071 and CsA on anti-CD3/anti-CD28–mediated proliferation (A) and IL-2 secretion (B) in mouse CD3^+ T cells. Electrophoretic mobility shift assays were performed in the presence or absence of AEB071 or CsA using radiolabeled probes containing NF-κB and NFAT binding-site sequences (insert). Experiments
were repeated at least two times with similar results. (C) AEB071 further reduces IL-2 secretion in antibody-stimulated PKC$\theta^{+/\text{c}}$ T cells.

**Figure 7.** Effect of AEB071 on cell adhesion. Phorbol ester--induced adhesion responses of Jurkat cells (A) or of the B cell-lymphoblastoid line JY (B) to immobilized ICAM-1 were quantified in the presence of AEB071 as described in Materials and Methods. Each bar represents the mean ± SD of triplicates. A representative experiment out of four independent experiments is shown.
Figure 1

A. Chemical structure of the molecule.

3-(1H-indol-3-yl)-4-[2-(4-methyl-piperazin-1-yl)-quinazolin-4-yl]-pyrrole-2,5-dione

B. Table of PKC isotypes and their Ki values (nM).

<table>
<thead>
<tr>
<th>PKC isotype</th>
<th>Ki (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα</td>
<td>0.95</td>
</tr>
<tr>
<td>PKCβI</td>
<td>0.64</td>
</tr>
<tr>
<td>PKCδ</td>
<td>2.1</td>
</tr>
<tr>
<td>PKCe</td>
<td>3.2</td>
</tr>
<tr>
<td>PKCη</td>
<td>1.8</td>
</tr>
<tr>
<td>PKCθ</td>
<td>0.22</td>
</tr>
</tbody>
</table>

C. Graph showing IP: (p)T219 and INPUT profiles with AEB071 concentrations (nM) over time.

% (p)T219

DMSO control 50 125 250 500 1000

PKCθ

PKCθ
Figure 2

A. [H]-thymidine uptake (% of control)

B. [H]-thymidine uptake (% of control)

C. [H]-thymidine uptake (% of control)

D. Cytokine secretion (% of control)

E. IL-2 secretion (% of control)

F. Luciferase activity (% of control)
Figure 4

### Jurkat T cells

#### A.

![Graph showing relative fluorescence units over time](image)

- **A10**: PKA Substrate Ab #9621
- **A12**: P-Akt Substrate Ab #9611
- **A15**: 42/44 P-p44/42 MAP Kinase (T202/Y204) Ab #9101
- **A17**: 74 P-Rat (S259) Ab #9421
- **A18**: 42 P-44/42 MAP Kinase Ab #9102
- **A23**: 45/64 P-ERK (T202/Y204) Ab #9401
- **A19**: 45 MEKII/II Ab #9102
- **A24**: 43 P-CREB (S133) Ab #9191
- **A26**: 46/54 P-SAPK/JNK (T183/Y185) Ab #9251
- **A28**: 46 P-SEK1/MKK4 (T223) Ab #9151
- **A29**: 43/50 P-c-Jun (S63) II Ab #9261
- **A33**: 43 P-p38 MAP Kinase (T180/Y182) Ab #9211
- **A45**: 32 P-Akt (S473) Ab #9211
- **A46**: 32 S6 Ribosomal Protein Ab #9221
- **A50**: 69-97 P-FKBP12 (S256) Ab #9461
- **A53**: 68-97 P-S6 Ribosomal Protein (S235/236) Ab #9211
- **A58**: 115 P-PDK/PKCh (S744/748) #2054
- **A65**: 70-95 P-p70 S6 Kinase (T202/Y204) Ab #9204
- **A67**: 78-90 P-PKC (pan) Ab #9371
- **A84**: 41 P-IkB (S32) Ab #9241
- **A7**: (Ser) PKC Substrate Ab #2261
- **A88**: 17-20 Cleaved Caspase-3 (Asp175) #9661
- **A89**: 85, 87 P-IKKa (S180) / IKKb (S181) #2881
- **A90**: 85 P-NF-xB p65 (S536) Ab #3031
- **B110**: 100-120 NF-AT1c

#### B.

![Western blot of medium](image)

#### C.

![Western blot of CD3/CD28](image)

#### D.

![Western blot of CD3/CD28 + AEB071](image)
Figure 5

Jurkat T cells

A.

Luciferase activity (% inhibition)

0 20 40 60 80 100

NF-κB

0.46 1.4 2.3 7 11 33 1000 AEB071 (nM)

B. PMA + ionomycin

<table>
<thead>
<tr>
<th>NS</th>
<th>control</th>
<th>10</th>
<th>30</th>
<th>100</th>
<th>300</th>
<th>1000</th>
<th>3000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AEB071 (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>NF-κBα</td>
<td>p-NF-κBα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mouse CD3+

C. Nuclear extracts

- cFos
- p50NF-κB
- NFATc
- DNA-polymerase δ

CD3/CD28 - + + + AEB071 - - +

D. EMSA

- cFos
- AP-1
- p50NF-κB

CD3/CD28 - + + + + AEB071 - - - + +
Mouse CD3⁺

A. [Bar graph showing thymidine uptake with [³H]-thymidine uptake (10³ cpm) for Medium and CD3/CD28 treatments.]

B. [Bar graph showing IL-2 levels with medium, CD3/CD28, 25 nM AEB071, and 10 nM CaA conditions.]

C. [Bar graph showing IL-2 levels with PKCε⁻/⁻ treatments and CD3/CD28 and AEB071 concentrations.]