

## Effect of E6060

(4-{5-[7-fluoro-4-(trifluoromethyl)benzo[*b*]furan-2-yl]-1*H*-2-pyrrolyl}benzoic acid), a novel subtype-selective retinoid, on lupus-like nephritis in female (NZBxNZW)F1

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**Abbreviations:** RAR, retinoic acid receptor; B/WF1 , NZBxNZW F1; SLE, systemic lupus erythematosus; BUN, blood urea nitrogen; ATRA, all-trans retinoic acid; DNP-KLH, dinitrophenylated keyhole limpet hemocyanin.

## Abstract

Disease amelioration by retinoids in various nephritic models has been reported from either immunological or pathophysiologic viewpoints. It has been also reported that retinoids exert immunosuppressive effects via retinoic acid receptor (RAR)- $\alpha$  dependent manner. In particular, synthetic retinoid agonists with selectivity to RAR- $\alpha$  have been reported to have remarkable disease ameliorating effect in some immune disease models via their potent immunosuppressive activities. However, there has been no report in which the effect of RAR- $\alpha$  selective agonists in the nephritic models was examined. In this report, we investigated the effect of a newly synthesized RAR- $\alpha$  selective retinoid agonist, E6060 (4-{5-[7-fluoro-4-(trifluoromethyl)benzo[*b*]furan-2-yl]-1*H*-2-pyrrolyl}benzoic acid), on the disease progression in a murine lupus nephritis model. Female (NZBxNZW)F1 mice were prophylactically treated with E6060 from 5 months of age, and their nephritic (proteinuria, BUN) and immunological parameters (serum anti-DNA autoantibodies, and total serum immunoglobulins) were monitored with age up to 10 months old. E6060 at 0.03 and 0.1 mg/kg (once daily, p.o.) significantly improved survival rate and prevented the development of proteinuria in (NZBxNZW)F1. Anti-DNA autoantibodies and total serum IgG were also significantly reduced in the E6060-treated mice. Among IgG isotypes, IgG2a was substantially reduced by E6060 treatment, indicating reduced Th1 responses in E6060-treated mice. In accordance with this possibility, elevation of serum interleukin-12 (p40) in old female B/W F1 was significantly inhibited by E6060 treatment. Our data suggest that RAR- $\alpha$  selective retinoid, E6060, is a promising candidate of new remedy for lupus nephritis in systemic lupus erythematosus patients.

## Introduction

Retinoids are vitamin A derivatives used for the treatment of vitamin A deficiency and dermatological disorders, as well as for chemoprophylaxis and therapy of certain cancers. Retinoids are ligands for retinoic acid receptor (RAR) and/or retinoid X receptor (RXR), both of which belong to the steroid receptor superfamily (Mangelsdorf and Evans, 1995). RAR and RXR each have 3 subtypes,  $\alpha$ ,  $\beta$ , and  $\gamma$ . Retinoids act by modulating the transcription of target genes through interaction with these RAR and RXR subtypes, thereby interfering with cellular regulation, growth and differentiation processes.

Retinoids, such as 4-hydroxyretinamide, all-*trans*-retinoic acid (ATRA), and 13-*cis*-retinoic acid, were reported to be effective in some immunological disease models, such as rat adjuvant arthritis (Brinckerhoff et al., 1983) and experimental allergic encephalomyelitis (EAE) (Racke et al., 1995), which are believed to be caused through inappropriate cellular immune responses. Recently, Am80, a ligand with an affinity specific for RAR- $\alpha$ , was reported to inhibit collagen-induced arthritis concomitantly with a prominent reduction in anti-collagen IgG titers (Kuwabara et al., 1996; Nagai et al., 1999). This report was the first one to show that a retinoid could reduce antibody production and thereby prevent rat collagen-induced arthritis. We have also synthesized and reported RAR- $\alpha$  subtype selective retinoid agonists (Yoshimura et al., 2000; Kikuchi et al., 2000, Seino et al., 2000). Among them, E6060 is one of the most potent RAR- $\alpha$ -selective ligands so far reported, and it exerts a potent immunosuppressive effect in murine models (Yoshimura et al., 2000).

Lupus nephritis is a devastating disease state associated with 40-50% of systemic lupus erythematosus (SLE) patients. Even nowadays, renal dysfunction due to lupus nephritis results in the death of a significant proportion of SLE patients. There are few effective therapies for this disease, and most of them provoke severe toxicity. To date, some synthetic retinoids have been prescribed for SLE patients with refractory dermal complications (Shornick et al., 1991).

These retinoids remarkably ameliorated the dermal disorders of the patients without apparent toxicity, but their effect on the autoimmune parameters was not mentioned in those reports. (NZBxNZW)F1 mouse (B/WF1) is a well-known model for lupus nephritis in SLE patients. This mouse resembles numerous autoimmune features of SLE patients, such as abnormal autoantibody formation and deposition of immune complexes (consisting of autoantibodies and components of complement) in the nephrons (Dixon et al., 1971), vasculitis, lymphadenopathy, and splenomegaly. Kinoshita et al. recently reported that administration of ATRA remarkably inhibited autoimmune nephritis in this mouse lupus model through its profound immunomodulating activities (Kinoshita et al., 2003). In addition, Lehrke and Liebler showed disease amelioration by various retinoids in anti-Thy-1.1-induced rat nephritis, a model of mesangioproliferative glomerulonephritis (Lehrke et al., 2002; Liebler et al., 2004). Their studies demonstrated that RAR- and RXR-selective retinoids suppressed the glomerular injury through inhibition of mesangial cell proliferation, glomerular infiltration of monocytes/macrophages, and accumulation of extracellular matrix components. By referring those findings, we investigated whether our RAR- $\alpha$ -selective ligand would inhibit the pathology of murine nephritis models. We prophylactically treated female B/WF1 with E6060, and found its remarkable disease ameliorating effect on various autoimmune parameters in this model.

## Materials and Methods

### *Animals*

Female (NZBxNZW)F1 (B/WF1) mice were obtained from Japan SLC Inc. (Shizuoka, Japan) at 15 weeks old. NZW mice were also purchased from SLC Inc., and served as non-lupus controls in the histopathological analysis. Throughout the experiment, these animals were bred in our animal facility under constant temperature and humidity. All animal experimentations were performed according to the internal guidelines for animal experimentation.

### *Drug treatment*

E6060 (4-{5-[7-fluoro-4-(trifluoromethyl)benzo[*b*]furan-2-yl]-1*H*-2-pyrrolyl}benzoic acid) was synthesized at Eisai Tsukuba Research Laboratories. At 5 month of age, female B/WF1 mice were divided into 5 groups each consisting of 12 animals. E6060 (0.01, 0.03 and 0.1 mg/kg) was suspended in 0.5 % methylcellulose (MC) solution, and orally administered once a day, 5 times a week from 5 to 10 month of age. Blood and urine samples were collected every other week, from 6 to 10 months of age, and subjected to assay. At the end of the study, B/WF1 and NZW were sacrificed for histological evaluation of the kidneys.

### *Anti-double stranded (ds) and single stranded (ss) DNA IgG ELISA*

We determined anti-*ds* and *ss*DNA by means of ELISA according to the previous report (Early et al., 1996) with slight modifications. Plates were pre-coated with 10 µg/ml poly-L-lysine (Sigma, St. Louis, MO), then coated with 50 µL of 5 µg/ml poly(dA-dT) / poly(dA-dT) dsDNA (Sigma) or 10 µg/ml heat-denatured calf thymus DNA (*ss*DNA, Sigma). Wells were blocked, then incubated with serum samples or standard serum for 2 h. The plates were washed and 50 µL of biotinylated goat anti-mouse IgG (Amersham International, Buckinghamshire, UK) was added to each well. Anti-DNA Ab was detected with 50 µL of POD-labeled streptavidin (Amersham) and developed with 50 µL of substrate (400 µg/ml *o*-phenylenediamine). The

amount of either anti-*ss*DNA or *ds*DNA IgG in each sample was expressed as units/ml, with reference to the standard serum containing 1000 U/ml of each class of anti-DNA.

#### *IL-12 ELISA*

Unlabeled or biotin-labeled anti-mouse IL-12 (p40/p70) was purchased from PharMingen (San Diego, USA). Mouse IL-12 heterodimer was obtained from R&D Systems (Minneapolis, MN). Assays were performed according to the procedure previously described (Gately, 1991).

#### *Immunoglobulin isotype ELISA*

We measured serum immunoglobulins with specific ELISA systems. We used unlabeled or peroxidase (POD)-conjugated goat IgG fraction of anti-mouse IgG Fc, IgG1 ( $\gamma$ 1), IgG2a ( $\gamma$ 2a) or IgM ( $\mu$ ) (all purchased from ICN Pharmaceuticals, Aurora, OH) for the first and second antibodies, respectively. The procedure was almost the same as aforementioned, except for the use of POD-labeled second antibodies, instead of the biotin-labeled ones. Each immunoglobulin concentration was calculated with reference to the subtype standard (IgG, IgG1, IgG2a, and IgM, all purchased from Zymed Laboratories, San Francisco, CA).

#### *Blood urea nitrogen (BUN)*

Serum BUN concentration was determined with BUN B test Wako (Wako Pure Chemical, Osaka, Japan), according to the manufacturer's instruction.

#### *Proteinuria*

We determined urinary protein content with Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA). Urinary protein was scored according to the following criteria ; 0 : < 30 mg/dL, 1<sup>+</sup>:  $\geq$  30 mg/dL, 2<sup>+</sup>:  $\geq$  100 mg/dL, 3<sup>+</sup>:  $\geq$  300 mg/dL, and 4<sup>+</sup>:  $\geq$  1000 mg/dL. Incidence of proteinuria represents the number of mice whose urinary protein score reached 1<sup>+</sup> or more.

#### *Renal histopathology*

Left kidney from each mouse was removed at the end of the study (at 10 months of age), and fixed in phosphate-buffered 10% formalin and embedded in paraffin. Five-micrometer

sections were stained with hematoxylin-eosin (HE) or periodic acid-Schiff (PAS). An observer blinded as to source of each section scored all glomeruli and classified the lesion as reported elsewhere (Raji et al., 1984; Uehara et al., 1991). The scoring system was as follows; 0: no lesion, 1<sup>+</sup>: ≤ 25 %, 2<sup>+</sup>: ≤50 %, 3<sup>+</sup>: ≤ 75 %, 4<sup>+</sup>: ≤100 % of glomeruli were involved. Injury score for an animal was calculated according to the following formula; glomerular injury score =  $\Sigma[(\text{degree of lesion}) \times (\% \text{ of the glomeruli with the same degree of lesion})]$ .

### *Immunohistochemistry*

To detect deposition of IgG and the third component of complement (C3) in glomeruli, right kidneys were frozen at -80°C until use. Five micrometer cryostat sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and C3 (ICN) diluted at 1:500 for 2h at room temperature under dark condition. After rinsing with PBS thoroughly, fluorescein positive deposits were observed with a fluorescent microscopy (Microphot-FZA, Nikon, Tokyo) equipped with a camera (FX-35DX, Nikon). For negative control, non-immune FITC-conjugated goat IgG (ICN) was used.

### *Statistics*

Unless otherwise mentioned, data are expressed as mean ± SEM. Parameters for drug-treated groups were compared with those for control B/WF1 by means of analytical methods indicated in footnotes under each figure or table. The dose-responsiveness of the effect of E6060 was evaluated using Jonckheere's or Tarone's test. A probability value of 5 % (2-sided) was considered statistically significant in all cases. All statistical analysis was conducted using the software package, SAS 6.12 (SAS Institute Japan, Tokyo, Japan).

## Results

### *RAR subtype selectivity of E6060*

As described in our previous report (Yoshimura et al., 2000), the values of binding affinity ( $K_i$ ) of E6060 to RAR- $\alpha$ , RAR- $\beta$ , and RAR- $\gamma$  were 0.2, 51, and 143 nM, respectively. In accordance with this, its  $EC_{50}$  values in RAR- $\alpha$ , RAR- $\beta$ , and RAR- $\gamma$ -mediated reporter transactivation assays were 0.2, 7, and 40 nM, respectively. E6060 did not induce reporter transactivation through any of the RXR homodimers (RXR- $\alpha$ , RXR- $\beta$ , and RXR- $\gamma$ ) at concentrations up to 3000 nM. In parallel experiments, *all-trans* retinoic acid (ATRA), an authentic endogenous retinoid, had almost the same affinity toward each RAR subtype in our binding and transactivation assays. Thus, E6060 is a selective RAR- $\alpha$  agonist, without any affinity for RXRs.

### *Survival Rate of B/WF1*

Female B/WF1 mice were orally treated with either E6060 (0.01, 0.03 and 0.1 mg/kg) from 5 to 10 months of age and their survival was monitored during that period. The survival rate among control B/WF1 started to decrease from around 7 months of age. Only 4 out of 12 control B/WF1 were alive at 10 months of age. E6060 improved their survival in a dose-dependent manner, and its effect at 0.03 and 0.1 mg/kg was statistically significant (Fig 1a;  $p < 0.05$  by Log-rank test followed by Bonferroni adjustment). E6060-treated animals looked healthy and gained body weight normally unless they developed nephritis. As indicated in Fig 1b, we didn't observe any body weight loss or inhibition of body weight gain in any E6060-treated groups.

In another experiment, in which B/WF1 mice were therapeutically treated with E6060 at 0.1 mg/kg for 6 months, blood samples were collected from the animals that survived to perform both blood chemical and hematological analysis. Even after the 6-month treatment,

E6060 did not show any toxicity as shown by the evaluation on hepatic markers (i.e. serum aspartate aminotransferase and alanine aminotransferase), nor anemia, which is known to be a common complication after usual retinoid treatment (data not shown). Teratogenicity is another severe toxicity with retinoid treatment. Although it is a preliminary finding, E6060 has been found to be a potent teratogen even at its pharmacological dose (i.e. 0.1 mg/kg) in our preliminary reproductive safety tests like common retinoids. This issue will be investigated more thoroughly in future safety evaluations.

#### *Proteinuria and BUN*

The number of mice with proteinuria was also monitored at various assay points and is shown in Fig. 2a. The incidence of proteinuria represents the number of mice whose urinary protein score reached 1<sup>+</sup> or more (over 30 mg/dL of protein concentration). Proteinuria-positive B/WF1 was observed in the control group already at 6.5 months of age, and all control mice had proteinuria by 10 months of age. E6060 prevented proteinuria development in a dose-dependent manner, and its effect was statistically significant at 0.03 and 0.1 mg/kg. In addition, E6060 prevented the elevation of BUN in B/WF1, and the effect was statistically significant from 0.03 mg/kg. Mean BUN in the control and E6060 0.01 mg/kg groups declined at the latest stage of the experiment. This is due to the fact that many animals in both groups with high BUN level died during that period, and they were excluded from the calculation of mean BUN thereafter.

#### *Renal Histopathology of B/WF1*

As shown in Fig3a, renal sections from control B/WF1 revealed remarkable proliferation and PAS-positive deposits in the mesangial region, massive mononuclear cell infiltrates in interstitium, vasculopathy and destruction of capillaries. Their glomeruli were injured much more severely than in age- and sex-matched NZW, which served as the non-nephritic control.

Due to these histopathological changes, the mean glomerular injury score in control B/WF1 ( $244.8 \pm 27.3$ ) was significantly higher than that of age-matched female NZW ( $125.4 \pm 15.1$ ). E6060 improved the glomerular injury in a dose-dependent manner, and the glomeruli from the animals treated with E6060 at 0.1 mg/kg looked almost normal (Fig3a). E6060 at the doses of 0.01, 0.03 and 0.1 mg/kg decreased the scores to  $223.9 \pm 28.2$ ,  $162.4 \pm 15.9$  and  $133.9 \pm 12.8$ , respectively, and the effect of 0.1 mg/kg of E6060 was statistically significant (Fig. 3b). In addition, E6060 markedly reduced IgG and complement deposition in the glomeruli compared to vehicle-treated B/W F1 (Fig3a).

#### *Anti-DNA IgG Antibody Production*

Serum anti-*ds*DNA IgG in B/WF1 controls gradually increased just after initiation of the study, and remained elevated until the end of the study. E6060 inhibited the anti-*ds*DNA IgG production, and its effect was statistically significant at 0.03 and 0.1 mg/kg. At 0.1 mg/kg, it almost completely inhibited the elevation of this parameter throughout the study period (Fig. 4a). In addition to anti-*ds*DNA, E6060 dose-dependently inhibited the production of anti-*ss*DNA, and its effect was statistically significant at 0.1 mg/kg (Fig. 4b).

#### *Immunoglobulin Production*

In control B/WF1, serum IgG was remarkably elevated within the 3-month period, from 5 to 8 months of age, while serum IgM was not. Among IgG subtypes, IgG2a was preferentially elevated compared to IgG1 in our B/WF1 controls. E6060 prevented the elevation of serum IgG in a dose-dependent manner, and its effect was statistically significant at 0.03 and 0.1 mg/kg (Table 1). It also significantly inhibited serum IgG2a elevation in B/WF1 at the same doses. Conversely, E6060 did not reduce, but rather significantly increased serum IgG1 at 0.1 mg/kg. It was reported that *all-trans* retinoic acid also suppressed IG2a production, but augmented IgG1 production via a reduction of IFN- $\gamma$  production by CD4<sup>+</sup> T-cells (Carman and

Hayes, 1991). Taken together, it suggests that E6060 exerts its effect by affecting murine antibody production in a similar manner as *all-trans* retinoic acid does. E6060 did not significantly alter serum IgM of B/WF1 throughout the study. According to these data, selective inhibition of IgG2a production by E6060 mainly contributed to its significant inhibition of total serum IgG elevation in this strain. Since murine IgG2a production and IgG1 production are known to be selectively induced by Th1- and Th2-cytokines, respectively, we next investigated the effect of E6060 on the serum cytokine levels in B/WF1 .

#### *Serum IL-12*

Serum IL-12 (p40) in B/WF1 controls at 10 months of age was significantly higher than that in age-matched female NZW (B/WF1 :  $863.9 \pm 101.2$  pg/ml vs. NZW:  $113.9 \pm 23.2$  pg/ml,  $p < 0.05$  by Mann-Whitney test). E6060 prevented elevation of serum IL-12 (p40) in B/WF1 in a dose-dependent manner, and its effect was statistically significant at 0.03 and 0.1 mg/kg (Fig. 5). We could not detect any elevation of other cytokines, i.e. IL-2, IL-4 and interferon- $\gamma$ , in our B/WF1 mice (data not shown).

## Discussion

E6060, a newly synthesized retinoid used in the present study, prevented the development of lupus-like nephritis in female B/WF1 mice and prolonged their life span. It remarkably suppressed abnormal elevation of antibody production in those mice, and this effect seems to contribute, at least partly, to its anti-nephritic effect. Recently, an RAR- $\alpha$  selective synthetic retinoid, Am80, was reported to inhibit type II-collagen-induced arthritis with a prominent reduction in anti-collagen IgG titer (Kuwabara et al., 1996; Nagai et al., 1999). Similarly, our RAR- $\alpha$  selective retinoid substantially inhibited not only anti-DNA antibody production (Fig. 4a), but also total IgG and IgG2a elevation in aged female B/WF1 (Table 1). In addition, our recent data indicated that E6060 significantly inhibited the antibody production against T-dependent antigen at 0.1 mg/kg, p.o. (data not shown). Hence, prominent inhibition of antibody production may be a common feature among RAR- $\alpha$  selective retinoids.

Plasma concentration of E6060 after oral dosing even at 0.03 mg/kg (the minimum effective dose) was enough beyond its EC<sub>50</sub> values to cause transcriptional activation via each RAR subtype. Thus, E6060 may have activated all of RAR subtypes in B/WF1, and it is not clear through which RAR subtype(s) E6060 exerted its effect in this study, even though it has higher affinity to RAR- $\alpha$  than the other RAR subtypes. Nevertheless, it has been reported that retinoid inhibits murine B lymphocyte proliferation via RAR- $\alpha$  dependent manner (Apfel et al., 1992). In addition, our preliminary data indicated that ATRA and other retinoids remarkably inhibit proliferation of alloantigen-stimulated murine T lymphocytes, and their effect is completely reversed by RAR- $\alpha$  selective antagonist (Kikuchi et al., 2001). Taking these findings in consideration, it seems possible to speculate that E6060 exerted its immunosuppressive effects in this model, at least partly, through RAR- $\alpha$  dependent manner.

Amongst the various autoantibodies, we measured anti-ss and dsDNA IgG, because these antibodies, especially the latter, are reliable markers of disease progression in SLE patients

(Hahn, 1998). To date, various cytokines have been proposed to be involved in this type of autoantibody production in B/WF1 (Druet et al., 1996). Among them, monoclonal antibody (mAb) against T helper-2 (Th2) cytokines, i.e. IL-4 (Nakajima et al., 1997), IL-6 (Finck et al., 1994) and IL-10 (Ishida et al., 1994), suppressed autoantibody production and improved the lupus nephritis in those mice. Additionally, treatment of the mice with recombinant IL-10 enhanced the autoantibody production and exacerbated the renal injury (Ishida et al., 1994). Besides the Th2-type cytokines, Th1-type cytokines are also shown to be involved in these processes. Anti-IL-12 mAb treatment substantially reduced anti-*dsDNA* IgG production, especially of IgG2a and IgG2b subclasses, in aged B/WF1, while it hardly prevented autoimmune nephritis in these mice (Nakajima et al., 1997). In addition, Appleby reported that IL-12 was an exacerbating factor in murine experimental nephritis models (Appleby et al., 1993). Anti-IL-12 mAb ameliorated anti-glomerular basement membrane (GBM)-induced renal lesions, and anti-GBM IgG2a production (Kitching et al., 1999). In agreement with that study, we observed that E6060 selectively reduced serum level of IgG2a and IL-12 both elevated in aged B/WF1. Marked inhibition of IL-12 production by our retinoid is consistent with the recent report showing that retinoids inhibit IL-12 production from activated macrophages through functional interactions between their receptors (RXR and RAR) and NF $\kappa$ B, a crucial transcription factor for IL-12 gene expression (Na et al., 1999). Taking these findings together, it is conceivable that the inhibition of IL-12 production by our retinoid contributed to substantial inhibition of autoantibody and serum IgG2a production in female B/WF1. Recently, Takano and his colleagues (Takano et al., 1999) reported that elevated serum IL-12 in SLE patients, apparently associated with the degree of their organ involvement. In accordance with that finding, E6060 remarkably reduced the renal injury score in aged B/WF1 (Fig. 3). We consider that IL-12 may play an important role not only in elevated antibody production, but also in developing renal involvement in B/WF1.

In addition to IL-12, IFN- $\gamma$  is regarded as an important cytokine in this model. IFN- $\gamma$  markedly up-regulates IgG2a production in mice (Finkelman et al., 1990). Anti-IFN- $\gamma$  mAb or recombinant soluble IFN- $\gamma$  receptor (IFN- $\gamma$ R) inhibits both autoantibody production and lupus nephritis in B/WF1 (Ozmen et al., 1995; Jacob et al., 1987). In IFN- $\gamma$ R knock-out MRL/lpr mice with lupus nephritis, both autoantibody production and renal injury were remarkably abrogated (Haas et al., 1997). Retinoid has been reported to be an intrinsic factor necessary to inhibit IFN- $\gamma$  production (Abb and Deinhardt, 1987; Carman and Hayes, 1991). Retinoid-mediated suppression of human IFN- $\gamma$  production is exerted at the transcriptional level (Cippitelli, 1996). More recently, Kinoshita reported remarkable disease-ameliorating effect of ATRA in B/WF1 mice (Kinoshita et al., 2003). In his report, ATRA preferentially inhibited the production of anti-DNA IgG2a, a subclass thought to be predominantly nephritogenic in B/WF1 (Park et al., 1983), presumably through its predominant inhibition of IFN- $\gamma$  production by splenic CD4<sup>+</sup> T cells. In this context, we speculate that E6060 also suppressed IFN- $\gamma$  production in B/WF1, and this effect contributed to the substantial improvement of the autoimmune disease. The fact that E6060 inhibited IL-12 production in our B/WF1 mice likely supports this notion, since IL-12 is a potent inducer of IFN- $\gamma$  production (Hsieh et al., 1993). However, very few mice in this study had a detectable level of serum IFN- $\gamma$  when they were sacrificed at 10 months of age, so we could not assess the effect of E6060 on this parameter. Instead, our preliminary observation reveals that E6060-treated B/WF1 had fewer IFN- $\gamma$ -producing CD4<sup>+</sup> T lymphocytes in their spleens than control mice did (data not shown). Another preliminary study also shows E6060 inhibited the upregulation of antigen-induced *ex vivo* IFN- $\gamma$  production and spleen T-cell IFN- $\gamma$  mRNA expression in DNP-KLH-immunized BALB/c mice (data not shown). Hence, inhibition of IFN- $\gamma$  production by E6060 may also contribute, at least in part, to its remarkable disease-modifying effect in B/WF1. This possibility should be investigated more directly in future experiments.

Recently, retinoids with various receptor subtype selectivity were reported to ameliorate the renal injury in anti-Thy1.1 nephritis model, a model of mesangioproliferative glomerulonephritis (Lehrke et al., 2002; Liebler et al., 2004). According to those reports and others (Wagner et al., 2000; Schaier et al., 2001), retinoids prevent renal damage through inhibition of mesangial cell proliferation, reducing extracellular matrix accumulation, lowering number of infiltrating mononuclear cells, and reducing inducible NO synthase expression by mesangial cells. It is suggested that these actions are mediated, at least in part, through activation of RARs locally expressed in glomerular tissues (Liebler et al., 2004). Like other retinoids, E6060 might have those direct actions on local renal tissues, although we don't have any direct evidences on this point and this possibility remains to be clarified. To investigate this possibility more directly, we have started to analyze the effect of the drug on rat Masugi's nephritis model, in which renal disease is induced by passive immunization of nephritogenic antibody, suggesting this nephritis is not dependent on antibody formation in the host animals.

Lupus nephritis is a life-threatening disease. Although the survival rate of SLE patients has improved significantly over time, even in the 1990's 10-20% of patients lost their lives every 5 years, and the biggest cause of death was renal dysfunction due to lupus nephritis. In addition, there are few approved treatments for lupus nephritis at this time, and more effective drugs are highly desirable. E6060 is one of the most potent compounds which have been tested in female B/WF1 (Corna et al., 1997). Moreover, E6060 did not induce any apparent toxicity up to 0.1 mg/kg, at which dose it completely prevented the disease progression. However, our preliminary studies indicate that it is a potent teratogen at this dose. Considering that SLE is most common in women at childbearing age, its teratogenic effects should be thoroughly investigated in additional preclinical studies. Accordingly, clinical trial of E6060 in SLE patients should be considered by carefully referring those safety data, and might be rationalized only if potential benefits from the treatment seem to outweigh its

teratogenic risk, and appropriate contraceptive measures for the patients could be successfully introduced.

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## Footnotes

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## Figure Legends

**Figure 1** Effect of E6060 on the survival rate and body weight gain of female B/WF1. Groups of 12 B/WF1 were treated with vehicle control (●), E6060 0.01 mg/kg (◇), 0.03 mg/kg (△), or 0.1 mg/kg (□) from 5 to 10 months of age. **a)** survival rate of B/WF1 mice. Significant difference from vehicle controls, \* $p < 0.05$  as compared by log-rank test followed by Bonferroni adjustment. **b)** Mean body weight in each treatment group is indicated in a time dependent manner. No statistically significant differences were observed between the control and any E6060-treatment groups at any time points.

**Figure 2** Effect of E6060 on incidence of proteinuria, and BUN level in B/WF1. B/WF1 were treated with vehicle (●), E6060 0.01 mg/kg (◇), 0.03 mg/kg (△), or 0.1 mg/kg (□) as indicated in Fig1. **a)** Incidence of proteinuria represents the number of mice whose urinary protein content score reached at 1<sup>+</sup> (30 mg protein/dL). **b)** BUN data are shown as the mean  $\pm$  SEM of mice surviving at each assay point. \* $p < 0.05$  as compared with control by log-rank test followed by Bonferroni adjustment, and # $p < 0.05$  as compared with control by repeated-measures ANOVA, followed by Dunnett-type multiple comparison.

**Figure 3** E6060 significantly ameliorated the glomerular injury in B/WF1 mice. **a)** Renal histopathology for B/WF1 treated with vehicle or E6060 at 0.1 mg/kg. Remarkable enlargement of mesangial area and mesangial cell proliferation along with mesangial immune deposition is seen in vehicle-treated B/WF1. In addition, IgG and C3 depositions are also detected in control glomeruli by immunofluorescence microscopy. All of these lesions are remarkably attenuated in the glomeruli from E6060-treated animals. All bars in **a** = 50  $\mu$ m. **b)** Glomerular injury score was determined as described in the Methods section, and expressed as

the mean  $\pm$  SEM. The number of mice surviving at the sacrifice point is indicated within parentheses. Significant difference from vehicle B/WF1 controls, \* $p < 0.05$  as compared by Kruskal-Wallis test, followed by Dunnett-type multiple comparison test,  $^{\S}p < 0.05$  by Mann-Whitney test.

**Figure 4** Effect of E6060 on anti-DNA IgG autoantibody production in female B/WF1. B/WF1 mice were treated with vehicle (●), E6060 0.01 mg/kg (◇), 0.03 mg/kg (△), 0.1 mg/kg (□) from 5 to 10 months of age, and anti-*ds*DNA (**a**) and *ss*DNA (**b**) IgG were determined in ELISA assays as described in the Methods section. Data are shown as the mean  $\pm$  SEM in mice surviving at each assay point. Significant difference from vehicle controls, \* $p < 0.05$  as compared by repeated-measures ANOVA, followed by Dunnett-type multiple comparison.

**Figure 5** Effect of E6060 on IL-12 production in female B/WF1. Serum IL-12 level was determined by ELISA specific for IL-12 (p40), at 10 months of age. Data are expressed as the mean  $\pm$  SEM of the group. The number of mice surviving at the assay is indicated within parentheses above the columns. Significant difference from B/WF1 vehicle controls, \* $p < 0.05$  as compared by one-way ANOVA, followed by Dunnett-type multiple comparison, and  $^{\S}p < 0.05$  by Mann-Whitney test.

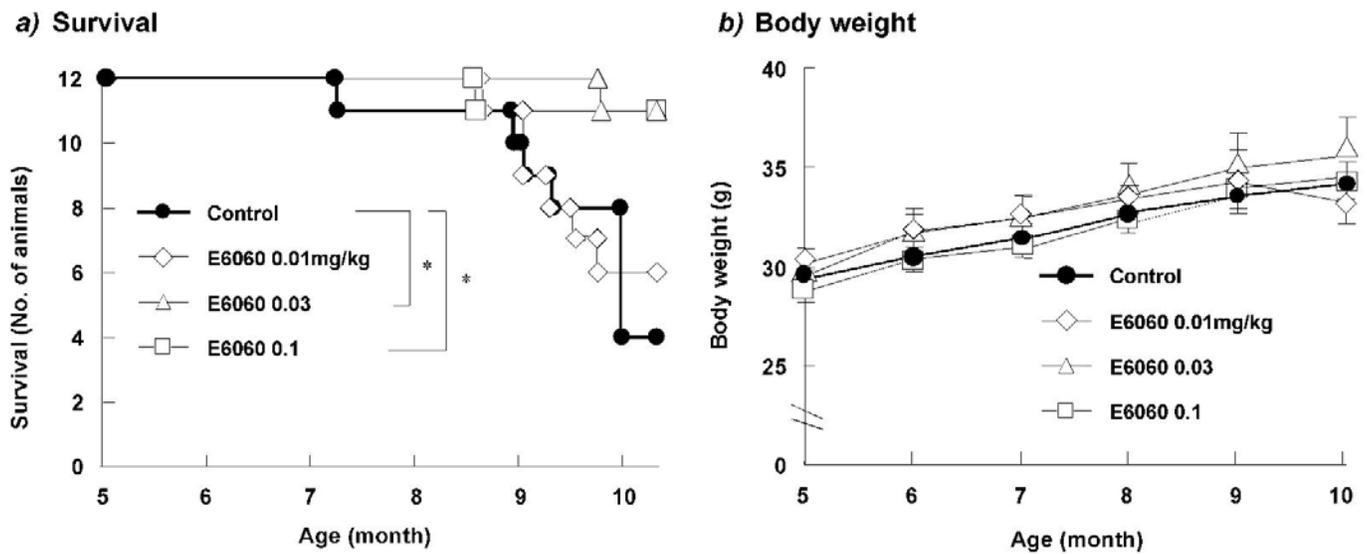
**Table 1** Serum immunoglobulin levels in female B/WF1 treated with E6060

Treatment	Dose (mg/kg)	Age (month)	n	Serum immunoglobulin ( $\mu\text{g/mL}$ )			
				IgM	IgG	IgG1	IgG2a
Vehicle control	-	5	12	4647 $\pm$ 223	2677 $\pm$ 196	274 $\pm$ 14	861 $\pm$ 89
		8	11	3726 $\pm$ 207	5242 $\pm$ 591	287 $\pm$ 34	2320 $\pm$ 218
E6060	0.01	5	12	4633 $\pm$ 308	2958 $\pm$ 233	334 $\pm$ 31	862 $\pm$ 103
		8	12	4042 $\pm$ 399	4702 $\pm$ 677	330 $\pm$ 58	2053 $\pm$ 302
E6060	0.03	5	12	4395 $\pm$ 283	2657 $\pm$ 252	313 $\pm$ 34	796 $\pm$ 87
		8	12	3475 $\pm$ 287	3377 $\pm$ 500*	369 $\pm$ 32	1241 $\pm$ 213*
E6060	0.1	5	12	4758 $\pm$ 307	3230 $\pm$ 298	331 $\pm$ 38	980 $\pm$ 119
		8	12	4746 $\pm$ 918	1518 $\pm$ 234*	853 $\pm$ 297*	351 $\pm$ 44*

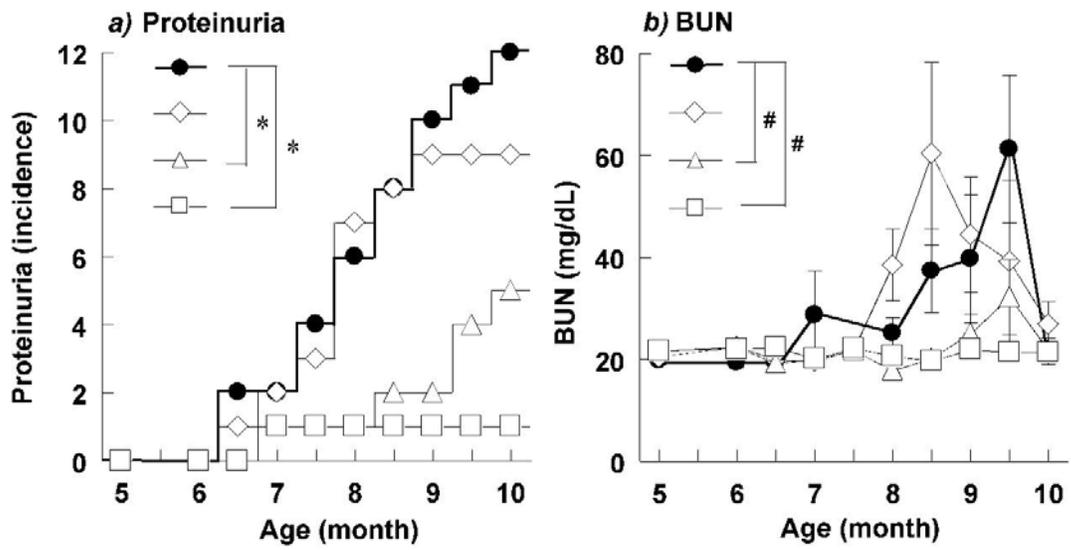
Serum IgM, IgG, IgG1, and IgG2a levels were determined by ELISA at the ages of 5 and 8 months old.

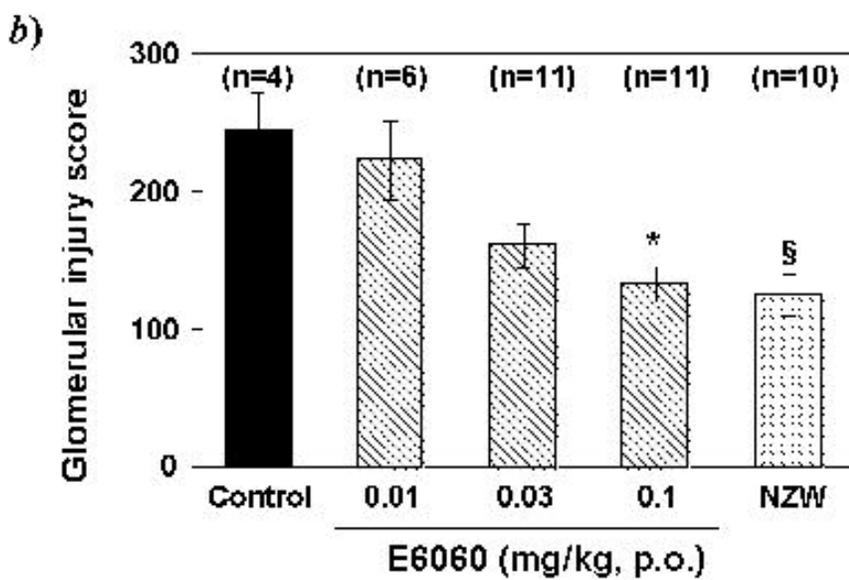
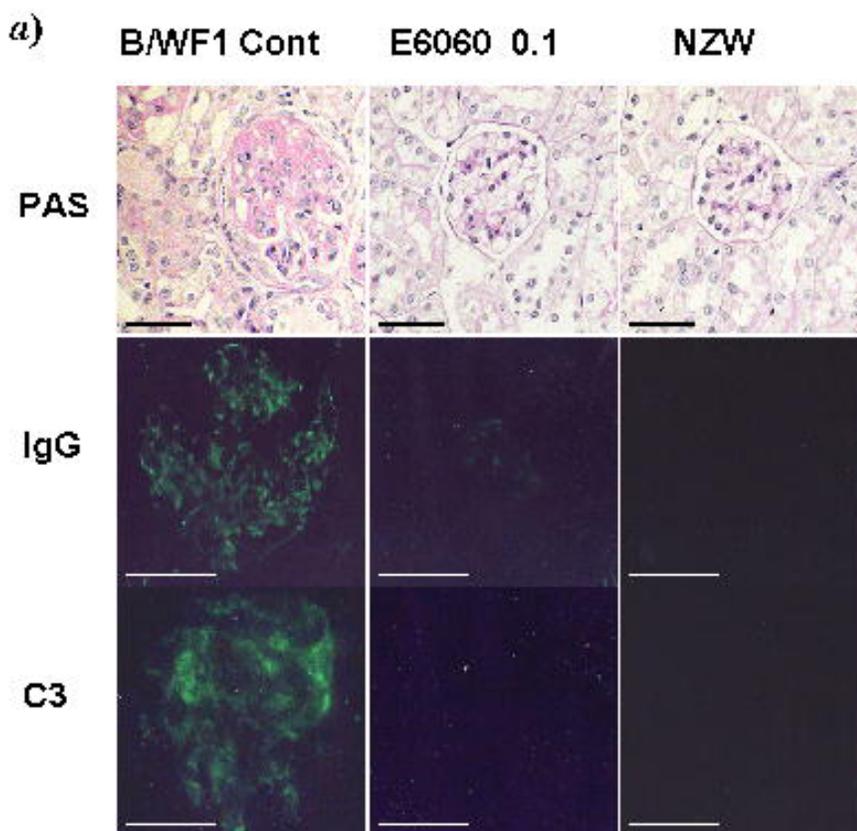
Values are indicated as the mean  $\pm$  SEM of the mice survived at each assay point. Significant difference from age-matched vehicle control, \* $p < 0.05$  as compared by one-way ANOVA followed by Dunnett multiple comparison.

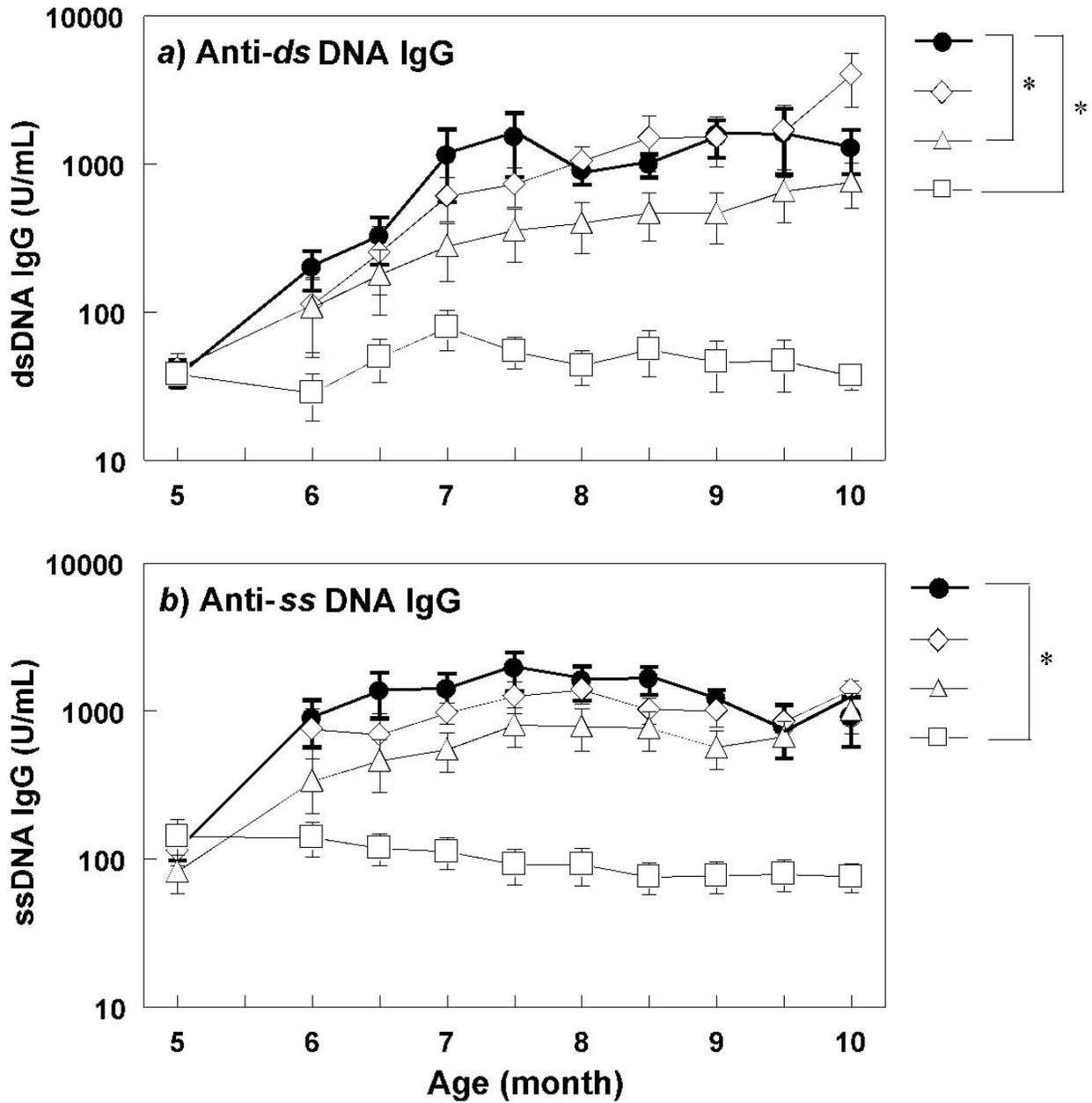
JPET#75598 Figure 1



JPET#75598 Figure 2







JPET#75598 Figure 5

