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ORIGINAL ARTICLE

# Effects of the novel protein kinase C inhibitor AEB071 (Sotrastaurin) on rat cardiac allograft survival using single agent treatment or combination therapy with cyclosporine, everolimus or FTY720

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**Keywords**
AEB071, animal, immunosuppressant, nonclinical, protein kinase C inhibitor, sotrastaurin, transplantation.

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## Summary

NVP-AEB071 (AEB, sotrastaurin), an oral inhibitor of protein kinase C (PKC), effectively blocks T-cell activation. The immunosuppressive effects of oral AEB were demonstrated in a rat local graft versus host (GvH) reaction and rat cardiac transplantation models. T-cell activation was suppressed by 95% in blood from AEB-treated rats, with a positive correlation between T-cell inhibition and AEB blood concentration. In GvH studies, AEB inhibited lymph node swelling dose-dependently (3–30 mg/kg). BN and DA cardiac allografts were acutely rejected within 6–10 days post-transplantation in untreated LEW rats. AEB at 10 and 30 mg/kg b.i.d. prolonged BN graft survival to a mean survival time of 15 and >28 days, and DA grafts to 6.5 and 17.5 days, respectively. In the DA to LEW model, combining a nonefficacious dose of AEB (10 mg/kg b.i.d.) with a nonefficacious dose of cyclosporine, everolimus or FTY720 led to prolonged median survival times (26 days, >68 days and >68 days, respectively). Pharmacokinetic monitoring excluded drug–drug interactions, suggesting synergy. In conclusion, these studies are the first to demonstrate that AEB prolongs rat heart allograft survival safely as monotherapy and in combination with nonefficacious doses of cyclosporine, everolimus or FTY720. Thus, AEB may have the potential to offer an alternative to calcineurin inhibitor-based therapies.

## Introduction

Current anti-rejection drugs exert potent immunosuppressive effects thereby increasing graft and patient survival. At the same time, however, they can induce severe side effects. For example, T-cell-suppressive calcineurin inhibitors can lead to nephrotoxicity, hypertension and dyslipidemias [1,2]. To reduce these limitations but potentiate their immunosuppression, calcineurin inhibitors are often combined with drugs exerting different mechanisms of action. An alternative strategy to improve the long-term clinical outcome is the search for new immunosuppressants with an improved therapeutic index and a novel mode of action offering different efficacy/or side effect profiles. New low molecular weight inhibitors of early T-cell activation are of particular interest as they may have the potential to replace calcineurin inhibitors.

T-cells are key mediators in the process of allograft rejection and consequently, represent an important immunosuppressive target [3]. For T-cells to become effector lymphocytes, however, they must undergo complex activation cascades triggered by alloantigen binding to T-cell receptors in combination with co-stimulation signals involving a number of intracellular signaling

 

proteins such as Lck, ZAP70, LAT, Vav1, protein kinase C (PKC) kinases, and transcription factors including NFAT and NFκB [4].

Our research has focused on the PKC family of serine/threonine kinases. These comprise:

**1** the conventional PKCs (α, β and γ) which are activated by diacylglycerol (DAG), phosphatidylserine and calcium ions;

**2** the novel PKC isoforms (δ, ε, η and θ) that are calcium ion-insensitive; and

**3** the atypical PKCs (ι/λ and ζ) that require neither DAG nor calcium [5].

The potential immunomodulatory roles of PKC isoenzymes have been addressed previously in cellular systems and experimental animal models including knockout mice [6–16]. At least three PKC isoforms (α, β, θ) have been shown to be critical for T- and B-cell activation [17,18]. PKCα is up-regulated during early T-cell activation, and PKCα-knockout mice show a pronounced reduction in IFNγ production [19]. PKCβ knockout mice are immunodeficient due to reduced humoral and cellular responses [20]. Mature T-cells from PKCθ knockout mice show impaired TCR-dependent early T-cell activation, consistent with the involvement of PKCθ in the IL-2 pathway and its role in immunological synapse formation [21–23]. Subsequently, it has been demonstrated that PKCθ-knockout mice are markedly less susceptible to antigen-induced arthritis, with $CD4^+$ T-cells from PKCθ-knockout mice expressing lower levels of Th1 cytokines [16]. These, and a number of other studies into the effects of PKC isoforms, suggest that PKC inhibition may form the basis for new therapeutic options that interfere with early T-cell activation and thus control inappropriate immune responses.

Compared to current immunosuppressive strategies [24], the inhibition of PKC isoforms by low molecular weight compounds [25] is attractive because of the involvement of certain PKC isoforms in early T- and B-cell signaling [10–13,18,21,23,26]. We have embarked on a large synthesis program of low molecular weight PKC inhibitors, testing their target specificity and therapeutic potential using *in vitro* and *in vivo* immunological assays. The novel T-cell suppressant NVP-AEB071 (AEB, INN: sotrastaurin; STN) is a potent oral inhibitor of classical and novel PKC isoforms that blocks early T-cell activation [27].

We report here the efficacy of AEB in a rat graft versus host (GvH) model and in two rat models of cardiac allograft rejection in which AEB was administered alone or in combination with standard immunosuppressant agents. The relation between AEB pharmacokinetics and pharmacodynamic effects was also characterized *in vivo*.

## Materials and methods

### Animals

For the GvH experiments, male Wistar/F rats and (Wistar × Fisher) F1 rats were used (Iffa Credo, L'Arbresle, France). For transplantation studies, inbred male Brown-Norway (BN, $RT1^n$ haplotype), Dark-Agouti (DA, $RT1^a$ haplotype) and Lewis rats (LEW, $RT1^l$ haplotype) of approximately 200–280g body weight were used (Harlan Olac, AD Zeist, the Netherlands). LEW rats acted as recipients throughout, while BN or DA rats served as donors for allogeneic transplantation. These donor–recipient combinations represent major histocompatibility complex (MHC) mismatches which are moderate for the BN-to-LEW and strong for DA-to-LEW strain combination. Rats were housed with free access to water and standard chow and were allowed to adapt to the local environment for at least 1 week before any procedures. Handling and care were in compliance with the Swiss Federal law for animal protection.

### Whole blood *ex vivo* stimulation and flow cytometry analysis

For *ex vivo* pharmacodynamic analyses, blood from AEB-treated male Lewis rats was collected in Eppendorf tubes precoated with sodium heparin (B. Braun, Melsungen, Germany; 5000 IU/ml), to obtain 100 U/ml final concentration. Per sample, 100 µl rat blood was plated out and activated with 10 µl of PMA/aCD28 mAb at a final concentration of PMA of 50 ng/ml and aCD28 mAb of 1 µg/ml [28]. Cultures were set up in duplicates. Control samples were left unstimulated. The final sample volume was adjusted with medium to 195 µl, and one hour after activation, 5 µl brefeldin A was added to a final concentration of 10 µg/ml. As medium served DMEM high glucose (Animed cat# 1-26F01-I) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 50 mg/ml dextran 40 and 5% FCS (Fetaclone I, Gibco #10270-106). All components were mixed thoroughly and plates were incubated at 37 °C, 5% $CO_2$ for further 16 h. Then samples were washed with CellWash (BD Biosciences, San Jose, CA, USA) and cells re-suspended in the remaining 100 µl buffer.

For detection of intracellular and surface markers, cells were permeabilized and nonspecific binding of antibody was blocked by incubation with heat-aggregated mouse IgG. Fluorescent-labeled antibodies in wash buffer were then added and incubated for 30 min in the dark. All antibodies were obtained from BD Biosciences. Samples were stained with APC-labeled anti-rat CD3 mAb and PE-Cy5 (CyChrome)-labeled anti-rat CD4 mAb to allow gating of T-cells and the $CD4^+$ subpopulation in

fluorescent-activated cell sorting (FACS) analysis. In addition, samples were incubated with fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD25. Cells were fixed and data was acquired on a FACScalibur flow cytometer (BD Biosciences) using Cellquest Plus software. Lymphocytes were gated in the FSC/SSC dot plot according to size and granularity, and further analyzed for expression of CD3, CD4 and the activation marker CD25. Data were calculated from dot plots or histograms as percentage of cells positively stained for CD25 in the $CD4^+$ $CD3^+$ subpopulation.

### Pharmacokinetic analysis

AEB was administered to LEW rats orally or intravenously ($n = 3$ in each case) for pharmacokinetic analysis and blood was collected at 2, 6 and 24 h postadministration. Drug concentrations were determined using reversed phase micro-HPLC coupled to a mass spectrometer (LC/MS) for detection. The lower limit of quantification was 5 ng/ml.

### Local GvH reaction (Popliteal lymph node assay)

This GvH model was described previously [29]. Briefly, 20 million spleen cells from male donor Wistar/F rats were injected into the right footpads of recipient male (Wistar × Fisher) F1 rats on day 1. Rats received AEB at oral doses of 3, 10 and 30 mg/kg twice daily (b.i.d.) or once daily (q.d.) in 5 ml/kg vehicle from day 1 to day 5. On day 8, recipients were sacrificed by $CO_2$ inhalation, the right and left popliteal lymph nodes removed and weighed. The weight difference between the right and left lymph node indicates the strength of the GvH reaction. Five rats were used per treatment group. Control groups were given vehicle alone.

### Heterotopic vascular heart allotransplantation

Heterotopic cardiac transplantation was performed as described previously [30]. Briefly, the cooled and heparinized donor heart was removed following ligation of all vessels except the ascending aorta and the right pulmonary artery. These vessels were then anastomosed end-to-side to the recipient's abdominal aorta and inferior vena cava. After release of the clamps, the heart started to beat within less than 2 min. Graft function was assessed daily by palpation for ventricular contraction. Hearts in which ventricular motion had ceased were considered rejected, which was confirmed subsequently by histology. Body weight was monitored regularly.

Recipient rats received the following treatments: vehicle, AEB (10 mg/kg and 30 mg/kg p.o., b.i.d.), cyclosporine (2.5 mg/kg p.o., q.d.), everolimus (0.3 mg/kg p.o., q.d.) or FTY720 (0.1 mg/kg p.o., q.d.), all as monotherapy, or AEB071 (10 mg/kg p.o., b.i.d.) in combination with cyclosporine (2.5 mg/kg p.o., q.d.), everolimus (0.3 mg/kg p.o., q.d.) or FTY720 (0.1 mg/kg p.o., q.d.). AEB was mixed in D(+)-glucose and dissolved in polyethylenglycol 400, 100 mM HCl and distilled water before administration. Micro-emulsion preconcentrates of cyclosporine (Neoral®) and everolimus (Novartis Pharma AG, Basel, Switzerland) were diluted with distilled water and administered orally. FTY720 (Novartis Pharma AG, Basel, Switzerland) was dissolved in distilled water and also given orally. All treatments were performed by oral gavage using rat-feeding needles. All monotherapy treatments were performed using a volume of 3 ml/kg, whereas the combination treatments consisted in twice 2 ml/kg given one after the other, with AEB always applied first. Treatments started immediately after transplantation, with the exception of FTY720 treatments initiated at 1 day pretransplant. The experiments were terminated at rejection or at predefined termination points. For ethical reasons (3R-compliance), most of the treatment groups were limited to $n = 5$–7. An increase in group size up to 10 was allowed to take into consideration a high degree of variability in the results. This was the case for the evaluation of the dose-related AEB monotherapy treatments in the DA-to-LEW model.

For histological examination, cardiac allografts were fixed in 10% buffered formalin, processed according to standard procedures and embedded in paraffin. Tissue blocks were cut to 3-μm sections that underwent staining with hematoxylin and eosin (H&E) and trichrome. The degree of acute cardiac rejection was scored according to Stewart [31]: 0R, no rejection; 1R, mild; 2R, moderate; 3R, severe. In addition, the grade of cardiac allograft vasculopathy (CAV) was scored: 0, no CAV; 1, mild (<25% lumen occlusion); 2, moderate (26–50% occlusion); 3, severe (>50% occlusion).

### Statistical analysis

In the GvH studies, data from different treatment groups were assessed for statistical significance by two-way ANOVA. Transplantation survival analysis was performed using Systat Version 10.2, SPSS Inc., Chicago, IL, USA. Since the duration of the experiment was restricted such that graft survival could not be monitored up to rejection time, Kaplan–Meier survival analysis was applied and the log-rank test was used for comparisons between two survival curves. The level of significance was set at $P < 0.05$.

 

## Results

### Pharmacokinetics and pharmacodynamics of AEB

Following intravenous administration ($n = 3$), the plasma half-life of AEB in LEW rats was 3.2 h and drug clearance was 3070 ml/h/kg (Table 1). Importantly, tissue distribution studies in rats revealed that AEB was distributed preferentially to lymphatic tissues. Area under the curve (AUC) for the first 24 h after oral administration of AEB 30 mg/kg was 31 μg/g·h (spleen), 21 μg/g·h (mesenteric lymph nodes), 13 μg/g·h (thymus) and 1.9 μg/g·h (blood). Pharmacokinetic analysis after intravenous and oral ($n = 3$) administration indicated an oral bioavailability of 34%, compatible with oral administration for *in vivo* testing.

The effect of AEB on T-cell activation was addressed by *ex vivo* stimulation of diluted blood from Lewis rats treated with vehicle or a single oral dose of AEB (10 or 30 mg/kg). Blood lymphocytes were stimulated via direct PKC activation using phorbol-12-myristate-13-acteate (PMA) without additional $Ca^{2+}$ ionophores. To increase specificity of the activation for T lymphocytes an activating anti-CD28 antibody was used in combination with PMA (PMA/aCD28). Activation was visualized by assessing the expression of CD25 (IL-2R) on $CD4^+$ $CD3^+$ T-cells by flow cytometry. With AEB 10 mg/kg, CD25 expression was inhibited by about 75% at 2 h post-treatment with full recovery at 6 h (Fig. 1a). At the higher dose of AEB (30 mg/kg) almost complete inhibition of CD25 expression was achieved at 2 h, with >60% inhibition remaining 6 h after administration (Fig. 1b). Blood levels were time-dependent and correlated positively with inhibition of CD25 expression (Fig. 1b). AEB did not have a discernable effect on the numbers or relative proportions of lymphocyte subpopulations. The dose- and time-dependent pharmacodynamic effects of oral AEB 10 mg/kg and 30 mg/kg indicated that both doses were suitable for further profiling in rats.

**Table 1.** Pharmacokinetic parameters of AEB in rats after administration of a single oral or intravenous dose. Levels were measured in whole blood by LC/MS.

| | |
|---|---|
| Oral administration | |
| Dose (mg/kg) | 20 |
| $C_{max}$ (ng/ml) | 623 |
| $T_{max}$ (h) | 0.5 |
| AUC (ng/g·h) | 2209 |
| Intravenous administration | |
| Dose | 5 |
| AUC (ng/g·h) | 1629 |
| $t_{½}$ E (h) | 3.2 |
| Clearance (ml/h/kg) | 3070 |
| Oral bioavailability (%) | 34 |

### Rat GvH model

In the GvH model, oral AEB strongly inhibited the allo-antigen-driven weight increase of draining popliteal lymph nodes in a dose-dependent manner (Fig. 2). This inhibition was highly significant for all doses 3, 10, and 30 mg/kg when administered twice daily (b.i.d.), and also for single daily doses (q.d.) of 10 and 30 mg/kg. Thus, AEB is a potent inhibitor of the allogeneic response in the rat GvH model.

### Effect of AEB monotherapy on rat cardiac allograft survival

In vehicle-treated recipient rats ($n = 5$), BN grafts were acutely rejected on days 7 and 8 (Fig. 3a). Oral AEB at 10 mg/kg and 30 mg/kg b.i.d. showed a dose-dependent immunosuppressive effect (Table 2) leading to pronounced

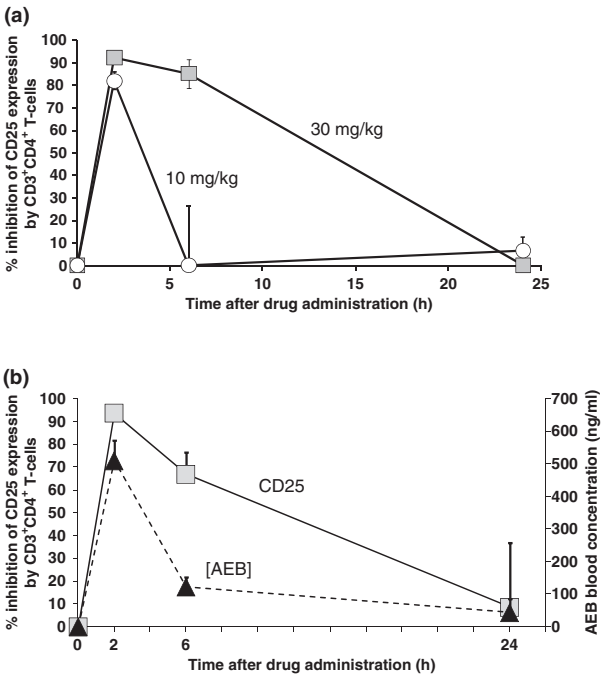


**Figure 1** Dose- and time-dependent inhibition of CD25 and correlation with AEB exposure. (a) Four rats received a single oral dose of AEB 10 mg/kg (circles) or 30 mg/kg (squares). Blood was sampled after 2, 6 and 24 h and whole blood was activated with PMA/anti-CD28 mAb. Expression of CD25 by $CD3^+$ $CD4^+$ cells was determined by multicolor FACS analysis. Data show % inhibition of CD25 expression compared to vehicle-treated control. (b) Four rats received a single oral dose of AEB 30 mg/kg. Blood was sampled and activated as in (a). At the same time, blood exposure of AEB was determined. Data show % inhibition of CD25 expression compared to vehicle-treated control (squares, solid line) and AEB blood concentration (triangles, dotted line).

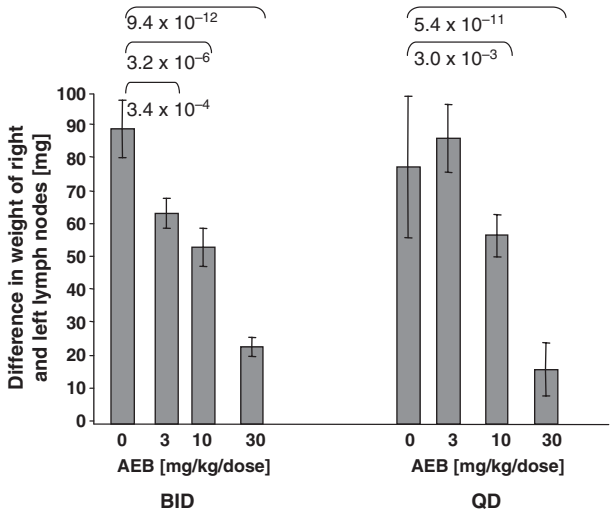


**Figure 2** AEB dose-response study in the rat local GvH model. Spleen cells ($2 \times 10^7$) from Wistar/F donor rats were injected into the right foot pads of (Fisher344xWistar/F) F1 recipient rats. From the day of injection (day 1) until day 4, oral AEB was administered twice daily (b.i.d.) or once daily (q.d.). Popliteal lymph nodes were removed and weighed on day 8. Data are differences in weight of right and left lymph nodes. Bars represent mean ± SD of five rats per group. Numbers underneath connected bar graphs indicate *P* values obtained by two-way ANOVA (Bonferroni correction for multiple comparisons confirmed significant differences).

prolongation of heart allograft survival. The median survival time (MST) of BN-to-LEW allografts was 15 days (range 10–17 days) with AEB 10 mg/kg b.i.d. ($n = 7$) and >28 days with AEB 30 mg/kg b.i.d. ($n = 5$). Histological examination revealed moderate to severe acute cellular rejection (Grades 2R–3R) in all cases, indicating that graft rejection was ongoing at time of necropsy (Fig. 3b). Using the stringent DA-to-LEW strain combination, allografts were rejected in vehicle-treated recipient rats on days 6–10 (MST 6 days) post-transplant ($n = 8$). Treatment with oral AEB 10 mg/kg and 30 mg/kg b.i.d. ($n = 10$ each) resulted in MST of 6.5 days (range 6–8 days) and 17.5 days (range 11–28 days), respectively (Table 2). Histological examination revealed severe rejection (Grade 3R) and moderate to severe rejection (Grades 2R–3R), respectively. Thus, in two major MHC-mismatched rat models of allotransplantation, oral AEB monotherapy at 30 mg/kg b.i.d. prolonged cardiac allograft survival with statistical significance.

### Effect of AEB combination therapy on rat cardiac allograft survival

Cyclosporine, everolimus and FTY720 were titrated in pilot transplantation experiments to define suboptimal (i.e. nonefficacious) doses for combination therapy with AEB (data not shown). Here, in DA-to-LEW transplants, cyclosporine at the suboptimal dose of 2.5 mg/kg q.d. prolonged cardiac allograft survival to a MST of 11 days ($n = 6$). Everolimus 0.3 mg/kg q.d. or FTY720 0.1 mg/kg q.d. both failed to affect graft survival times at these doses (MST 7 days and 7.5 days, respectively) (Table 2). Severe acute rejection was confirmed histologically in all animals.

The combination of a low dose of AEB (10 mg/kg b.i.d.), previously shown to be ineffective as monotherapy in the DA-to-LEW strain combination, with cyclosporine at 2.5 mg/kg q.d. prolonged MST to 26.5 days (range 16 to >50 days, $n = 6$). Severe acute rejection (Grade 3R) without signs of chronic allograft vasculopathy was observed in all animals at the end of the treatment period (Fig. 3c). AEB 10 mg/kg b.i.d. in combination with everolimus 0.3 mg/kg q.d. resulted in prolonged allograft survival of MST >69 days (range 36 to >100 days, $n = 6$). A similar prolongation (MST >69 days, range 11 to >100 days, $n = 6$) was observed for AEB 10 mg/kg b.i.d. combined with FTY720 0.1 mg/kg q.d. (Table 2). All grafts rejected before day 100 showed histologically-confirmed severe acute rejection (Grade3R). Grafts of recipient rats that were terminated on day 100 for scheduled necropsy ($n = 3$ each from the everolimus and FTY720 combination group) showed mild to moderate acute interstitial rejection (Grades 1R–2R) and mild to severe chronic allograft vasculopathy in five out of six animals (CAV 1–3, Fig. 3d).

All animals in whom the allograft survived beyond the first week gained body weight during the evaluation period. In terms of hematological parameters, rats receiving FTY720 alone or in combination with AEB showed the well-documented decrease in peripheral lymphocytes. AEB at 10 and 30 mg/kg b.i.d. alone and in combination with cyclosporine or everolimus also resulted in a slight decrease in peripheral lymphocyte counts. No relevant changes in blood chemistry (serum creatinine, alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase) were observed with any treatment regimen.

### Pharmacokinetics of AEB during combination therapy

Pharmacokinetic analysis of blood from transplanted rats showed that AEB 10 mg/kg resulted in an AUC of 1281 ng/ml·h ($n = 10$). Co-administration of cyclosporine, everolimus and FTY720 induced a small to moderate decrease in AEB exposure compared to AEB monotherapy (Fig. 4). Slight increases in cyclosporine and everolimus blood levels were observed during combination treatment with AEB, but there was no marked change in the concentration of FTY720 or its active metabolite FTY720-phosphate compared to FTY720 monotherapy.

 

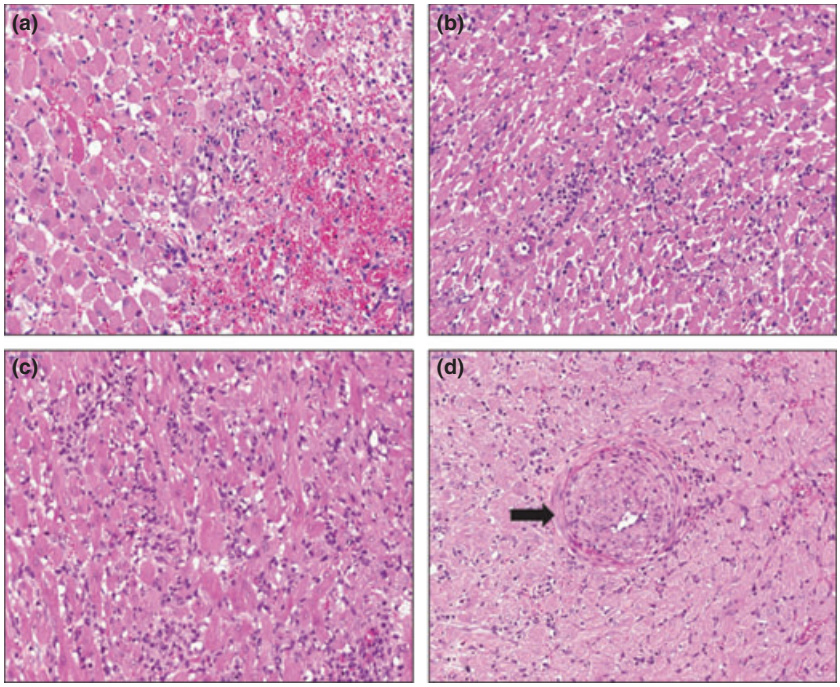


**Figure 3** Histology of the transplanted heart. (a) Severe acute rejection (grade 3R) with intimal arteritis and multifocal interstitial hemorrhage in vehicle-treated animal in BN-to-Lew model, 8 days post-tx (H&E). (b) Moderate acute cellular rejection (grade 2R) in AEB 30 mg/kg b.i.d.-treated animal in BN-to-Lew model, 28 days post-tx (H&E). Multifocal mononuclear cell infiltration with myocyte damage was visible. (c) Severe acute cellular rejection (grade 3R) in AEB 10 mg/kg b.i.d. and cyclosporine 2.5 mg/kg/day-treated animal in DA-to-Lew model, 50 days post-tx (H&E). Diffuse mononuclear cell infiltration with vasculitis and disruption of normal architecture was visible. (d) Mild acute cellular rejection (grade 1R) and severe chronic allograft vasculopathy (arrow) with nearly complete intramural arterial occlusion in AEB 10 mg/kg b.i.d. and FTY720 0.1 mg/kg/day-treated animal in DA-to-Lew model, 100 days post-tx (H&E).

**Table 2.** Effect of oral AEB alone and when combined with oral cyclosporine, everolimus or FTY720 on survival of heart allograft.

| Strain combinations | Treatment | Dose (mg/kg b.i.d.) | Dose (mg/kg q.d.) | Graft survival (days) | Median survival time (days) | Acute cellular rejection scores |
|---|---|---|---|---|---|---|
| BN-to-LEW | Control | 0 | | 7, 7, 7, 8, 8 | 7 | 3R, 3R, 3R, 3R, 3R |
| | AEB | 10 | | 10, 11, 15, 15, 16, 16, 17, | 15* | 3R, 3R, 3R, 3R, 3R, 3R, 3R |
| | AEB | 30 | | >28, >28, >28, >28, >28 | >28* | 3R, 2R, 2R, 2R, 2R |
| DA-to-LEW | Control | 0 | | 6, 6, 6, 6, 6, 7, 7, 10 | 6 | 3R, 3R, 3R, 3R, 3R, 3R, 3R, 3R |
| | AEB | 10 | | 6, 6, 6, 6, 6, 7, 7, 7, 7, 8 | 6.5 | 3R, 3R, 3R, 3R, 3R, 3R, 3R, 3R, 3R, 3R |
| | AEB | 30 | | 11, 14, 15, 16, 17, 18, 18, 20, 23, >28 | 17.5* | 3R, 3R, 3R, 3R, 3R, 3R, 3R, 3R, 3R, 2R |
| | Cyclosporine | | 2.5 | 7, 9, 9, 13, 14, 14 | 11 | 3R, 3R, 3R, 3R, 3R, 3R |
| | AEB + cyclosporine | 10 | 2.5 | 16, 17, 26, 27, 28, >50 | 26.5* | 3R, 3R, 3R, 3R, 3R, 2R |
| | Everolimus | | 0.3 | 7, 7, 7, 7, 8, 8, | 7 | 3R, 3R, 3R, 3R, 3R, 3R |
| | AEB + everolimus | 10 | 0.3 | 36, 37, 37, >100, >100, >100 | >68.5* | 3R, 3R, 3R, 3R†, 2R†, 1R† |
| | FTY720 | | 0.1 | 7, 7, 7, 8, 8, 8 | 7.5 | 3R, 3R, 3R, 3R, 3R, 3R |
| | AEB + FTY720 | 10 | 0.1 | 11, 26, 37, >100, >100, 100 | >68.5* | 3R, 3R, 3R, 3R†, 1R†, 3R |

*$P < 0.01$ versus Control.
†Chronic allograft vasculopathy present.

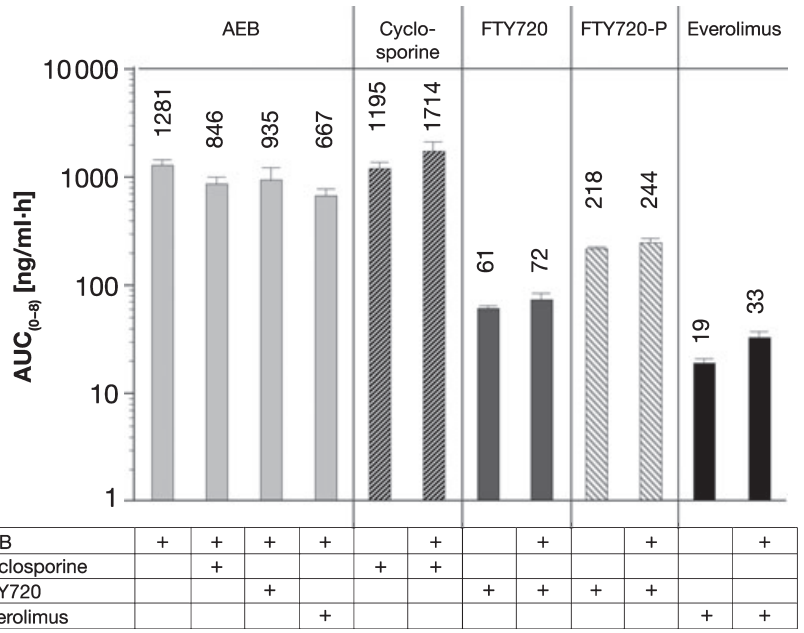


**Figure 4** Comparison of drug exposure as monotherapy or combination therapy in transplanted animals. AUC (0–8 h postadministration, $AUC_{0-8}$) was determined at the end of the experiment, following the linear trapezoidal rule. All drugs were administered orally, at the following doses: AEB 10 mg/kg b.i.d., cyclosporine 2.5 mg/kg/day, everolimus 0.3 mg/kg/day and FTY720 0.1 mg/kg/day. Since FTY720 is rapidly activated *in vivo* by phosphorylation, $AUC_{0-8}$ for the corresponding phosphate (FTY720-P) was also determined. + indicates that treatment was administered.

## Discussion

The potent PKC inhibitor AEB was selected from a large series of maleimide-type kinase inhibitors generated as part of a systematic chemical optimization program [27]. Comprehensive *in vitro* studies using isolated enzymes and various cellular systems have demonstrated AEB to be a highly selective inhibitor of the immunologically relevant PKC isoforms α, β, and θ [27]. In the current study we investigated:

**1** the pharmacokinetics of AEB in relation to its inhibitory effects on the ex vivo activation of T cells; and

**2** the immunosuppressive activity of AEB in rodent models of graft versus host reaction and solid organ transplantation.

Early pharmacokinetic studies in rats demonstrated that AEB is orally bioavailable and distributes preferentially to lymphatic tissues. A low molecular weight immunosuppressant that is orally available and acts through a novel mode of action has the potential to expand the range of drugs currently used to manage allograft rejection and possibly autoimmune diseases. The distribution properties of maleimide-type PKC inhibitors formed part of the selection criteria when identifying candidate inhibitors for further profiling. The observation that oral administration of efficacious AEB doses resulted in high drug concentrations in both the spleen and lymph nodes supported the decision to undertake additional *in vivo* testing since high local drug levels in lymphatic tissue may contribute to efficacy. The role of high lymphatic levels of AEB warrants further investigations in other species, particularly large animals, to form the basis for modeling drug distribution patterns in man.

Expression of the T-cell activation marker CD25, monitored with the goal to predict efficacious doses of AEB in rat transplant models, was found to be inhibited in a dose- and time-dependent manner by AEB. The oral dose of AEB (30 mg/kg b.i.d.) almost completely prevented up-regulation of CD25 on peripheral T cells and was therefore predicted to exert relevant immunosuppressive effects in rat models. The pharmacodynamic profiles of AEB also demonstrate that a single oral dose of AEB can lead to sustained but reversible immunosuppression. Further studies are ongoing to test the effects of AEB on a range of other immune cell markers in animal models. This will support the potential for use of T-cell activation markers as clinical biomarkers of pharmacodynamic changes induced by AEB.

The pharmacokinetic and pharmacodynamic properties observed with AEB (Fig. 1) are consistent with its potent inhibition of the local parent-to-F1 GvH reaction in the rat. This mechanistic model of allogeneic immune responses is based on donor allogeneic T-cell interaction with host B-cells, leading to a swelling of local lymph nodes [29]. Inhibition of early T-cell activation by AEB [27] can explain the observed reduction in popliteal lymph node swelling. This inhibition was efficacious and dose-dependent for both the once-daily and twice-daily dosing regimen. The exception was the lowest dose (3 mg/kg), which was only efficacious when administered twice daily, indicating that AEB 3 mg/kg q.d. is inadequate to maintain continuous immunosuppressive blood

levels. Thus, beyond a certain threshold dose level once daily oral dosing of AEB may be sufficient to achieve a therapeutic effect. Conventional immunosuppressive drugs such as cyclosporine have previously been shown to be highly active in rat GvH [24,29]. The marked activity of oral AEB in the rat GvH model (Fig. 2) suggests that AEB has the potential to interfere with immune responses and thereby prevent graft rejection. Furthermore, efficacy in this model provides a hint that AEB may be useful in autoimmune diseases where T-cell-mediated pathology is dominant. In fact, recent clinical studies demonstrated activity of AEB in the treatment of psoriasis [32].

Oral AEB monotherapy was efficacious in rat solid organ transplantation at doses found to be active in the pharmacokinetic/pharmacodynamic and GvH studies. Based on heartbeat as functional readout the study shows that oral AEB at a dose of 30 mg/kg prevented rejection of BN cardiac allografts in LEW recipient rats over the 28-day treatment period; a less pronounced suppression of allograft rejection was obtained in the more stringent DA-to-LEW strain combination. Histologically confirmed rejection in grafts that were collected at the predetermined endpoint of the observation period indicates that this monotherapy regimen did not provide a full protection against graft rejection over the entire treatment period. This is in line with the findings from the pharmacokinetic/pharmacodynamic and GvH models, in which this dose achieved a marked but incomplete inhibitory effect. However, treatment with AEB in combination with cyclosporine, everolimus or FTY720, using sub-therapeutic doses of each combination drug, led to a marked increase of allograft survival times. The most pronounced effects were observed in the everolimus and FTY720 combination groups, with median survival times of >69 days.

To explore the type of drug interaction governing these combinations, drug levels were monitored at the time of graft rejection or at the point of reaching the predefined endpoint of the study (day 100). In all drug combinations tested, the blood levels of AEB dropped to 53–73% of monotherapy levels while the blood level of all other drugs increased slightly compared to single-agent treatments. While further pharmacokinetic studies are needed, based on these data it is unlikely that the slight rise in blood levels of FTY720 (FTY720-P) and everolimus in combination with AEB contributes significantly to the nine- to 10-fold increase in cardiac allograft survival. Importantly, in the DA-to-LEW strain combination, everolimus monotherapy at a three-fold higher dose of 1 mg/kg has been shown previously at our center to produce inferior allograft survival to that obtained with everolimus 0.3 mg/kg together with AEB, despite the fact that everolimus AUC at 1 mg/kg was twice as high as in the combination arm (data not shown). Thus, the data suggest a synergistic pharmacodynamic effect rather than a pharmacokinetic interaction of AEB in combination with FTY720 and everolimus.

The immunosuppressive effects of AEB described here in rodent mechanistic and transplantation models are mediated through inhibition of classical and novel PKC isoforms as demonstrated in biochemical and cellular studies published elsewhere [27]. The PKC isoforms known to contribute to T-cell activation, namely PKCα, PKCβ and PKCθ [10–13,18,21,23,26] are all potently inhibited by AEB. The further profiling of AEB in batteries of tyrosine, lipid and Ser/Thr kinases showed no relevant inhibitory activity of AEB outside the PKC family [27]. Further studies should address whether specific inhibition of a certain PKC isoform or the broad inhibition of several PKC isoforms is triggering the immunosuppressive effects observed *in vivo*. Moreover, it is possible that the relative contribution of PKC isoform inhibition may differ between models. In view of the broad expression profile of PKCs and their regulatory role in cell activation, growth, differentiation and apoptosis, cytoskeletal functions and gene expression, it is of note that AEB was very well tolerated in the *in vivo* models described for the entire observation period of up to 100 days post-transplant.

In conclusion, the studies reported here suggest a therapeutic potential of the potent pan PKC inhibitor AEB for the treatment of various T-cell-dependent conditions including transplant rejection.

## Authorship

Christian Beerli performed PK studies, collected and analyzed pharmacokinetic data; Christian Bruns designed research/study, analyzed data and wrote the paper; Christoph Burkhart performed the pharmacodynamic analysis; Barbara Metzler designed research, collected and analyzed data and contributed to the manuscript; Randall E. Morris contributed to the design and interpretation of *in vivo* studies; Charles Pally performed all transplantations; Juergen Wagner headed the PKC project that led to the discovery of AEB071 and contributed to planning of the *in vivo* experiments; Gisbert Weckbecker designed and analyzed *in vivo* studies and wrote the paper; Grazyna Wieczorek performed histopathological examination, contributed to the manuscript.

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## References

1. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 2004; **351**: 2715.
2. Lechler R, Sykes M, Thomson AW, Turka LA. Organ Transplantation – how much of the promise has been realized? *Nat Med* 2005; **11**: 605.
3. Hall BM. Cells mediating allograft rejection. *Transplantation* 1991; **51**: 1141.
4. Kane LP, Lin J, Weiss A. Signal transduction by the TCR for antigen. *Curr Opin Urol* 2000; **12**: 242.
5. Parker PJ, Murray-Rust J. PKC at a glance. *J Cell Science* 2004; **117**: 131.
6. Tan S-L, Parker PJ. Emerging and diverse roles of protein kinase C in immune cell signaling. *Biochem J* 2003; **376**: 545.
7. Salonen T, Sareila O, Jalonen U, Kankaanranta H, Tuominen R, Moilanen E. Inhibition of classical PKC isoenzymes downregulates STAT1 activation and iNOS expression in LPS-treated murine J774 macrophages. *Brit J Pharmacol* 2006; **147**: 790.
8. Humphries MJ, Limesand KH, Schneider JC, Nakayama KI, Anderson SM, Reyland ME. Suppression of apoptosis in the protein kinase Cδ-null mouse in vivo. *J Biol Chem* 2006; **281**: 9728.
9. Klecha AJ, Genaro AM, Gorelik G, *et al.* Integrative study of hypothalamus–pituitary–thyroid–immune system interaction: thyroid hormone-mediated modulation of lymphocyte activity through the protein kinase C signaling pathway. *J Endocrinol* 2006; **189**: 45.
10. Sumoza-Toledo A, Eaton AD, Sarukhan A. Regulatory T cells inhibit protein kinase Cθ recruitment to the immune synapse of naive T cells with the same antigen specificity. *J Immunol* 2006; **176**: 5779.
11. Manicassamy S, Gupta S, Huang Z, Sun Z. Protein kinase C-θ-mediated signals enhance $CD4^+$ T cell survival by up-regulating Bcl-xL. *J Immunol* 2006; **176**: 6709.
12. von Essen M, Nielsen MW, Bonefeld CM, *et al.* Protein kinase C (PKC)α and PKCθ are the major PKC isotypes involved in TCR down-regulation. *J Immunol* 2006; **176**: 7502.
13. Hermann-Kleiter N, Thuille N, Pfeifhofer C, *et al.* G PKCθ and PKA are antagonistic partners in the NF-AT transactivation pathway of primary mouse $CD3^+$ T lymphocytes. *Blood* 2006; **107**: 4841.
14. Pardo OE, Wellbrock C, Khanzada UK, *et al.* FGF-2 protects small cell lung cancer cells from apoptosis through a complex involving PKCε, B-Raf and S6K2. *EMBO J* 2006; **25**: 3078.
15. Hayashi K, Altman A. Filamin A is required for T cell activation mediated by protein kinase C-theta. *J Immunol* 2006; **177**: 1721.
16. Healy AM, Izmailova E, Fitzgerald M, *et al.* PKC-theta deficient mice are protected from Th1-dependent antigen-induced arthritis. *J Immunol* 2006; **177**: 1886.
17. Baier G. The PKC gene module: molecular biosystematics to resolve its T cell functions. *J Immunol. Reviews.* 2003; **192**: 64.
18. Guo B, Su TT, Rawlings DJ. Protein kinase C family functions in B-cell activation. *Curr Opin Immunol* 2004; **16**: 367.
19. Pfeifhofer C, Gruber T, Letschka T, *et al.* Defective IgG2a/2b class switching in PKCα−/− mice. *J Immunol* 2006; **176**: 6004.
20. Leitges M, Schmedt C, Guinamard R, *et al.* Immunodeficiency in protein kinase C beta-deficient mice. *Science* 1996; **273**: 788.
21. Altman A, Isakov N, Baier G. Protein kinase C θ: a new essential superstar on the T-cell stage. *Immunol Today* 2000; **21**: 567.
22. Sun Z, Arendt CW, Ellmeier W, *et al.* PKC-θ is required for TCR-induced NFκB activation in mature but not immature T lymphocytes. *Nature* 2000; **404**: 402.
23. Pfeifhofer C, Kofler K, Gruber T, *et al.* Protein kinase C theta affects $Ca^{2+}$ mobilization and NFAT cell activation in primary mouse T cells. *J Exp Med* 2003; **197**: 1525.
24. Dumont FJ. Immunosuppressive strategies for prevention of transplant rejection. *Exp Opin Ther Patents* 2001; **11**: 377.
25. Hu H. Recent discovery and development of selective protein kinase C inhibitors. *DDT* 1996; **1**: 438.
26. Wilkinson SE, Nixon JS. T-cell signal transduction and the role of protein kinase C. *Cell Mol Life Sci* 1998; **54**: 112.
27. Evenou JP, Wagner J, Zenke G, Brinkmann V, Wagner K, Kovarik J. The potent protein kinase C selective inhibitor AEB071 (Sotrastaurin) represents a new class of immunosuppressive agents affecting early T cell activation. *J Pharmacol Exp Ther* 2009; **330**: 792.
28. Weckbecker G, Bruns C, Fischer KD, *et al.* Strongly reduced alloreactivity and long-term survival times of cardiac allografts in Vav1- and Vav1/Vav2-knockout mice. *Transplant Intl* 2007; **20**: 353.
29. Rolstad B. The popliteal lymph node graft-versus-host (GvH) reaction in the rat: a useful model for studying cell interactions in the immune response? *Immunol Rev* 1985; **88**: 153.

30. Ono K, Lindsey ES. Improved technique of heart transplantation in the rat. *J Thorac Cardiovasc Surg* 1969; **57**: 225.
31. Stewart S, Winters GL, Fishbein MC, *et al.* Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. *J Heart Lung Transplant* 2006; **24**: 1710.
32. Skvara H, Dawid M, Kleyn E, *et al.* The PKC inhibitor AEB071 may be a therapeutic option for psoriasis. *J Clin Invest* 2008; **118**: 3151.


 