

## Effects of KW-3635, a Specific Thromboxane A<sub>2</sub>-Receptor Antagonist, on the Development of Lupus Nephritis in NZB × NZW F1 Mice

Michiyo Kawakage, Hideaki Mizumoto, Etsuko Nukui, Soichiro Sato and Akira Karasawa

*Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Shimotogari 1188, Nagaizumi-cho, Sunto-gun, Shizuoka 411, Japan*

*Received June 14, 1993 Accepted September 1, 1993*

**ABSTRACT**—We examined the effect of KW-3635, a specific thromboxane A<sub>2</sub> (TXA<sub>2</sub>)-receptor antagonist, on the development of lupus nephritis in NZB × NZW F1 mice. KW-3635 was orally given once a day for 33 weeks beginning at eight weeks of age. In the control group, the mice began to die at 39 weeks of age, showing severe proteinuria and histopathologic abnormality in the renal glomeruli. Administration of KW-3635 (30 mg/kg/day) significantly reduced urinary protein excretion ( $1.7 \pm 0.9$  vs.  $8.5 \pm 2.4$  mg/6 hr/mouse,  $P < 0.01$ ), mortality (1/18 vs. 6/19,  $P < 0.05$ ) and the histopathologic score of the kidney examined at 41 weeks of age. Thus, chronic administration of KW-3635 markedly attenuated the renal disease in NZB × NZW F1 mice, suggesting that TXA<sub>2</sub> is an important mediator of the pathogenesis in this murine model of lupus nephritis.

**Keywords:** Thromboxane A<sub>2</sub>-receptor antagonist, KW-3635, Kidney, Nephritis

Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) is a labile derivative of arachidonic acid metabolism and exerts multiple actions within the kidney. These actions include constriction of glomerular afferent and efferent arterioles, intraglomerular platelet aggregation, contraction of glomerular mesangial cells and increase of mesangial cell matrix synthesis (1, 2). Increased renal TXA<sub>2</sub> biosynthesis has been documented in a variety of animal models of renal disease (3–5) and in patients with systemic lupus erythematosus (SLE) (6). These results suggest that TXA<sub>2</sub> plays an important role in the progression of renal diseases.

It was demonstrated that patients with SLE had significantly higher urinary TXB<sub>2</sub> (stable product of TXA<sub>2</sub>) and lower 6-keto-PGF<sub>1 $\alpha$</sub>  (stable product of PGI<sub>2</sub>) as compared with those in normal subjects. In the patients with SLE, a significant inverse correlation was found between the urinary TXB<sub>2</sub>/6-keto-PGF<sub>1 $\alpha$</sub>  ratio and creatinine clearance (Ccr) (6). Moreover, recent studies have shown that a thromboxane A<sub>2</sub>-receptor antagonist (TXRA) improves renal function in patients with lupus nephritis (7, 8). Renal TXA<sub>2</sub> production is also increased in NZB × NZW F1 mice that spontaneously develop an autoimmune disease with glomerulonephritis, which resembles human SLE (9). The effects of thromboxane synthase inhibitors (TXSIs) have been investigated in NZB × NZW F1 mice. Most of the studies (10, 11) indicated that TXSI did not protect NZB × NZW F1 mice against the development of

lupus nephritis, although Parbtani et al. (12) reported that a TXSI had some beneficial effects in this strain of mice. On the other hand, few researchers have investigated the possible protective effects of TXRA in NZB × NZW F1 mice (13).

KW-3635 is a novel TXRA (14, 15). We reported that KW-3635 potentiates the diuretic action of furosemide in saline-loaded normal rats, suggesting that it inhibits TXA<sub>2</sub> within the kidney (16). In the present study, we determined the effect of KW-3635 on the development of murine lupus nephritis in NZB × NZW F1 mice to clarify the role of TXA<sub>2</sub> in this renal disease.

### MATERIALS AND METHODS

#### *Animals*

Female NZB × NZW F1 mice (Charles River Japan, Inc., Yokohama) were used. Food and water were provided ad libitum.

#### *Experimental protocols*

Beginning at 8 weeks of age, eighteen NZB × NZW F1 mice in each group were randomized to receive either KW-3635 (30, 100 mg/kg/day) or its vehicle (0.3% sodium carboxymethylcellulose). KW-3635 at 30 or 100 mg/kg/day was orally given to mice once a day. These doses were selected by a preliminary experiment examining the

effect of the drug on the sudden death induced by the TXA<sub>2</sub> analogue U-46619 (0.5 mg/kg, i.v.) in mice. The mortalities in the 30 and 100 mg/kg (p.o.) of KW-3635-treated groups decreased to 75% (15/20) ( $P < 0.05$ ) and 55% (11/20) ( $P < 0.01$ ), respectively, as compared with 100% (20/20) in the vehicle-treated group. The mortality was not improved by 10 mg/kg of KW-3635 (19/20). The doses that blocked the response to U-46619 were chosen for the chronic treatment studies.

Effects of KW-3635 were determined by measuring urinary protein excretion, renal histopathologic changes, anti-DNA antibody levels in sera and the mortality. For the measurement of urinary protein, groups consisting of 9 mice in each were used. The urine was periodically collected for 6 hr following oral administration of saline (0.5 ml/10 g), and the concentration of urinary protein was measured by an autoanalyzer (AU510; Olympus, Tokyo).

Histopathologic changes in the glomeruli were evaluated at 41 weeks. The kidney was removed and fixed with 10% buffered-formalin (pH 7.25). Transverse kidney slices were embedded in paraffin, sectioned and stained with hematoxylin and eosin. The specimens of the kidney were examined by light microscopy. Individual abnormalities in tissue sections were graded on a scale from 0 to 4+, where 0 was no abnormality, and 1+, 2+, 3+, and 4+ represented mild, moderate, moderately-severe, and severe abnormalities, respectively, according to the report of Spurney et al. (17).

Anti-DNA antibody levels in sera were measured by ELISA according to the method of Mihara et al. (18). Briefly, after coating with ssDNA at a concentration of 2  $\mu$ g/ml overnight at 4°C, the plates were washed 3 times with PBS containing Tween 20 and BSA (PBS-T). Serial dilutions of sera in PBS-T were added to each well and incubated. The plates were again washed, followed by the addition of peroxidase-conjugated goat anti-mouse Ig diluted 1/500 in PBS-T. After washing with PBS-T, the plates were incubated with a substrate solution of *o*-phenylenediamine. The absorbance at 490 nm was measured using a Immuno Reader NJ-2000 (Inter Med Japan Co., Ltd., Tokyo). Data are expressed as optical density (OD) units calculated.

#### Drugs used

Sodium (*E*)-11-[2-(5,6-dimethyl-1-benzimidazolyl)ethylidene]-6,11-dihydrodibenz[*b,e*]oxepine-2-carboxylate monohydrate (KW-3635) was synthesized in our laboratories. Single strand DNA (ssDNA) (Sigma Chemical Co., St. Louis, MO, USA) and peroxidase-conjugated goat anti-mouse immunoglobulins (Ig) (Cappel Research Products, Durham, NC, USA) were used to measure anti-DNA antibody levels in sera.

#### Statistical analyses

Data are presented as means  $\pm$  S.E. Results were statistically evaluated with a Scheffe-type test following the Kruskal-Wallis test. For histopathologic studies and incidence of mortality, the statistical significance was tested by the  $\chi^2$ -test.

## RESULTS

At the beginning of the experiment, eighteen NZB  $\times$  NZW F1 mice were used in each group. In the vehicle treated group, the mice began to die from 39 weeks of age. The mice treated with KW-3635 at 30 mg/kg/day had a significantly reduced mortality at 40 and 41 weeks of ages (Fig. 1). In the group treated with a high dose (100 mg/kg/day) of KW-3635, the mortality was sig-

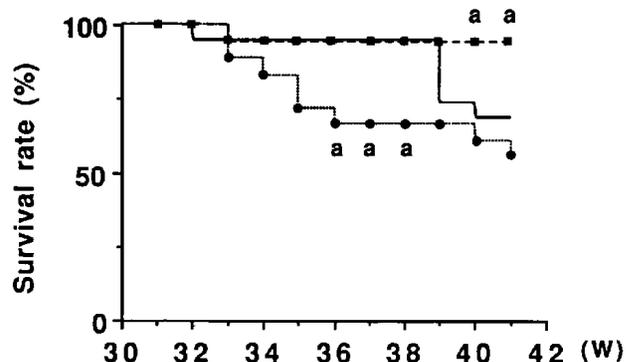


Fig. 1. Mortality in NZB  $\times$  NZW F1 mice treated with vehicle or KW-3635 (30, 100 mg/kg/day, p.o.). Eighteen animals in each group were used at the beginning of the experiment. <sup>a</sup>: Significant difference from the vehicle group at  $P < 0.05$ . No symbol: vehicle, ■: 30 mg/kg/day, ●: 100 mg/kg/day.

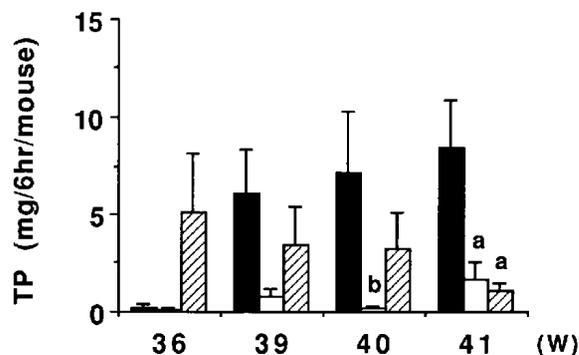
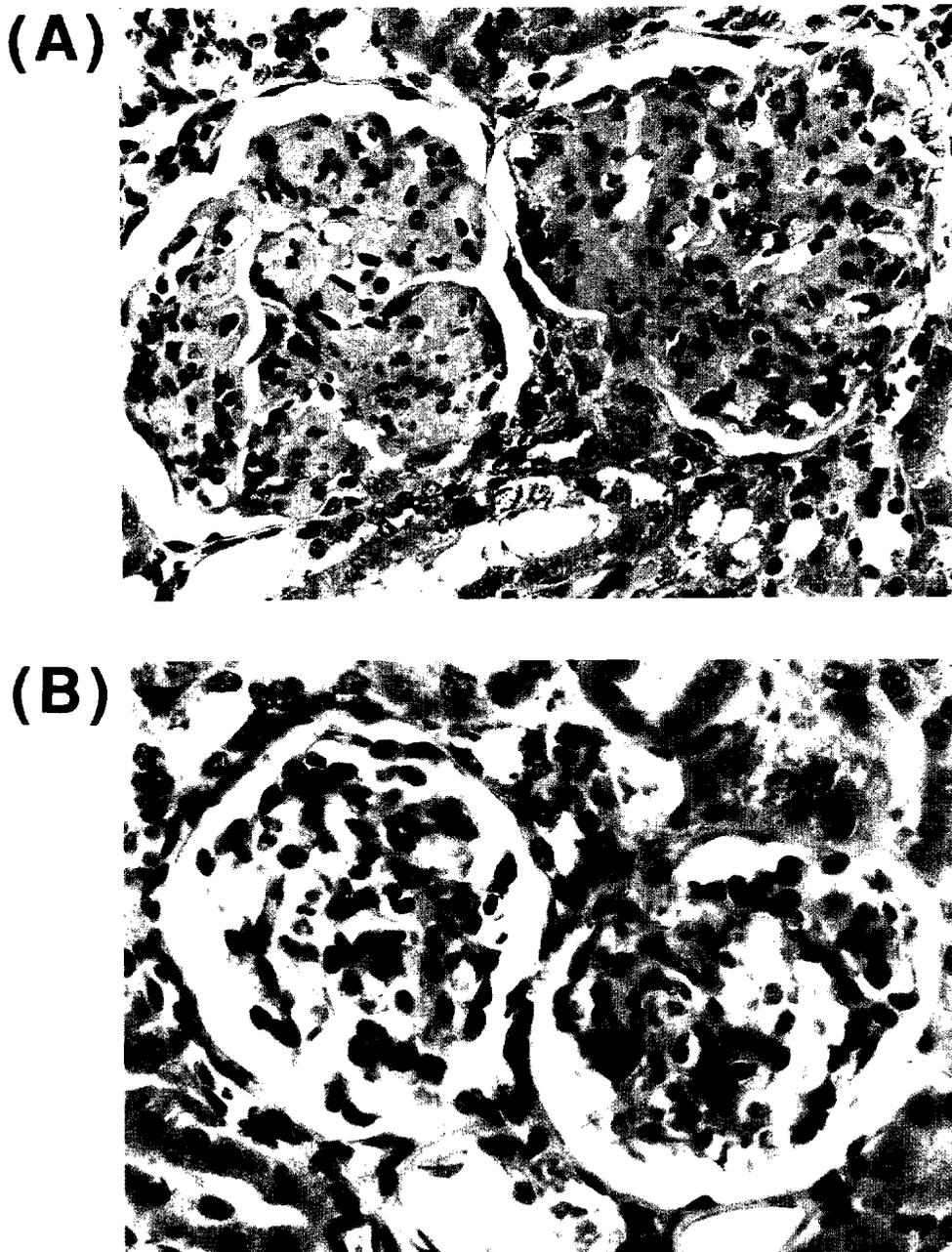


Fig. 2. Effect of KW-3635 on urinary protein excretion. NZB  $\times$  NZW F1 mice were treated with vehicle (solid bars) or KW-3635 (30 mg/kg: opened bars; 100 mg/kg: hatched bars). Each bar represents the mean  $\pm$  S.E. of 6–9 animals. <sup>a, b</sup>: Significant difference from the vehicle group at  $P < 0.05$  and  $P < 0.01$ , respectively. TP: total protein.

**Table 1.** Biochemical parameters in sera at the age of 41 weeks

	N	TG (mg/dl)	TC (mg/dl)	TP (g/dl)	ALB (g/dl)	UN (mg/dl)
Vehicle	12	119.2±27.2	302.9±67.2	5.74±0.30	2.47±0.22	61.3±20.4
KW-3635						
30 mg/kg	17	87.6±16.4	174.7±16.2	6.49±0.20	3.02±0.13	29.3± 5.4
100 mg/kg	10	110.8±41.0	156.7±14.7	6.66±0.28	2.87±0.23	36.5±11.7

Values are means±S.E. TG: triglyceride, TC: total cholesterol, TP: total protein, ALB: albumin, UN: urea nitrogen.



**Fig. 3.** Microphotographs of the kidney from NZB×NZW F1 mice. A: vehicle, B: KW-3635 (30 mg/kg/day). The magnification is 100×.

nificantly higher at 36 to 38 weeks of ages as compared with that in the control mice (Fig. 1). Proteinuria occurred from 35 weeks of age, although there were individual variations. At 40 and/or 41 weeks of age, the mice treated with KW-3635 at 30 and 100 mg/kg exhibited significantly decreased proteinuria as compared with the vehicle-treated control (Fig. 2). The mice in all the groups showed similar urine volume (data not shown). Table 1 shows the biochemical parameters in the sera at 41 weeks of age. In the vehicle group, triglyceride and urea nitrogen exhibited higher values. KW-3635 tended to reduce these parameters, although the effects were not statistically significant.

Thereafter, we evaluated the effect of KW-3635 on renal histologic abnormalities at 41 weeks of age. Figure 3 shows the representative photomicrographs of the kidney from NZB  $\times$  NZW F1 mice given either the vehicle or KW-3635 (30 mg/kg/day). The kidneys from the vehicle treated mice typically showed severe proliferative glomeruli, glomerulosclerosis and lymphoid cell infiltration, which are compatible with the histopathologic changes in SLE. The histopathologic abnormality in the KW-3635 (30 mg/kg/day)-treated group was significantly decreased as compared with that in the vehicle control (Table 2).

Anti-ssDNA antibody levels in the sera are shown in Table 3. There was no significant difference in anti-ssDNA antibody levels between the KW-3635 treated mice and the vehicle control, indicating that immuno-suppres-

sive action is not involved in the beneficial effect of KW-3635.

## DISCUSSION

NZB  $\times$  NZW F1 mice serve as an excellent animal model for the study of SLE (9). In NZB  $\times$  NZW F1 mice, renal TXA<sub>2</sub> production is increased, and the enhanced renal TXA<sub>2</sub> production correlates with urinary protein excretion as well as the severity of renal histopathology (9). In the present study, we examined the effects of KW-3635, a TXRA, on urinary protein excretion, histopathologic changes in glomeruli and mortality in this model. KW-3635 at a dose of 30 mg/kg/day decreased urinary protein excretion and reduced the histopathologic changes and the mortality. The reduced mortality, however, cannot entirely be ascribed to the ameliorated renal injury, since it may also be related to the systemic blockade by KW-3635 of various TXA<sub>2</sub> responses. Stegmeier et al. reported, in an abstract, that BM13,505, another TXRA, delayed the manifestation of lupus nephritis in NZB  $\times$  NZW F1 mice (13). Our results, as well as the result reported by Stegmeier et al., suggest that TXA<sub>2</sub> is an important mediator of the development of lupus nephritis in NZB  $\times$  NZW F1 mice.

Cyclooxygenase inhibitor (9) and most of the TXSI (10, 11) failed to show any beneficial effects on lupus nephritis in NZB  $\times$  NZW F1 mice. For example, TXSI had no apparent effect on urinary protein excretion and mortality in spite of a 40% reduction of urinary TXB<sub>2</sub> excretion (11). This may be due to the incomplete suppression of renal synthesis of TXA<sub>2</sub>. Indeed, since urinary TXB<sub>2</sub> is derived from both the kidney and the extrarenal sources in lupus nephritis (6, 19), a 40% reduction of urinary TXB<sub>2</sub> may not reflect the suppression of renal synthesis of TXA<sub>2</sub>. Alternatively, prostaglandin endoperoxides, which cannot be attenuated by TXSI, might have aggravated the lupus nephritis. Even if TXSI can completely inhibit renal synthesis of TXA<sub>2</sub>, it can not inhibit the synthesis of prostaglandin endoperoxides, which may act as an agonist on the thromboxane receptor (20). Therefore, TXRA has some therapeutic advantages over TXSI for the treatment of thromboxane-dependent disorders.

Inhibition of TXA<sub>2</sub> (and prostaglandin endoperoxides) may improve renal injury by a variety of direct and indirect mechanisms. In the kidney, TXA<sub>2</sub> induces the decrease of glomerular filtration rate via the reduction of renal blood flow (21) and the contraction of mesangial cells (2, 22). Thus, TXRA could improve the depressed renal function induced by TXA<sub>2</sub>, the synthesis of which is increased in NZB  $\times$  NZW F1 mice (9). On the other hand, TXA<sub>2</sub> causes intraglomerular platelet aggregation and releases a variety of growth factors which stimulate mesangi-

**Table 2.** Frequency of specific histologic abnormalities in NZB  $\times$  NZW F1 mice at 41 weeks of age

	N	Glomerular change				
		0	1+	2+	3+	4+
Vehicle	13	0	2	6	2	3
KW-3635						
30 mg/kg	17	7	4	4	2	0
100 mg/kg	10	3	2	4	1	0

a

<sup>a</sup>: Significant difference from the vehicle group at  $P < 0.05$ .

**Table 3.** Anti-DNA antibody levels in sera of NZB  $\times$  NZW F1 mice at 41 weeks of age

	N	Anti-ssDNA
Vehicle	12	0.83 $\pm$ 0.12
KW-3635		
30 mg/kg	17	1.05 $\pm$ 0.09
100 mg/kg	10	1.00 $\pm$ 0.10

Data are presented at OD units.

al cells proliferation (22). Moreover, TXA<sub>2</sub> can enhance immune complex deposition (3), stimulate production of extracellular matrix protein (23) and increase glomerular capillary permeability, leading to the renal degeneration and inflammation. KW-3635 might have inhibited these deleterious actions of TXA<sub>2</sub>, resulting in the improvement of lupus nephritis in NZB×NZW F1 mice.

In the present study, KW-3635 at a high dose (100 mg/kg/day) did not improve or rather aggravated the lupus nephritis in NZB×NZW F1 mice. TXA<sub>2</sub> is one of the factors modulating the tubuloglomerular feedback response (24) and may be important to maintain glomerular filtration rate in some cases. In fact, it has been reported (25) that TXA<sub>2</sub> could contribute to the increase of glomerular filtration rate in the hypoperfused kidney. Therefore, it is possible that KW-3635 at the high dose might have completely inhibited even the beneficial renal response of TXA<sub>2</sub>, resulting in the decrease in glomerular filtration rate. Thus, KW-3635 at the high dose might not have improved lupus nephritis in NZB×NZW F1 mice. The exact mechanism for the ineffectiveness at the high dose of KW-3635 awaits further investigation.

In summary, chronic TXA<sub>2</sub> receptor blockade showed the improvement of lupus nephritis in NZB×NZW F1 mice. Treatment with KW-3635 decreased urinary protein excretion, histopathologic changes and the mortality. These data suggest that TXA<sub>2</sub> is an important mediator of the development of lupus nephritis in NZB×NZW F1 mice.

#### Acknowledgment

We wish to thank Dr. Nishimura for his help as a pathologist.

#### REFERENCES

- 1 Levenson, D.J., Simmons, C.E., Jr. and Brenner, B.M.: Arachidonic acid metabolism, prostaglandins and kidney. *Am. J. Med.* **72**, 354–374 (1982)
- 2 Mene, P., Pugliese, F., D'Agostino, A. and Cinotti, G.A.: Thromboxane A<sub>2</sub>, prostaglandins, and mesangial cell proliferation. *Kidney Int.* **41**, 554–556 (1992)
- 3 Remuzzi, G., Imberti, L., Rossini, M., Carminati, C., Cattaneo, G.M. and Bertani, T.: Increased glomerular thromboxane synthesis as a possible cause of proteinuria in experimental nephrosis. *J. Clin. Invest.* **75**, 94–101 (1985)
- 4 Carven, P.A., Melhem, M.F. and DeRubertis, F.R.: Thromboxane in the pathogenesis of glomerular injury in diabetes. *Kidney Int.* **42**, 937–946 (1992)
- 5 Benigni, A., Chiabrando, C., Piccinelli, A., Perico, N., Gavinelli, M., Furci, L., Patino, O., Abbate, M., Bertani, T. and Remuzzi, G.: Increased urinary excretion of thromboxane B<sub>2</sub> and 2,3-dinor-TXB<sub>2</sub> in cyclosporin A nephrotoxicity. *Kidney Int.* **34**, 164–174 (1988)
- 6 Patrono, C., Ciabattani, G., Remuzzi, G., Gotti, E., Bombardieri, S., DiMunno, O., Tartarelli, G., Cinotti, G.A., Simonetti, B.M. and Pierucci, A.: Functional significance of renal prostacyclin and thromboxane A<sub>2</sub> production in patients with systemic lupus erythematosus. *J. Clin. Invest.* **76**, 1011–1018 (1985)
- 7 Cinotti, G.A., Pierucci, A. and Mene, P.: Experimental and clinical action of thromboxane A<sub>2</sub> receptor antagonists. *Contrib. Nephrol.* **81**, 270–278 (1990)
- 8 Pierucci, A., Simonetti, B.M., Pecci, G., Mavrikakis, G., Feriozzi, S., Cinotti, G.A., Patrignani, P., Ciabattani, G. and Patrono, C.: Improvement of renal function with selective thromboxane antagonism in lupus nephritis. *N. Engl. J. Med.* **320**, 421–425 (1989)
- 9 Kelley, V.E., Sneve, S. and Musinski, S.: Increased renal thromboxane production in murine lupus nephritis. *J. Clin. Invest.* **77**, 252–259 (1986)
- 10 Archer, R.L., Cunningham, A.C., Moore, P.F., Potter, J.A., Bliven, M.L. and Otterness, I.G.: Effects of dazmegrel, piroxicam and cyclophosphamide on the NZB/W model of SLE. *Agents Actions* **27**, 369–374 (1989)
- 11 Clark, W.F., Parbtani, A., McDonald, J.W.D., Taylor, N., Reid, B.D. and Kreeft, J.: The effects of a thromboxane synthase inhibitor, a prostacyclin analog and PGE<sub>1</sub> on the nephritis of the NZB/W F1 mouse. *Clin. Nephrol.* **28**, 288–294 (1987)
- 12 Parbtani, A., Clark, W.F. and McDonald, J.W.D.: Reversal of proteinuria and reduction of mortality in NZB/W F1 lupus mice by prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), iloprost and a thromboxane synthase inhibitor (TSI) (abstract). *Kidney Int.* **33**, 321 (1988)
- 13 Stegmeier, K., Mertin, J., Pill, J. and Hartig, F.: Effects of thromboxane receptor blocker BM13.505 on the development of proteinuria in autoimmune NZB/W mice. *Thromb. Haemost.* **58**, 262 (1987)
- 14 Karasawa, A., Kawakage, M., Shirakura, S., Higo, K., Kubo, K., Ohshima, E. and Obase, H.: Antiplatelet effects of the novel thromboxane A<sub>2</sub> receptor antagonist sodium (*E*)-11-[2-(5,6-dimethyl-1-benzimidazolyl)ethylidene]-6,11-dihydrodibenz[*b,e*]oxepine-2-carboxylate monohydrate. *Arzneimittelforschung* **41**, 1230–1236 (1991)
- 15 Miki, I., Kishibayashi, N., Nonaka, H., Ohshima, E., Takami, H., Obase, H. and Ishii, A.: Effects of KW-3635, a novel dibenzoxepin derivative of a selective thromboxane A<sub>2</sub> antagonist, on human, guinea pig and rat platelets. *Japan. J. Pharmacol.* **59**, 357–364 (1992)
- 16 Kawakage, M., Ishihara, N., Karasawa, A. and Kase, H.: Effects of KW-3635 on the diuretic action of furosemide in rats. *Folia Pharmacol. Japon.* **101**, 33–38 (1993) (Abs. in English)
- 17 Spurney, R.F., Fan, P.-Y., Ruiz, P., Sanfilippo, F., Pisetsky, D.S. and Coffman, T.M.: Thromboxane receptor blockade reduces renal injury in murine lupus nephritis. *Kidney Int.* **41**, 973–982 (1992)
- 18 Mihara, M., Ohsugi, Y., Saito, K., Miyai, T., Togashi, M., Ono, S., Dobashi, K., Hirayama, F. and Hamaoka, T.: Immunological abnormality in NZB/NZW F1 mice; Thymus-independent occurrence of B cell in the development of autoimmune disease, as evidenced by analysis of the athymic nude individuals. *J. Immunol.* **141**, 85–90 (1988)
- 19 Spurney, R.F., Bernstein, R.J., Ruiz, P., Klotman, P.E., Pisetsky, D.S. and Coffman, T.M.: Physiologic role for enhanced renal thromboxane production in murine lupus nephritis. *Prostaglandins* **42**, 15–28 (1991)
- 20 Fitzgerald, G.A., Reilly, I.A.G. and Pedersen, A.K.: The biochemical pharmacology of thromboxane synthase inhibition

- in man. *Circulation* **72**, 1194–1201 (1985)
- 21 Baylis, L.P.: Effect of administered thromboxanes on the intact, normal rat kidney. *Renal Physiol.* **10**, 110–121 (1987)
- 22 Shultz, P.J., DiCorleto, P.E., Silver, B.J. and Abboud, H.E.: Mesangial cells express PDGF mRNAs and proliferate in response to PDGF. *Am. J. Physiol.* **255**, F674–F684 (1988)
- 23 Klahr, S., Schreiner, G. and Ichikawa, I.: Progression of renal disease. *N. Engl. J. Med.* **318**, 1657–1666 (1988)
- 24 Welch, W.J. and Wilcox, C.S.: Modulating role for thromboxane in the tubuloglomerular feedback response in the rat. *J. Clin. Invest.* **81**, 1843–1849 (1988)
- 25 Goto, F., Jackson, E.K., Ohnishi, A., Herzer, W. and Branch, R.A.: Effect of cyclooxygenase and thromboxane synthase inhibition on the response to angiotensin II in the hypoperfused canine. *J. Pharmacol. Exp. Ther.* **234**, 799–803 (1987)