Elucidation of Teratogenic Mechanisms of Immunosuppressant Leflunomide in Mice

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Elucidation of Teratogenic Mechanisms of Immunosuppressant Leflunomide in Mice

マウスにおける免疫抑制剤 **Leflunomide** の催奇形性メカニズムの解明

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

In 1957, thalidomide was introduced as a sedative, pregnant women were frequently treated with thalidomide for their nausea of pregnancy. Four years later, as thalidomide's teratogenic effects became apparent all over the world, it was withdrawn from the market. Following this chemical hazard, the governments of USA, EU and Japan prepared the guidelines to strictly regulate the preclinical toxicity studies for developing medicine. Now, the teratogenicity is the one of the most important items in the safety assessment of medicines, various types of studies to examine its teratogenicity have been conducted in pharmaceutical companies. In this way, establishment of the present system for the preclinical safety assessment in every candidate medicines was inspired by the thalidomide case. However, about 5000 papers have been published during the past 40 years specifically concerning thalidomide teratogenicity and at least 30 hypotheses concerning the mechanism of action of this drug have been advanced, the teratogenic mechanism of action has remained elusive (Stephens *et al*, 2000).

At present day, in spite of the clear warning that the medicines which are determined to have the teratogenic potency in animals or are suspected the potential risk in human pregnancy, should not to be prescribed for pregnant women, it has been often reported that women took these drugs during pregnancy and it resulted in the birth of babies with congenital malformations. This is due to the fact that about half of pregnancies are unplanned (Henshaw, 1998), and overall fewer than 50% of women recognized they are pregnant by the fourth week in gestation (Floyd *et al*, 1999), leading to the common occurrence of inadvertent exposure to a medication of unknown safety during a critical

period in embryonic development (Chambers *et al*, 2006). This reproductive risk of embryonic exposure heightens our need to identify the mechanism of the teratogenic action of drugs. By understanding the embryopathic mechanism of these drugs, we may be able to develop a non-teratogenic analogue of the drug with pharmacological activity to treat adult disease.

1-1. Pharmacological actions of Leflunomide

Leflunomide [N-(4'-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide] is a disease-modifying antirheumatic drug which reduces the signs and symptoms of inflammatory arthritis and delays the radiological progression of rheumatoid arthritis (RA) (Goldenberg, 1999). Leflunomide is rapidly converted to its active metabolite, A77 1726 [2-cyano-3 hydroxy-N-(4-trifluoromethylphenyl)-crotonamide], possibly in the gut wall, plasma and liver. A77 1726 inhibits cell proliferation in activated lymphocytes in patients with RA but its precise mechanism of action is unclear. In the *in vitro* experiments, Prakash and Jarvis indicated that the drug inhibits protein tyrosine kinase activity and dihydroorotate dehydrogenase (DHODH) activity, which is the fourth enzyme of pyrimidine nucleotide biosynthesis, in actively dividing cells (Prakash and Jarvis, 1999).

Which mechanism contributes mainly to the pharmacological action of Leflunomide? Some researchers gave some suggestions about the potency of Leflunomide on its immunosuppressive efficacy in *in vitro* studies using the murine and human T cell lines. Up to 12.5 μM A77 1726, addition of uridine, precursor of pyrimidine nucleotides, completely reversed the inhibition of cell proliferation caused by A77 1726, indicating that pyrimidine biosynthesis is the target, since inhibition of cell proliferation correlates with a reduction in pyrimidine nucleotide

levels and uridine coadministration prevents the inhibitory effect. At 150 μM of A77 1726, uridine no longer reverses the inhibition of proliferation even through pyrimidine nucleotide levels are restored (Elder *et al*., 1997, Rückemann *et al*., 1998). These results suggested that the second mechanism for inhibition of proliferation is probably inhibition of protein tyrosine kinases, since these higher concentration of A77 1726 inhibit IL-2-induced tyrosine phosphorylation of Jak1 and Jak3, the protein tyrosine kinases initiating signaling by the IL-2R (Elder *et al*., 1997). In the clinical trials, plasma concentration of A77 1726 in human singly administered 100 mg (clinical dose) of Leflunomide was up to 28.09 μM at 12 h after dosing (Arava® FDA web site, 1998). Davis *et al* reported the comparative study between the inhibitory effects of Leflunomide on DHODH and protein tyrosine kinases. Comparing the *Ki* values obtained for DHODH with the reported IC_{50}s for A77 1726 inhibition of protein tyrosine kinases, it is found that this compound is some 100-1000-fold more potent as a DHODH inhibitor (Davis *et al*, 1996). These reports suggest that Leflunomide-mediated pharmacological actions largely depend on the inhibition of DHODH activity at clinical dose.

1-2. Teratogenicity of Leflunomide in animals and humans

Preclinical and clinical studies pertaining to the reproductive risks of preconception and postconception exposures to Leflunomide became available for analysis and interpretation. Using the basic science principles of teratology and the preclinical reproductive toxicity studies, an attempt was made to estimate the reproductive risks of Leflunomide.

The administration of excess Leflunomide to pregnant mammals can cause various malformations in their offspring. In the teratogenicity studies in rats and in rabbits, Leflunomide

induced embryo toxic (growth retardation, embryo lethality) and teratogenic. In rats, Leflunomide caused multiple malformations when administered during organogenesis at a dose of 10 mg/kg/day (Sanofi-Aventis, 2003). In other report, when administered orally to pregnant rats during organogenesis at a dose of 15 mg/kg/day, Leflunomide was teratogenic (most notably producing anophthalmia or microphthalmia and internal hydrocephalus) (Goldenberg, 1999). In rabbits, Leflunomide induced fused and incomplete ossification of the sternebra when pregnant rabbits were treated with 10 mg/kg/day Leflunomide during fetal organogenesis (Sanofi-Aventis, 2003).

No observed adverse effect level for teratogenicity in rats was 1 mg/kg, which is equivalent to 67.80 μM as a plasma concentration of A77 1726 (Brent, 2001). Described above, highest plasma concentration of A77 1726 in human was 28.09 μM at the clinical dose (100 mg/man) (Arava® FDA web site, 1998). Furthermore, the inhibitory effect of Leflunomide on DHODH activity is 40 times, and its antiproliferative effect on cell cultures is 300 times stronger in rat than in man (Brent, 2001). In a prospective controlled study of RA medications in pregnancy, 43 Leflunomide-exposed women were compared to 78 women with RA who did not use Leflunomide and a second group of 47 women without RA. Based on very small numbers, rates of major birth defects were similar between the groups. However, infants exposed to Leflunomide were significantly more likely than non-diseased comparison infants to be born prematurely and were significantly smaller in birth weight, there were no significant differences on these two measures between the Leflunomide-exposed group and the RA comparison group without Leflunomide treatment, suggesting that the underlying disease and/or other medications used to treat RA are likely related to these adverse outcomes (Chambers *et al*, 2006). In this study condition, no evidence showed the risk of teratogenicity in human administered Leflunomide at the clinical dose. However, it is regarded that the teratogenic risk of Leflunomide is unknown because of the clinical data of shortage in human pregnancy. Furthermore, since the preclinical study data cannot

determine whether the human embryo or fetus is more sensitive than the adult, these species differences and possible consequences on safety margins cannot be considered for estimating the reproductive risk of Leflunomide in humans (Brent, 2001). Accordingly, Leflunomide has been assigned an FDA pregnancy category X based on its mechanism of actions in human.

1-3. Objectives

As described above, based on the comparative study about pharmacological actions of Leflunomide, inhibition of DHODH mainly contributes to Leflunomide's immunosuppressive efficacy. However, it is suspected that DHODH inhibition may be also responsible for the embryo toxic and teratogenic effects of Leflunomide in animals, the mechanisms of teratogenicity remain to be examined (Brent, 2001).

In mice, there is much information available with respect to their molecular and cellular biology, and many reports have been published about the effects of Leflunomide treatment on the immune system, DHODH and protein tyrosine kinase activities. In the course of the work on this dissertation, I conducted the teratogenicity studies to examine the effects on embryo lethality, intrauterine growth and teratogenicity of Leflunomide in mice and the critical periods of those malformations, and the some studies to elucidate the mechanisms related with teratogenicity in mice treated with this compound using the biochemical and global gene expression analysis techniques.

CHAPTER 2

THE EFFECTS OF LEFLUNOMIDE ON EMBRYO/FETAL DEVELOPMENT IN MICE ADMINISTERED DURING ORGANOGENESIS

Teratogenicity study of the dihydroorotate-dehydrogenase inhibitor and protein tyrosine kinase inhibitor Leflunomide in mice

2-1. Introduction

Leflunomide, an isoxazol derivative, is an immunosuppressive agent (Brent, 2001). It has been demonstrated that inhibition of T-lymphocyte activation and production of IgM and IgG contribute to the immunosuppressive potential of Leflunomide (Prakash and Jarvis, 1999). Leflunomide is a potent inhibitor of protein tyrosine kinase and of DHODH, which is the fourth enzyme in the de novo pathway of pyrimidine nucleotide synthesis (Elder *et al*, 1997, Huang *et al*, 2002, Pinschewer *et al*, 2001, Siemasko *et al*, 1998, Silva *et al*, 1996). This pathway is needed for T-cell activation and proliferation through a generalized requirement of ribonucleotide precursors (Goldenberg, 1999). Leflunomide may serve as an immunomodulatory agent by specifically arresting activated lymphocytes *via* its action on DHODH. Another pharmacological action is the inhibition of protein tyrosine kinase that is known to play essential roles in signal transduction by the cytokine receptors (Siemasko *et al*, 1998, Elder *et al*, 1997, Waldman *et al*, 2001, Mattar *et al*, 1993).

Since the differentiation and proliferation of T-cells are dependent on signaling from cytokines, inhibition of intracellular tyrosine phosphorylation by Leflunomide may contribute to inhibiting the activation of T-cells (Xu *et al*, 1995).

The teratogenicity of Leflunomide has been reported in rats and rabbits. In rats, Leflunomide induced multiple malformations including cranioschisis and exencephaly when administered during organogenesis (days 7-17 of pregnancy) (Sanofi-Aventis, 2003). In another study, malformations of the head, rump, vertebral column, ribs and limbs were observed (Brent, 2001). In rabbits, Leflunomide induced fused and incomplete ossification of the sternebra when rabbits were treated with Leflunomide during fetal organogenesis (Sanofi-Aventis, 2003). Although inhibition of DHODH and/or protein tyrosine kinase is suspected to be involved in the teratogenicity of Leflunomide, the mechanism remains unclear. In mice, there is much information available with respect to their molecular and cellular biology, and many reports have been published about the effects of Leflunomide treatment on the immune system, DHODH and protein tyrosine kinase activities. As a first step in elucidating the teratological mechanisms of Leflunomide, the present study was performed to investigate the embryo toxicity and teratogenicity of Leflunomide in mice.

2-2. Materials and methods

2-2-1. Animals

CD-1 (ICR) mice (Charles River Japan Inc., Kanagawa, Japan), 10-week-old males and 9-week-old females, were utilized in this study. Use of animals in this study was approved by the animal care and use committee of Shionogi & Co., Ltd. The temperature of the animal room

ranged from 23 ± 3 °C, and the relative humidity from 30 to 70 %. Animals were allowed *ad libitum* access to sterilized tap water and diet (Certified Rodent Diet: CRF-1 Oriental Yeast Co., Ltd. Tokyo, Japan). A 12-h light/dark photocycle (08:00 light on) was maintained. Estrous virgin females were mated with males overnight on a one-to-one basis. The morning of copulation plug observation was designated gestation day 0 (GD0). On GD5, the plug-positive females were assigned to four groups, *i.e.*, 14 animals to a control group (vehicle) and 7 animals each to three Leflunomide-treatment groups (10, 30 or 70 mg/kg/day) using body weight-stratified sequenced randomization.

2-2-2. Test substance and treatment

Leflunomide (purity 99.5%), purchased from Sigma-Aldrich (St. Louis, MO, USA.) was suspended in 1.0 wt/vol% carboxymethylcellulose (CMC; Nacalai Tesque, Kyoto, Japan) solution. Dams were treated with Leflunomide suspended in CMC at a dose of 10, 30 or 70 mg/kg/day by gavage from GD6-15. These dose levels were selected from the results of dose-range finding study, in which pregnant mice were administered with Leflunomide at doses of 1, 10 and 100 mg/kg/day from GD6-15. At 1 and 10 mg/kg, no adverse effects were found, whereas at 100 mg/kg, body weight gain of dams decreased significantly and all fetuses were resorbed in all litters. The control dams received CMC. Dosing volume was 10 ml/kg.

2-2-3. Evaluation of embryo toxicity and teratogenicity

All dams were weighed on GD0, and GD5 to 18. On GD18, all dams were euthanized with exsanguination from caudal vena cava under the anesthesia with diethyl ether and hysterectomized. The uterine contents were examined for the number of implantations, resorptions, living and dead fetuses, fetal weight, placental weight and fetal external abnormalities. Every

second fetus per litter was preserved in Bouin's solution for subsequent free-hand sectioning to examine visceral anomalies. The remaining fetuses were removed skin, thoracic and visceral organs and brown fat, fixed in 70 % ethanol for skeletal examination, following euthanasia under the overdosing of pentobarbital sodium. After fixation, fetuses were macerated with potassium hydroxide, stained with Alizarin red S (Sigma-Aldrich, St. Louis, MO, USA) in order to detect skeletal anomalies.

2-2-4. Plasma concentration analysis of A77 1726 and Leflunomide

Plasma concentrations of Leflunomide and A77 1726 were analyzed on first day and last day of administration period from GD6 to GD15. For analysis on GD6, plug-positive dams were dosed Leflunomide at 30 mg/kg on GD6 singly. For analysis after treatment on GD15, dams were dosed from GD6 to GD15 daily. Dams were anesthetized with diethyl ether and laparotomized. Approximately 1.0 ml blood per time point per animal was drawn from the caudal vena cava, using heparinized disposable needle-tipped syringes,1, 2, 4, 8, 24 and 48 h after dosing on GD6 and 0 (not dosed on GD15), 1, 2, 4, 8, 24 and 48 h after dosing on GD15. Blood samples were transferred to the microtubes and immediately centrifuged (720 g, 15 min, 4°C) to obtain plasma. A77 1726 and Leflunomide were extracted from 30 μl of mouse plasma by precipitation with a mixture of acetonitrile/methoanol (1:1, vol/vol). The samples were analyzed by High-performance liquid chromatography (HPLC) using an LC-10A LCsolution (Shimadzu, Kyoto, Japan) with the CapcellpaK C18 MGII (5μL, 150 × 2.1 mm i.d., Shiseido, Tokyo, Japan) column and detection at a wavelength of 281 nm for Leflunomide and 300 nm for A77 1726, switching at 8.5 min into the run. A mixture of acetonitrile/2 wt/vol% phosphoric acid/heptafluorobutyric acid mixture (550:450:2, vol/vol/vol) was used as mobile phase. As parameters, C_{max} (μg/ml), T_{max} (h) and $AUC_{0\text{-}48h}$ (μg•h/ml) were calculated with Microsoft Excel®.

2-2-5. Statistics

Fetal data was pooled by litter, which served as the unit of analysis. Continuous data include maternal body weights, the number of implantations, post-implantation loss, number of live fetuses, sex ratio, fetal weight, placental weight, incidence of external, skeletal and visceral anomalies. These parameters were analyzed by Dunnett's parametric multiple *t*-test to compare each of the Leflunomide-treated groups with the control group. Binary data including incidence of dams with fetuses showing external, skeletal and visceral malformations were analyzed by Dunnett type multiple comparison for a response rate to compare each of the Leflunomide-treated groups with the control group. All pairwise statistical tests were conducted as two-sided tests. A difference from the control group was considered statistically significant at a value of $p < 0.05$.

2-3. Results

2-3-1. Body weights of dams

Absolute maternal body weights in the 30 and 70 mg/kg treatment groups were significantly lower than the control (vehicle) group from the mid- to late stages of pregnancy (Fig. 2-1). Although maternal body weight was similar in all groups during the early dosing (GD6 to 9), loss of body weight was observed from GD10 onwards at 70 mg/kg. No significant changes in maternal body weight were observed at the 10 mg/kg dose level.

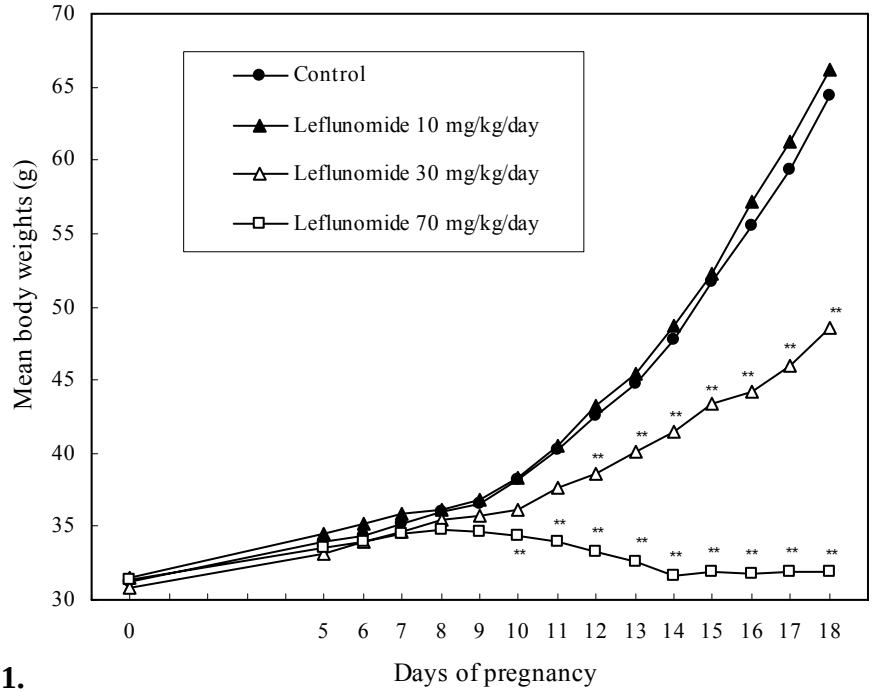


Figure 2-1.

Changes in body weights of dams treated with Leflunomide.

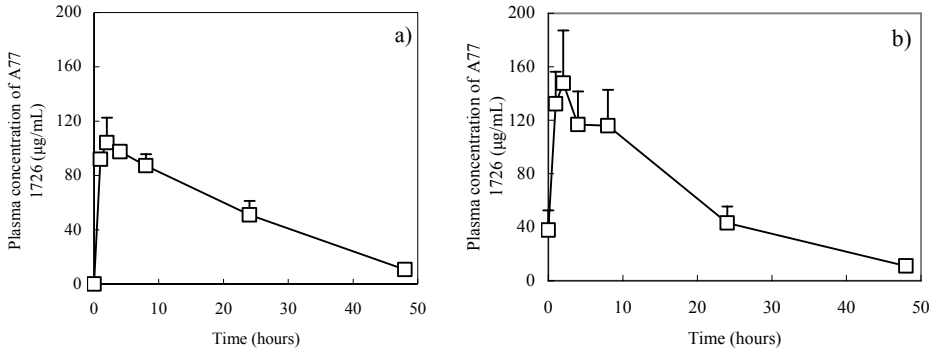
** p <0.01

2-3-2. Plasma concentration analysis of A77 1726 and Leflunomide

Data on the plasma concentration of A77 1726 are summarized in Fig. 2-2. Since the concentrations of Leflunomide were below the limits of quantitation at any single time point (<0.1 μg/ml), these data were not shown. A77 1726 was detected in the maternal plasma 24 and 48 h after administration on GD6 (single treatment) and on GD15 (daily exposure, GD6 to GD15). The mean values of the C_{24h} and C_{48h} of A77 1726 were 47.4% and 10.0% of the value of the C_{max} on GD6, respectively. On GD15, the mean values of C_{24h} and C_{48h} were 29.3% and 7.4% of the value of C_{max}, respectively. Meanwhile, the mean value of C_{24h} on GD6 was comparable to the value of C_{0h} on GD15 after dosing daily from GD6, and the values of C_{max} and $AUC_{0\text{-}48h}$ on GD15 were approximately 1.4 and 1.1 times of those on GD6, respectively. This data indicated that the plasma concentration of A77 1726 didn't increase by repeated dosing.

2-3-3. Fetal observations

Total litter resorption was observed in all dams in the 70-mg/kg group. These embryos appeared resorbed at an early stage of pregnancy, since only implantation scars were found in the uterus. Furthermore, maternal body weights started to decrease from GD9 (the 4th days of dosing), and the decrease continued until GD18 (end of the experiment). On GD18, the body weight fell to the level of GD0. Meanwhile, no maternal clinical signs were found. At 30 mg/kg, the number of live fetuses (42.4 % drop compared with control data), fetal weight (male: 34.5 % drop, female: 37.6 % drop) and placental weight (male: 41.5 % drop, female: 48.9 % drop) were significantly lower than the control group (Table 2-1).



		Plasma concentration of A77 1726 (μg/mL)							AUC_{0-48h}	C_{max}	T_{max}
		0h	1h	2h	4h	8h	24h	48h	(μg•hr/mL)	(μg/mL)	(hr)
GD6	Mean	-	92.0	104.1	97.5	87.2	50.9	10.7	2558.8	104.1	2.0
	SD	-	4.5	18.4	3.1	8.5	10.5	1.3	177.3	15.7	1.5
GD15	Mean	37.9	132.2	147.7	116.8	115.8	43.3	11.0	2879.0	147.8	2.0
	SD	14.8	24.0	39.5	24.8	26.9	12.2	1.6	467.5	34.6	2.8

Figure 2-2.

Plasma concentration-time profiles of A77 1726 in mice.

Each point represents the mean, and a vertical bar shows +SD. Plasma concentrations of Leflunomide were below the limit of quantitation (<0.1 μg/ml) at any time-point, not shown in figure. Plasma concentration of A77 1726 were analyzed after dosing Leflunomide at 30 mg/kg/day **a)** on GD6 singly, and **b)** on GD15 (repeated daily dosing from GD6 to GD15).

Table 2-1: Embryo toxicity attributed to Leflunomide treatment during period of organogenesis in mice

	Leflunomide (mg/kg/day)			
	0	10	30	70
No. of dams	11	6	6	7
No. of implantations	15.1 ± 1.9	16.2 ± 1.7	15.5 ± 2.7	14.7 ± 2.1
Post-implantation loss (%)				
Total	4.6 ± 5.0	10.3 ± 4.9	47.2 ± 33.6**	100.0 ± 0.0**
Resorptions	4.6 ± 5.0	7.3 ± 2.3	40.1 ± 37.4**	100.0 ± 0.0**
Macerations	0.0 ± 0.0	0.9 ± 2.3	1.1 ± 2.7	0.0 ± 0.0**
Dead	0.0 ± 0.0	2.2 ± 3.4	6.0 ± 5.4**	0.0 ± 0.0
No. of live fetuses	14.4 ± 1.6	14.5 ± 1.8	8.3 ± 5.2**	0.0 ± 0.0**
Sex ratio (M/M+F)	0.52 ± 0.13	0.45 ± 0.13	0.42 ± 0.28	
Fetal body weight (g)				
Male	1.39 ± 0.06	1.37 ± 0.07	0.91 ± 0.11**	
Female	1.33 ± 0.08	1.32 ± 0.07	0.83 ± 0.06**	
Placental weight (g)				
Male	0.094 ± 0.009	0.099 ± 0.008	0.055 ± 0.007**	
Female	0.088 ± 0.007	0.088 ± 0.011	0.045 ± 0.006**	

(mean ± SD)
** Significant difference at $p < 0.01$ compared with the control group

Leflunomide induced observable external, skeletal and visceral malformations at the 30 mg/kg dose level (Table 2-2 to 2-4). The incidence of fetuses with external, skeletal and visceral malformations were 77.2 ± 29.4 % (5/5; dams with abnormal fetuses/dams with live fetuses), 100 % (5/5), and 51.1 ± 42.3 % (4/5), respectively. The most characteristic external malformation was exencephaly (26.3 ± 29.5 %). Some dead fetuses also showed severe neural tube defects (NTDs) including anencephaly, as well as a split face (not shown). These observations demonstrate the potential for Leflunomide to adversely affect neural tube closure in early mouse embryos. Other external malformations observed here were cleft palate (49.3 ± 49.3 %), open eye (24.9 ± 29.5 %), short tail (23.3 ± 32.5 %) and kinked tail (38.9 ± 29.4 %). Shortened lower trunk and limb reduction defects were also observed at low incidence. Most skeletal malformations involved the axial skeleton, ribs, sternebrae and skull bones. The main malformations were as follows: cervical to sacral vertebrae (fused cervical arch, misshapen cervical arch, fused thoracic arch, fused thoracic centrum, fused lumber centrum, fused sacral arch and fused sacral centrum), fused ribs, misaligned sternebra and fused supraoccipital bone. In the limb, malpositioned forepaw phalanx and misshapen metacarpal were found in two fetuses. In the visceral examination, high frequencies of malformations of the heart, great vessels (34.5 ± 39.5 %) and head (22.6 ± 23.4 %) were observed. Malformations of the heart and great vessels included persistent atrioventricular canal, membranous ventricular septum defect, transposed great vessels and persistent truncus arteriosus. Fetuses having exencephaly as external anomalies showed multiple anomalies of the head in visceral examinations, *i.e.* misshapen cerebellum, cerebrum, rhinencephalon and absent rhinencephalon.

At 10 mg/kg, no effects of Leflunomide, including teratogenicity, were observed for any of the parameters.

Table 2-2: External malformation of fetuses

	Leflunomide (mg/kg/day)					
	0		10		30	
No. of dams	11		6		5	
No. of dams with fetuses with malformations	1	(9.1)	0	(0.0)	5	(100.0)**
No. of examined fetuses	158		87		50	
No. of fetuses with malformations	1	(0.8±2.5)	0	(0.0±0.0)	39	(77.2±29.4)**
Craniofacial region						
Anasarca	0	(0.0)	0	(0.0)	1	(1.7±3.7)
Exencephaly	0	(0.0)	0	(0.0)	10	(26.3±29.5)**
Misshapen head	0	(0.0)	0	(0.0)	1	(1.5±3.4)
Protruding head	0	(0.0)	0	(0.0)	4	(6.2±13.8)
Cleft palate	0	(0.0)	0	(0.0)	28	(49.3±40.1)**
Misshapen face	0	(0.0)	0	(0.0)	1	(1.5±3.4)
Microtia	0	(0.0)	0	(0.0)	1	(1.5±3.4)
Exophthalmos	0	(0.0)	0	(0.0)	1	(1.8±4.1)
Open eye	0	(0.0)	0	(0.0)	9	(24.9±29.5)**
Limb						
Bowed forelimb	0	(0.0)	0	(0.0)	1	(1.8±4.1)
Misshapen limb	0	(0.0)	0	(0.0)	1	(2.0±4.5)
Manus varus	0	(0.0)	0	(0.0)	1	(1.5±3.4)
Pes varus	1	(0.8±0.8)	0	(0.0)	5	(8.1±11.6)
Finger and Toe						
Ectrodactyly (Finger)	0	(0.0)	0	(0.0)	3	(6.0±13.4)
Brachydactyly (Finger)	0	(0.0)	0	(0.0)	3	(6.0±13.4)
Polydactyly (Finger)	0	(0.0)	0	(0.0)	1	(2.0±4.5)
Polydactyly (Toe)	0	(0.0)	0	(0.0)	3	(4.6±10.3)
Syndactyly (Finger)	0	(0.0)	0	(0.0)	5	(10.0±22.4)
Syndactyly (Toe)	0	(0.0)	0	(0.0)	1	(1.5±3.4)
Malpositioned toe	0	(0.0)	0	(0.0)	1	(2.0±4.5)
Tail						
Short tail	0	(0.0)	0	(0.0)	13	(23.3±32.5)*
Kinked tail	0	(0.0)	0	(0.0)	22	(38.9±29.4)**
Others						
Anal atresia	0	(0.0)	0	(0.0)	1	(2.0±4.5)
Short lower trunk	0	(0.0)	0	(0.0)	5	(8.1±11.6)*

Values: Number of fetuses with malformation (%: The incidence of fetuses with malformation)
Significantly different from Control group *: $p<0.05$ **: $p<0.01$

Table 2-3: Skeletal malformations in fetuses from dams treated with Leflunomide

	Leflunomide (mg/kg/day)					
	0		10		30	
No. of dams	11		6		5	
No. of dams with fetuses with malformations	2	(18.2)	2	(33.3)	5	(100.0)**
No. of examined fetuses	83		44		25	
No. of fetuses with malformations	2	(2.6±5.7)	2	(3.0±6.7)	25	(100.0)**
Absent of skull [(a)]	0	(0.0)	0	(0.0)	5	(30.0±41.5)**
Misshapen alisphenoid	0	(0.0)	0	(0.0)	5	(20.0±44.7)
Absent of palatine	0	(0.0)	0	(0.0)	9	(30.7±21.3)**
Split of palatine	0	(0.0)	0	(0.0)	4	(16.0±35.8)
Fusion between basioccipital bone and supraoccipital bone	1	(1.3±4.3)	0	(0.0)	11	(38.0±24.7)**
Fusion between supraoccipal bone and cervical arch	0	(0.0)	0	(0.0)	5	(16.7±16.7)**
Axial skeleton deformities						
Sternebrae	0	(0.0)	0	(0.0)	8	(42.7±37.5)**
Ribs	0	(0.0)	0	(0.0)	5	(23.3±19.0)**
Cervical vertebrae	0	(0.0)	0	(0.0)	17	(72.7±30.0)**
Thoracic vertebrae	1	(1.3±4.3)	2	(4.6±7.4)	10	(47.3±33.4)**
Lumbar vertebrae	0	(0.0)	0	(0.0)	6	(26.7±25.3)**
Sacral vertebrae	0	(0.0)	0	(0.0)	9	(30.0±41.5)*
Misshapen metacarpal	0	(0.0)	0	(0.0)	1	(4.0±8.9)
Malpositioned forepaw phalanx	0	(0.0)	0	(0.0)	1	(4.0±8.9)
Bent tibia	0	(0.0)	0	(0.0)	1	(4.0±8.9)

Values: Number of fetuses with the malformation (%: The incidence of fetuses with the malformation)
Significantly different from Control group *: $p<0.05$ **: $p<0.01$
Anomalies of vertebrae: Misshapen, misaligned, fused, and absent
(a): This skeletal malformation was seen in fetuses with exencephaly.

Table 2-4: Visceral malformations in fetuses from dams treated with Leflunomide

	Leflunomide (mg/kg/day)					
	0		10		30	
No. of dams	11		6		5	
No. of dams with fetuses with malformations	1	(9.1)	1	(16.7)	4	(80.0)
No. of examined fetuses	75		43		25	
No. of fetuses with malformations	1	(1.5±5.0)	1	(2.1±5.1)	14	(51.1±42.3)**
Anomalies of head [(a)]	0	(0.0)	0	(0.0)	5	(22.6±23.4)**
Anomalies of heart and great vesseles	0	(0.0)	0	(0.0)	11	(34.5±39.5)**
Cor triloculare	0	(0.0)	0	(0.0)	1	(3.3±7.5)
Persistent A-V canal	0	(0.0)	0	(0.0)	2	(6.2±8.5)*
Membranous ventricular septum defect	0	(0.0)	0	(0.0)	7	(21.0±31.7)*
Transposed great vessels	0	(0.0)	0	(0.0)	3	(10.7±15.3)*
Narrowed aortic arch	0	(0.0)	0	(0.0)	1	(3.3±7.5)
Absent truncus arteriosus	0	(0.0)	0	(0.0)	1	(2.9±6.4)
Persistent truncus arteriosus	0	(0.0)	0	(0.0)	6	(18.1±26.2)*
Narrowed pulmonary trunk	0	(0.0)	0	(0.0)	1	(2.9±6.4)
Retroesophageal subclavian artery	0	(0.0)	0	(0.0)	1	(2.9±6.4)
Misshapen thyroid	1	(1.5±5.0)	1	(2.1±5.1)	0	(0.0)
Malpositioned thymus	0	(0.0)	0	(0.0)	1	(3.3±7.5)
Small lung	0	(0.0)	0	(0.0)	4	(13.3±29.8)
Absent kidney	0	(0.0)	0	(0.0)	1	(2.9±6.4)
Small kidney	0	(0.0)	0	(0.0)	1	(3.3±7.5)
Misshapen kidney	0	(0.0)	0	(0.0)	1	(2.9±6.4)

Values: Number of fetuses with the malformation (%: The incidence of fetuses with the malformation)
Significantly different from Control group *: $p<0.05$ **: $p<0.01$
(a): Misshapen cerebellum, misshapen cerebrum, absent rhinencephalon and misshapen rhinencephalon. These visceral malformations were seen in fetuses with exencephaly.

2-4. Discussion

The present study indicated that Leflunomide, a potent DHODH and protein tyrosine kinase inhibitor, caused severe embryo/fetal toxicity and teratogenicity in the mouse. Treatment with Leflunomide at 70 mg/kg induced severe embryo toxicity; all embryos were absorbed in all dams. However no maternal clinical signs were found, the body weight gains reduced significantly. These results suggest that fetal losses can account for the reduction in maternal weight gain during pregnancy, not maternal adverse effects. At 30 mg/kg, embryo viability and intrauterine growth were suppressed, and various fetal malformations increased significantly. NTDs, including exencephaly were induced at relatively high incidence. Other malformations observed with high frequency were open eye, cleft palate, tail deformities, and anomalies of the axial skeleton, and of the heart and great vessels. Repeated administration of Leflunomide at 10 mg/kg/day to pregnant rats during fetal organogenesis has been reported to induce malformations, mainly cranioschisis (14/279, abnormal fetuses/examined fetuses), exencephaly (19/279), ventricular dilation (35/138), anophthalmia (22/138) and microphthalmia (16/138) (Sanofi-Aventis, 2003). In contrast with rats, we observed no effects of Leflunomide at 10 mg/kg in mice. Severe teratogenicity and embryo toxicity were induced at 30 mg/kg and higher. In toxicokinetics analysis, T_{max}, C_{max} and $AUC_{0\text{-}24h}$ of non-pregnant female rats (6 weeks old) treated with Leflunomide at the dose of 10 mg/kg were 1.0 h, 22.9 μg/ml and 319.5 μg·h/ml, respectively (Sanofi-Aventis, 2003). Each parameter in pregnant mice treated at 30 mg/kg measured in this study was about 5 times in C_{max} and about 10 times in $AUC_{0\text{-}24h}$ compared with rats treated at 10 mg/kg. From this result, it is assumed that exposure in rat is only slightly lower than mouse. Although the underlying reasons for cross-species differences in embryo toxicity and teratogenicity are not clear, such a difference may partially explain why the DHODH inhibitory effect of A77 1726 is about 5 times higher in rat than in

mice (IC_{50}, rat: 13 nM, mouse: 69 nM) (Kuo *et al*, 1996).

Plasma concentration of A77 1726 on human administered with Leflunomide has been reported (Arava®, FDA web site, 1998). Healthy male humans were administered a single 100 mg/man (clinical dose) of Leflunomide. Plasma samples were collected up to 36 days (864 h) after dosing. Each parameters of plasma concentration of A77 1726 were calculated as follows; C_{max}, 7.59 ± 1.42 μg/ml; T_{max}, 12.67 ± 8.87 h; $AUC_{0\text{-}864h}$, 2090.1 ± 448 μg·h/ml; and $T_{1/2}$, 7.76 ± 0.50 days. The value of C_{max} on mice administered Leflunomide at 30 mg/kg/day in our study was approximately 14 times that in humans administered a tablet of Leflunomide (100 mg/man), whereas the values of $T_{1/2}$ and T_{max} of A77 1726 in mice were markedly lower than humans. Hence, the values of $AUC_{0\text{-}48h}$ on mice and $AUC_{0\text{-}864h}$ on human were comparable.

Some immunosuppressive agents causing inhibition of *de novo* synthesis of pyrimidine nucleotides have been developed (***e.g.*** Leflunomide, brequinar, and malononitrilamides), but there has been no report on teratogenicity in any species, aside from Leflunomide. Teratogenicity has been reported for some immunosuppressive drugs that inhibit *de novo* synthesis of purine nucleotides. Mycophenolic acid (MPA, active metabolite of mycophenolate mofetil) is a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH), and inhibits the *de novo* synthesis of guanosine nucleotide (Cellcept®, Roche web site, 2008). In teratogenicity studies of MPA, fetal resorptions and malformations (anophthalmia, agnathia and hydrocephaly) were induced in rats, and diaphragmatic hernia in rabbits (Le Ray *et al*, 2004). Bredinin (as known as Mizoribin) is an imidazole nucleoside isolated from *Eupenicillium brefeldianium* with an immunosuppressive activity, known to be an enzyme inhibitor in the pathway of inosine mono phosphate (IMP) to guanosine 5'-mono phosphate (GMP) conversion (Kawai *et al*, 1993). The teratogenicity of Bredinin in rats and mice has been reported (Kobayashi *et al*, 1974, Okamoto *et al*, 1978). In a teratology study in which pregnant mice were treated with Bredinin during the period of fetal organogenesis, no neural

tube defects was detected, but multiple malformations were found, including face, limb and tail malformations. In an *in vitro* study, the proliferation of mouse embryonic cells was inhibited by Bredinin and rescued by co-addition of guanosine or GMP to the culture medium. In contrast, the inhibition was not altered by the addition of inosine or IMP (Kawai *et al*, 1993). This report suggests that the teratogenicity of Bredinin may be related to the inhibition of purine nucleotide *de novo* synthesis. In this study, we did not attempt to elucidate the mechanisms of teratogenicity for Leflunomide, although, as with inhibition of purine *de novo* synthesis, the pyrimidine *de novo* synthesis inhibition and anti-proliferative activity of Leflunomide can be attributed to teratogenicity in mouse fetuses.

2-5. Conclusion

The results of the present study demonstrated that the isoxazol derivative Leflunomide has embryo toxicity and teratogenicity at the minimum maternal toxic dose of 30 mg/kg/day in mice. Characteristic malformation was exencephaly, which can be attributed to abnormal development of the neural tube. Other malformations caused by Leflunomide treatment were cleft palate and deformities of the axial skeleton and the heart and great vessels.

CHAPTER 3

THE STUDY OF THE CRITICAL PERIODS FOR THE TERATOGENICITY OF LEFLUNOMIDE IN MICE

Critical periods for the teratogenicity of immune-suppressant Leflunomide in mice

3-1. Introduction

Leflunomide is an immunosuppressive drug that has shown to be a powerful antiproliferative agent for lymphocytes. It inhibits the enzymatic activity of protein tyrosine kinases and of DHODH, an enzyme involved in pyrimidine nucleotide *de novo* synthesis (Xu *et al*, 1995, Siemasko *et al*, 1998, Dimitrova *et al*, 2002). Leflunomide was found to be teratogenic when administered to rats and rabbits (Arava®, Sanofi-Aventis US web site, 2007, Sanofi-Aventis, 2003). In rats, cranioschisis, exencephaly, anophthalmia, microphthalmia, membranous ventricular septum defect, and malformations of the axial skeleton and the rib were found when the dams were administered Leflunomide at a dose of 10 mg/kg/day during organogenesis from gestation day (GD) 7 to GD17 (Sanofi-Aventis, 2003). In another study, Leflunomide caused anophthalmia, microphthalmia and internal hydrocephalus at a dose of 15 mg/kg/day (Arava®, Sanofi-Aventis US web site, 2007). In rabbits, treatment with Leflunomide from GD6 to GD18 causes malformations of the head and bilateral dysplasia of the spine of the scapula (Sanofi-Aventis, 2003). It is

demonstrated the teratogenicity of Leflunomide in mice (CHAPTER 2). Its administration at a dose of 30 mg/kg/day to pregnant mice from GD6 to GD15 caused multiple malformations over the entire body of the fetus. Malformations seen in this study were exencephaly, cleft palate, tail deformity, and anomalies of the sternebrae, ribs, cervical to sacral vertebrae and cardiovascular malformations (membranous ventricular septum defect, transposition of great vessels and persistent A-V canal).

Certain organs such as the palate often show more than one susceptible period; these periods are very likely to coincide with an early developmental event in that organ (Padmanabhan and Ahmed, 1997). Multiple susceptible periods call attention to the fact that there is more than one underlying embryological process involved in organogenesis. A given organ may show susceptible periods at two or three different times in response to as many agents. For example, Landauer produced the same type of long bone defects in chicks with boric acid, insulin and pilocarpine but the peaks of susceptibility were the 96th, 120th and 144th hours of incubation, respectively (Landauer, 1954). Since the susceptible period depends on the compounds tested and targeted organs, studying the susceptible periods to a teratogen should yield valuable information about the developmental toxicity of compounds.

In an attempt to gain details of the teratogenicity of Leflunomide, the present study was conducted to determine the critical periods for teratogenicity in mice by a single administration of Leflunomide on one of the days from GD6 to GD11 when the major susceptible period of Leflunomide-related malformations were expected to exist.

3-2. Material and methods

3-2-1. Animals

CD-1 (ICR) mice (Charles River Japan Inc.), 10-week-old females and 11-week-old males were maintained under laboratory conditions of 20 to 26°C, 30 to 70% relative humidity, a 12-h light / dark (photo) cycle (08:00 light on) and allowed *ad libitum* access to food (Certified Rodent Diet: CRF-1 Oriental Yeast Co., Ltd.) and tap water. The use of animals in this study was approved by the animal care and use committee of Shionogi & Co., Ltd. Females were mated with males overnight on a one-to-one basis. The morning on which a copulation plug was observed was designated as GD0.

3-2-2. Test substance and treatment

Leflunomide (purity 99.5%, Sigma-Aldrich) was suspended in 1.0 wt/vol% carboxymethylcellulose (CMC; Nacalai Tesque) solution. Dams received a single administration of Leflunomide by gastric intubation at a dose of 50 mg/kg on one of GD6 to GD11. The dosage level was selected based on the previous study on the teratogenicity of Leflunomide (CHAPTER 2). In this previous work, repeated dosing of dams from GD6 to GD11 at a dose of 70 mg/kg/day resulted in total litter resorptions in all dams. These embryos appeared to have been resorbed at an early stage of pregnancy, suggesting that a single administration at 70 mg/kg at an early stage of pregnancy may induce severe embryo toxicity. The dosing volume was calculated as 10 ml/kg based on the body weight on the day of administration. The control animals received 1.0 wt/vol% CMC daily from GD6 to GD11. Dams dosed 1.0 wt/vol% CMC from GD6 to GD15 showed no developmental toxicity.

3-2-3. Evaluation of embryo toxicity and teratogenicity

All dams were weighed on GD0 and on GD5 to 18. On GD18, dams were euthanized with exsanguinations from the caudal vena cava under diethyl ether anesthesia and hysterectomized. The number of implantations, absorbed embryos and live/dead/macerated fetuses were counted, and the body weights of live fetuses were measured. Following external observation, the fetuses for visceral examination were fixed in Bouin's solution for more than two weeks under anesthesia with diethyl ether. Visceral alterations were observed by stereoscopic microscopy. The head was examined by the free hand razor blade sectioning method. The thorax and abdomen were examined by microdissection method. The fetuses for skeletal examination were euthanized with an overdose of pentobarbital sodium and fixed in 70% ethanol after removing skin, thoracic and visceral organs and brown fat. After fixation for at least 2 weeks, the fetuses were macerated with potassium hydroxide and stained with Alizarin red S (Sigma-Aldrich).

3-2-4. Statistics

Fetal data was pooled by litter, which served as the unit of analysis. Continuous data noted below were analyzed using Dunnett's parametric multiple *t*-test to compare each of the Leflunomide-treated groups with the control group: maternal body weights, mean number of implantations, post-implantation loss, mean number of live fetuses, fetal body weights, and the incidences of external, skeletal, and visceral anomalies. Binary data including the number of dams totally resorbed, and the number of dams with fetuses showing external, skeletal and visceral malformations were analyzed by Dunnett type multiple comparison for the response rate to compare each of the Leflunomide-treated groups with the control group. All pairwise statistical tests were performed as the two-sided ones. A difference between control and treated group was regarded as statistically significant at the value of $p < 0.05$.

3-3. Results and discussion

3-3-1. Body weights of dams, fetal viability and intrauterine growth

The mean body weights of the group treated on GD7 were significantly lower than that of the control group from GD14 to 18 and those in GD8-treated group were also slightly lower (Fig. 3-1). The lower maternal body weights on GD7- and GD8-treated groups are suggested to be caused by fetal death, since no dams showed any abnormal clinical signs and the lower body weight gains of these groups were seen from the medium term of pregnancy, not from the next day of administration, and fetal viability was significantly low in the GD7 and GD8 treated groups compared with the control group (Table 3-1). Fetal body weights were significantly low in those treated on GD7 and GD9 compared with the control data. From these results, it is demonstrated that the single administration of Leflunomide on GD7, GD8 or GD9 caused severe embryo toxicity, *i.e.*, lethality and reduced intrauterine growth.

3-3-2. External malformations

The results of external malformations are shown in Table 3-2. The incidences of fetuses with external malformations were significantly high in the GD7, 9, 10 and 11 treated groups. In the GD7 treated group, exencephaly and cleft face were found in one fetus. Treatment on GD9 induced mainly cleft palate. On GD10, characteristic malformations were hematoma on forepaw/hindpaw, cleft palate, and limb and tail deformities. Malformations of the finger of forepaw/hindpaw observed in this group were ectrodactyly, polydactyly and syndactyly. In the GD11 treated group, open eye and deformities of the finger of forepaw/hindpaw and tail were mainly found. Deformities of the finger of forepaw/hindpaw in this group were ectrodactyly,

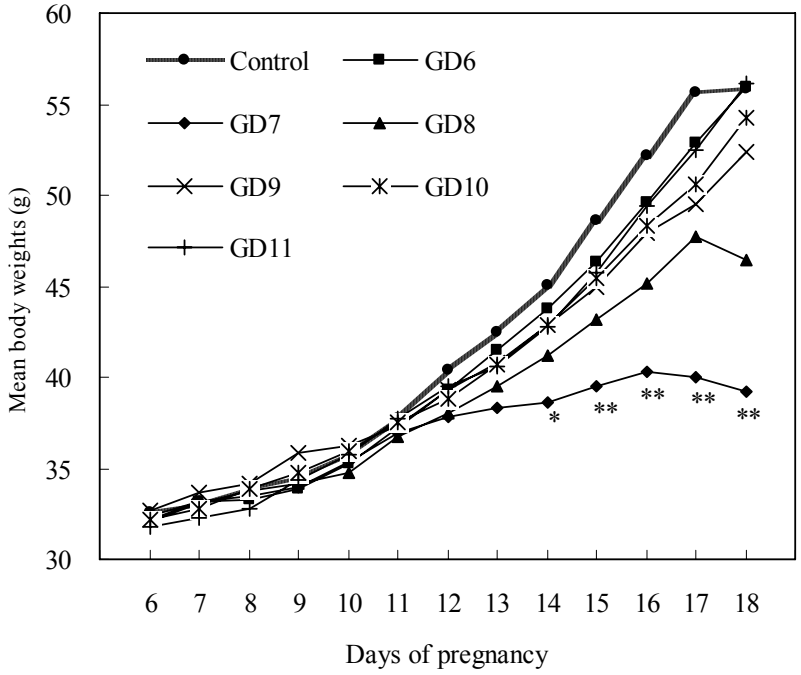


Figure 3-1.

Changes in mean body weights of dams treated with Leflunomide.

Dams were dosed 50 mg/kg of Leflunomide on one of GD6-11. In the control group, CMC was administered from GD6 to GD11. *p <0.05, **p <0.01

GD: gestation day administered Leflunomide

Table 3-1: Maternal and fetal effects of treatment with Leflunomide on one of GD6-11

Treatment day	GD6-11	GD6	GD7	GD8	GD9	GD10	GD11
Dose (mg/kg)	0	50	50	50	50	50	50
No. of dams	8	8	10	8	10	9	10
Mean No. of implantations	13.9±2.1	14.1±1.6	13.6±3.7	13.5±4.2	14.3±2.9	12.9±2.4	13.3±2.0
No. of dams totally resorbed	0	1	6 **	2	1	1	0
Post-implantation loss (%)							
Total	7.7±7.8	23.5±38.2	88.4±19.7 **	50.5±40.4 *	31.0±36.7	19.2±32.5	8.3±6.6
Resorption	7.7±7.8	23.5±38.2	87.6±21.3 **	49.7±41.0	31.0±36.7	19.2±32.5	6.2±6.3
Maceration	0.0	0.0	0.0	0.8±2.4	0.0	0.0	1.4±4.5
Dead	0.0	0.0	0.8±2.4	0.0	0.0	0.0	0.7±2.1
Mean No. of live fetuses	12.9±2.6	10.8±5.6	1.2±2.0 **	6.8±5.9 *	9.7±5.9	11.0±4.5	12.1±1.2
Fetal body weight (g)							
Male	1.36±0.09	1.34±0.08	1.08±0.23 *	1.36±0.14	1.17±0.16	1.18±0.17	1.24±0.15
Female	1.31±0.07	1.25±0.11	1.18±0.02	1.27±0.18	1.13±0.16 *	1.16±0.16	1.17±0.12

Mean±S.D.
* Significant differences at $p < 0.05$ compared with the control group
** Significant differences at $p < 0.01$ compared with the control group

brachydactyly and syndactyly. Only a few malformations were observed in the GD6 and GD8 treated groups.

Leflunomide caused exencephaly at a high incidence in mice with repeated dosing at 30 mg/kg/day during GD6 to GD15 (CHAPTER 2) and a single administration at a lower dose (30 mg/kg) on GD7 also induced exencephaly in one fetus of the 107 examined (data not shown). The incidence of exencephaly in current study was much lower than that in CHAPTER 2, however, this malformation was also seen in lower dosing group (30 mg/kg) on GD7, and the incidence of spontaneous exencephaly was lower than that in current study (Morita *et al*, 1987). These results suggest that exencephaly seen in the GD7 treated group was related to Leflunomide.

The repeated dosing of Leflunomide on GD6 to GD15 at a dose of 30 mg/kg/day caused various malformations on limb and finger of forepaw/hindpaw like bowed forelimb, pes varus, ectrodactyly, polydactyly, syndactyly at low incidences. Thus, a difference in frequency of malformations was noted between a single dosing and repeated dosing. Exposure at high concentration on the time of critical step of limb development and/or reducing embryo lethality by shortening the dosing duration may explain the difference in frequent types of fetal malformations.

3-3-3. Skeletal malformations

The results of skeletal examination are shown in Table 3-3. The incidence of fetuses with skeletal malformations in the groups given Leflunomide on GD7, 8, 9 or 10 was markedly higher than that in the control group. On GD7, the incidences of malformations of the skull bone, cervical to lumbar vertebrae and ribs were high. On GD8, the skeletal malformations of cervical to lumbar vertebrae, ribs and sternebrae were found. Treatment on GD9 induced absent palatine and anomalies of lumbar to caudal vertebrae, ribs and sternebrae. GD10 administration resulted in multiple malformations over the entire body. Found at particularly high incidence were absent

palatine, anomalies of thoracic to caudal vertebrae, ribs and sternebrae, and forelimb/hindlimb bones. Malformations of the vertebrae were fusion of the vertebral arch or body along the craniocaudal axis, fusion of the vertebral arch and body, discontinuous vertebral arch and misshapen vertebral body. Rib malformations consisted of fused, discontinuous and branched ribs. Anomalies of the limb bone mainly included fused or misshapen metacarpal, absent fibula and bent tibia. Malformations of the ischium and pubis were small. In the group dosed on GD6, there were no skeletal malformations.

Skeletal examination showed that as the dosing day switches from GD7 to GD9, the site of the malformation of vertebral bones transforms from cervical to caudal vertebrae. This is because the somites develop from the presomitic mesoderm, and somite formation progresses from the cranial end on GD7.75 and to the caudal site until GD13.5 in mouse embryo (Tam and Tan, 1992, DeSesso, 2006) and Leflunomide may affect the formation the somite level that is susceptible at the time of treatment.

3-3-4. Visceral malformations

The incidences of fetuses with visceral malformations in the groups treated with Leflunomide on GD7, 9 and 10 were markedly higher than that in the control group (Table 3-4). In the GD7-treated group, one fetus of the five examined showed cardiovascular malformation (cor triculare, persistent atrioventricular (A-V) canal, right-sided aortic arch and transposition of the great vessels). In the GD9-treated group, the incidence of cardiovascular malformations (membranous ventricular septum defect and persistent truncus arteriosus) was significantly higher than that of the control group. Treatment on GD10 caused absent accessory lobe of lung. In the GD6-, 8-, and

Table 3-2: External malformations in the mice fetuses following maternal treatment with Leflunomide on one of GD6-11

	Treatment with Leflunomide on selected gestation day (GD)						
Day treated	GD6-11	GD6	GD7	GD8	GD9	GD10	GD11
Dose (mg/kg)	0	50	50	50	50	50	50
No. of dams	8	7	4	6	9	8	10
No. of dams with fetuses with malformations	1 (12.5)	1 (14.3)	3 (75.0)	1 (16.7)	6 (66.7)	8 (100.0) **	10 (100.0) **
No. of fetuses examined	103	86	12	54	97	99	121
No. of fetuses with malformations	1 (1.0±2.7)	1 (1.1±2.9)	3 (45.8±41.7) *	1 (1.5±3.7)	40 (50.2±43.2) **	84 (85.1±29.7) **	104 (86.3±23.6) **
Misshapen cutis	0 (0.0)	0 (0.0)	1 (8.3±16.7) *	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Hematoma	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (3.7±11.1)	15 (17.6±30.2) *	5 (4.4±11.5)
Exencephaly	0 (0.0)	0 (0.0)	1 (12.5±25.0) *	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cleft face	0 (0.0)	0 (0.0)	1 (12.5±25.0) *	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cleft palate	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.5±3.7)	35 (45.8±43.2) **	38 (40.8±37.0) **	7 (5.9±9.7)
Exophthalmos	0 (0.0)	0 (0.0)	1 (8.3±16.7) *	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Open eye	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (5.7±9.7) **
Anomalies of limb	1.0±2.7	1.1±2.9	25.0±50.0	0 (0.0)	12.7±33.1	27.3±31.8	8.2±15.6
Hemimelia (Hindlimb)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	12 (11.5±32.6)	0 (0.0)
Bowed forelimb	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (3.8±10.9)	0 (0.0)
Bowed hindlimb	0 (0.0)	1 (1.1±2.9)	0 (0.0)	0 (0.0)	0 (0.0)	10 (9.6±21.7)	0 (0.0)
Manus vara	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (7.7±21.8)	0 (0.0)
Pes varus	1 (1.0±2.7)	0 (0.0)	1 (25.0±50.0)	0 (0.0)	2 (12.7±33.1)	13 (13.3±18.6)	10 (8.2±15.6)
Pes valgus	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (7.2±13.4) *	0 (0.0)
Anomalies of finger of fore/hindpaw	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	15.9±24.0	78.4±38.5 **	65.1±31.4 **
Ectrodactyly (forepaw)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	48 (46.3±33.3) **	15 (12.4±14.3)
Ectrodactyly (hindpaw)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6±16.7)	16 (18.0±27.8)	39 (31.9±32.5) **
Brachydactyly (forepaw)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.1±3.2)	6 (5.0±8.1) *
Brachydactyly (hindpaw)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6±16.7)	2 (1.9±5.4)	15 (12.6±14.9) **
Enlarged finger (forepaw)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.1±3.9) *	0 (0.0)
Polydactyly (forepaw)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (4.3±6.7) **	0 (0.0)
Polydactyly (hindpaw)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (10.4±20.7)	31 (30.3±21.0) **	13 (11.5±14.7)
Syndactyly (forepaw)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6±16.7)	31 (32.1±24.0) **	13 (10.7±10.7) *
Syndactyly (hindpaw)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.9±2.8)	26 (26.7±22.6) **	30 (24.9±17.8) **
Short tail	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6±16.7)	31 (31.4±44.0)	80 (65.5±37.5) **
Kinked tail	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (10.0±17.4)	48 (47.9±39.7) **	63 (53.5±30.9) **

Values: Number of fetuses with malformation (%: The incidence of fetuses with malformation)
* Significantly different from Control group $p < 0.05$
** Significantly different from Control group $p < 0.01$

Table 3-3: Skeletal malformations in the mice fetuses following maternal treatment with Leflunomide on one of GD6-11

	Treatment with Leflunomide on selected gestation day (GD)						
Day treated	GD6-11	GD6	GD7	GD8	GD9	GD10	GD11
Dose (mg/kg)	0	50	50	50	50	50	50
No. of dams	3	4	3	3	5	8	9
No. of dams with fetuses with malformations	0 (0.0)	0 (0.0)	3 (100.0) *	2 (66.7)	4 (80.0)	8 (100.0) **	7 (77.8)
No. of fetuses examined	17	22	6	19	27	53	57
No. of fetuses with malformations	0 (0.0)	0 (0.0)	4 (77.8±38.5) **	9 (47.6±50.2)	17 (72.0±43.8) **	47 (88.4±17.5) **	8 (14.0±9.9)
Anomalies of skull	0 (0.0)	0 (0.0)	2 (50.0±50.0) *	0 (0.0)	3 (8.6±19.2)	14 (25.7±31.6)	2 (3.4±6.9)
Misshapen zygomatic	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.9±6.4)	0 (0.0)	0 (0.0)
Misshapen basiophenoid	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6±9.6)	2 (13.3±29.8)	0 (0.0)	0 (0.0)
Absent palatine	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6±9.6)	10 (40.5±39.1)	16 (31.5±39.2)	1 (1.6±4.8)
Anomalies of sternebra	0 (0.0)	0 (0.0)	1 (11.1±19.2)	4 (22.2±38.5)	5 (20.7±32.8)	14 (26.0±35.2)	1 (1.6±4.8)
Anomalies of rib	0 (0.0)	0 (0.0)	3 (66.7±57.7) **	8 (42.9±51.5)	4 (19.5±21.9)	8 (14.1±29.6)	0 (0.0)
Anomalies of cervical vertebra	0 (0.0)	0 (0.0)	2 (44.4±50.9) *	6 (31.8±33.5)	1 (5.0±11.2)	6 (10.7±30.3)	0 (0.0)
Anomalies of thoracic vertebra	0 (0.0)	0 (0.0)	3 (44.4±50.9)	7 (38.1±54.1)	1 (6.7±14.9)	18 (32.0±35.5)	1 (1.9±5.6)
Anomalies of lumber vertebra	0 (0.0)	0 (0.0)	1 (33.3±57.7)	5 (27.8±48.1)	8 (38.6±41.5)	16 (28.9±32.6)	1 (1.9±5.6)
Anomalies of sacral vertebra	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	17 (72.0±43.8) **	15 (26.2±29.9)	2 (3.7±7.4)
Anomalies of caudal vertebra	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	10 (42.6±39.0)	20 (34.9±36.9)	0 (0.0)
Anomalies of forelimb	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	17 (32.1±28.9) **	0 (0.0)
Anomalies of ilium	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.8±5.1)	0 (0.0)
Anomalies of ischium and pubis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (12.5±35.4)	0 (0.0)
Anomalies of hindlimb	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (10.7±14.7)	12 (21.3±31.3)	0 (0.0)

Values: Number of fetuses with malformation (%: The incidence of fetuses with malformation)
* Significantly different from Control group $p < 0.05$
** Significantly different from Control group $p < 0.01$

Table 3-4: Visceral malformations in the mice fetuses following maternal treatment with Leflunomide on one of GD6-11

	Treatment with Leflunomide on selected gestation day (GD)						
Day treated	GD6-11	GD6	GD7	GD8	GD9	GD10	GD11
Dose (mg/kg)	0	50	50	50	50	50	50
No. of dams	8	7	3	6	9	8	10
No. of dams with fetuses with malformations	0 (0.0)	0 (0.0)	2 (66.7)	1 (16.7)	6 (66.7) *	5 (62.5) *	2 (20.0)
No. of fetuses examined	50	42	5	25	46	46	58
No. of fetuses with malformations	0 (0.0)	0 (0.0)	2 (44.4±50.9) *	1 (3.3±8.2)	12 (40.3±41.3) **	12 (26.6±33.6)	2 (3.3±7.0)
Hydrocephaly	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (8.3±23.6)	0 (0.0)
Anomalies of heart and great vessels	0 (0.0)	0 (0.0)	1 (33.3±57.7)	0 (0.0)	10 (32.9±41.9) *	1 (3.1±8.8)	0 (0.0)
Cor triculare	0 (0.0)	0 (0.0)	1 (33.3±57.7) **	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Membranous ventricular septum defec	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	10 (32.9±41.9) **	1 (3.1±8.8)	0 (0.0)
Persistent A-V canal	0 (0.0)	0 (0.0)	1 (33.3±57.7) **	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Right-sided aortic arch	0 (0.0)	0 (0.0)	1 (33.3±57.7) **	0 (0.0)	1 (3.7±11.1)	0 (0.0)	0 (0.0)
Persistent truncus arteriosus	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (25.9±37.4) **	0 (0.0)	0 (0.0)
Transposition of great vessels	0 (0.0)	0 (0.0)	1 (33.3±57.7) **	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Misshapen thyroid	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.3±8.2)	1 (1.9±5.6)	1 (2.5±7.1)	2 (3.3±7.0)
Absent accessary lobe of lung	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (11.1±23.6)	9 (18.9±28.8) *	0 (0.0)
Malpositioned ovary	0 (0.0)	0 (0.0)	1 (11.1±19.2) *	0 (0.0)	0 (0.0)	1 (2.1±5.9)	0 (0.0)
Malpositioned testis	0 (0.0)	0 (0.0)	1 (33.3±57.7) **	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Values: Number of fetuses with malformation (%: The incidence of fetuses with malformation)
* Significantly different from Control group $p < 0.05$
** Significantly different from Control group $p < 0.01$

11-treated groups, there was only a small incidence of fetuses with malformation. There were no abnormalities in the digestive organs in any groups.

Various cardiovascular malformations were induced by Leflunomide treatment on GD7 or GD9. Because the heart is the first organ to develop and embryonic growth cannot progress efficiently until the cardiovascular system becomes functional, the process of induction of cardiac development must occur at the early stage of fetal development. In mice, the simple tubular heart develops between GD7 and GD8. Thereafter, the endocardial cushion is formed, following tubular heart looping. Finally, the atrioventricular valves and interventricular septum develop from the endocardial cushion (Fishman and Chien, 1997, Christoffels *et al*, 2000, Song *et al*, 2000). Since GD7 and GD9 were within the developmental period of the tubular heart, looping and chamber formation, it was suggested that Leflunomide might disturb these processes, and result in causing severe cardiovascular malformations.

The immunosuppressive metabolite of Leflunomide, A77 1726, has two known biochemical effects. The first reported biochemical effect of A77 1726 is inhibition of protein tyrosine kinases, and the second one is inhibition of DHODH, the forth enzyme in the biosynthesis of pyrimidines (Xu *et al*, 1995, Siemasko *et al*, 1998, Dimitrova *et al*, 2002). Xu *et al* demonstrated that A77 1726 was effective at inhibiting the tyrosine kinase activity of platelet-derived growth factor (PDGF) receptor in C6 glioma cells and epidermal growth factor (EGF) receptor in A431 squamous carcinoma cells, and had no effect on the tyrosine kinase activity of the fibroblast growth factor receptor (Xu *et al*, 1999). Many researchers have reported that inhibition of this signaling causes various malformations. In several strains of mice, targeted disruption of the EGF receptor gene showed epithelial defects, open eyes, cleft palate and multiple organ failure (Abbott *et al*, 2005). And PDGF receptor and/or its ligand deficient cause renal, cardiovascular, hematological

abnormalities and cleft palate (Xu *et al*, 2005, Levéen *et al*, 1994). However, it has remained unclear whether Leflunomide inhibits PDGF and EGF receptor tyrosine kinases in mouse embryo, and whether the inhibition of tyrosine kinases relates to teratogenicity of Leflunomide in mouse or not. The other biological mechanism is the inhibition of DHODH. There are some reports about teratogenicity of compounds which have effects to inhibit the nucleotide synthesis. Mycophenolic acid, active metabolite of mycophenolate mofetil, which is a potent inhibitor of inosine monophosphate dehydrogenase and inhibits *de novo* synthesis of purine nucleotides, has been reported to cause embryonic death and congenital malformations (anophthalmia, agnathia and hydrocephaly) (Le Ray *et al*, 2004, Cellcept®, Roche web site, 2008). Bredinin (Mizoribin) is an imidazole nucleotide, an enzyme inhibitor in the pathway from inosine 5'-monophosphate to xanthosine 5'-monophosphate or xanthosine 5'-monophosphate to guanosine 5'-monophosphate in the purine nucleotide *de novo* synthesis (Kawai *et al*, 1993). It has been reported that this compound showed the embryonic lethality and the teratogenicity in rats and mice, it caused multiple malformations, including face, limb and tail deformities (Kobayashi *et al*, 1974, Okamoto *et al*, 1978). In the *in vitro* assay, the proliferation of mouse embryonic cells inhibited by Bredinin was rescued by co-administration of guanosine or guanosine monophosphate to the culture medium, suggesting that Bredinin may induce cytotoxicity by competition with guanosine in fetal cells and result in embryonic lethal and teratogenic actions (Okamoto *et al*, 1980). Comparing the *Ki* values obtained for DHODH with the reported IC_{50} for A77 1726 inhibition of protein tyrosine kinases, the potency of A77 1726 as a DHODH inhibitor is some 100-1000-fold greater than that reported for its inhibition of protein tyrosine kinases (Davis *et al*, 1996). This report suggests that an alternative explanation for the teratogenicity of Leflunomide may be the potent inhibition of DHODH by Leflunomide.

3-4. Conclusion

In current report, it is demonstrated that there are many critical periods and Leflunomide disturbs the normal development of the various organs. These results suggest that Leflunomide may inhibit broadly *de novo* synthesis of pyrimidine nucleotide and/or tyrosine phosphorylation on various organs at embryonic organogenesis. Identification of the critical periods for teratogenicity of Leflunomide offers valuable information for further studies to elucidate the mechanism of its teratogenicity.

CHAPTER 4

THE EVALUATION OF RELATIONSHIP BETWEEN DHODH INHIBITION AND TERATOGENICITY OF LEFLUNOMIDE IN MICE

Inhibiting the teratogenicity of the immunosuppressant Leflunomide in mice by supplementation of exogenous uridine

4-1. Introduction

Leflunomide, an immuno-suppressant drug, has been demonstrated to inhibit T-lymphocyte activation and production of IgM and IgG. The biochemical activity of Leflunomide includes inhibition of DHODH and protein tyrosine kinase. It has been demonstrated that A77 1726 binds to, and is a potent inhibitor of, DHODH, an enzyme in the de novo pyrimidine synthesis pathway that is important for DNA synthesis. Some researchers have suggested that the immunosuppressive effects of Leflunomide are related to a profound inhibition of pyrimidine nucleotide *de novo* synthesis (Dimitrova *et al*, 2002, Elder *et al*, 1997, Fox, 1998, Pinschewer *et al*, 2001, Silva *et al*, 1996). Uridine, which is the precursor substance of pyrimidine nucleotides, normalizes the concentration of pyrimidine nucleotides that have been reduced by Leflunomide *in vitro* (Rückemann *et al*, 1998) and in *in vivo* studies in rats (Chong *et al*, 1999), and antagonizes the inhibitory effect of Leflunomide on T-cell proliferation and activation of antiviral antibodies (Chong

et al, 1999). The other biochemical action of Leflunomide is the inhibition of protein tyrosine kinases. Leflunomide inhibits protein tyrosine phosphorylation in T cells, B cells, and CTLL cell lines (Siemasko *et al*, 1998, Xu *et al*, 1995). The potency of A77 1726 as a DHODH inhibitor is 100- to 1000-fold greater than that reported for its inhibition of protein tyrosine kinases (Davis *et al*, 1996, Xu *et al*, 1995). These data suggest that the immunosuppressive efficacy of A77 1726 might be mainly attributable to the potent inhibition of DHODH by this compound.

Leflunomide has been found to be teratogenic in rats, rabbits, and mice. In rats, 10 mg/kg/day of Leflunomide induces multiple malformations including cranioschisis and exencephaly when administered during organogenesis (GD7-17). Fused and incomplete ossification of the sternebra are seen when rabbits are treated with Leflunomide during fetal organogenesis at a dose of 10 mg/kg/day (Sanofi-Aventis, 2003). Leflunomide causes multiple malformations over the entire body of the fetus when administered at a dose of 30 mg/kg/day to pregnant mice during GDs6-15. Characteristic malformations are exencephaly, cleft palate, tail deformity, anomalies of the axial skeleton, and cardiovascular malformations (CHAPTER 2).

The mechanisms of teratogenicity of Leflunomide are unclear. We assumed that the inhibition of DHODH activity is related to the teratogenicity of Leflunomide. Pyrimidine nucleotides are synthesized through two pathways, the *de novo* synthesis pathway and a salvage pathway. In the former pathway, DHODH catalyzes the dehydrogenation of dihydroorotate to orotate. Orotate is converted to uridine monophosphate *via* ortidine monophosphate. In the latter pathway, uridine or thymidine degraded from nucleotides are turned into functional nucleotides without catalysis of DHODH (Breedveld and Dayer, 2000). If DHODH inhibition is related to the teratogenicity of Leflunomide, its inhibitory effect must be neutralized by co-administered of uridine, and the pyrimidine nucleotide level can be restored through the salvage pathway in embryonic tissues. In mice, there is much information available with respect to molecular and cellular biology,

and some reports have been published about the effects of Leflunomide treatment on the immune system, including DHODH and protein tyrosine kinase activities. In this study, we examined the effects of co-administration of Leflunomide and uridine on the developing mouse embryo, and the involvement of teratogenicity on the concentration of each nucleotide to clarify whether or not inhibition of DHODH is related to the teratogenicity of Leflunomide.

4-2. Materials and methods

4-2-1. Chemicals and reagents

Leflunomide (purity 99.5%), uridine (purity >99%), uridine 5'-triphosphate (UTP), cytidine 5'-triphosphate (CTP), adenosine 5'-triphosphate (ATP), and guanosine 5'-triphosphate (GTP) were purchased from Sigma-Aldrich. Carboxymethyl cellulose (CMC), diethyl ether, and trichloroacetic acid were from Nacalai Tesque. Tri-*n*-octylamine was from Wako Pure Chemical Industries (Osaka, Japan). Potassium dihydrogenphosphate was from Kanto Chemical (Tokyo, Japan). Leflunomide was suspended in 1.0 wt/vol% CMC solution.

4-2-2. Animals

CD-1 (ICR) mice (Charles River Japan Inc.), 10-week-old females and 11-week-old males, were maintained under laboratory conditions of 20 to 26°C, 30 to 70% relative humidity, a 12-h light/dark photocycle (08:00 light on) and were allowed to access food and tap water *ad libitum* (Certified Rodent Diet: CRF-1 Oriental Yeast Co., Ltd.). The use of animals in this study was approved by the animal care and use committee of Shionogi & Co., Ltd. Females were mated with males overnight on a one-to-one basis. The morning on which a copulation plug was observed was

designated as GD0.

4-2-3. Evaluation of embryo toxicity and teratogenicity

Dams in the Leflunomide-treated group received a single administration of Leflunomide by gastric intubation at a dose of 70 mg/kg in the morning of GD10. The dosage level was selected based on the previous study on the teratogenicity of Leflunomide (CHAPTER 3). A single dosing of dams on GD10 at a dose of 50 mg/kg causes mild embryonic lethality and multiple fetal malformations. The incidences of fetuses with malformations in this group are 85.1%, 88.4%, and 26.6% in external, skeletal, and visceral examinations, respectively. In the current study, animals were administered Leflunomide on GD10 at a dose of 70 mg/kg, at which it was expected that all fetuses would show multiple malformations. The dosing volume was calculated as 10 ml/kg based on the body weight just before administration. The control animals received 1.0 wt/vol% CMC on GD10. In the Leflunomide and uridine-combined administration groups, uridine was intraperitoneally administered at a dose of 1000 mg/kg/time at 0.5, 4, 9, and 24 h (four times) or 0.5 and 4 h (two times) after Leflunomide administration. The dosing volume was 5 ml/kg based on the body weight on the day of administration. In the preliminary study, dams treated with uridine 0.5, 4, 9, and 24 h after dosing of CMC on GD10 showed no differences from the control (CMC only) group in fetal viability, intrauterine development, and teratogenicity (data not shown).

On GD17, all dams were euthanized with exsanguinations from the caudal vena cava under diethyl ether anesthesia and hysterectomized. The number of implantations, absorbed embryos, and live/dead/macerated fetuses were counted, and the body weights of live fetuses were measured. Following external observation, half of the live fetuses per litter were fixed in Bouin's solution following diethyl ether anesthesia for subsequent free-hand sectioning to examine visceral anomalies. The remaining fetuses had their skin, thoracic and visceral organs, and brown fat removed, and were

fixed in 70% ethanol for skeletal examination, following euthanasia under intraperitoneal overdosing of pentobarbital sodium. After fixation, the fetuses were macerated with potassium hydroxide, and the fetal skeleton was stained with Alizarin red S (Sigma-Aldrich).

4-2-4. Extraction and quantitation of nucleotides from embryo

Nucleotides, *i.e.*, UTP, CTP, ATP, and GTP, were quantitated to examine whether the concentrations of pyrimidine nucleotides were altered by dosing of Leflunomide and to check whether the co-administered uridine was sufficient to normalize the concentration of pyrimidine nucleotides in embryonic tissues. The concentration of nucleotides extracted from whole embryo tissue (3 embryos were pooled per sample) was measured. Pregnant mice were administered 70 mg/kg of Leflunomide in the morning on GD10. In the group co-administered with Leflunomide and uridine, the dams were dosed with 1000 mg/kg/time uridine at 0.5, 4, and 9 h after Leflunomide treatment. The uridine-treated group was dosed with uridine at the same dosage and times as the group co-administered with Leflunomide and uridine after CMC dosing. The control group was only given CMC solution. The dams were hysterectomized under diethyl ether anesthesia and their embryos were collected 10 h after Leflunomide treatment. Tissue samples were lyophilized and weighed, and briefly homogenized with 200 μl of 16 mg/mL trichloroacetic acid. The homogenate was immediately centrifuged at 3360 x *g* for 5 min. The supernatant was collected and neutralized with a double volume of tri-*n*-octylamine/diethyl ether (1:4, vol/vol). After 30 min on ice, 50 μl of aqueous phase was placed directly on a ChemcoPak anion exchange column (2.1 x 250 mm i.d., Particil 10 SAX, Chemco Scientific, Osaka, Japan). The mobile phase was 0.5 M potassium dihydrogenphosphate. The corresponding peaks of the four nucleotides were detected at a wavelength of 254 nm by an HPLC system (SCL-10A, Shimadzu) with a liquid chromatograph (LC-10AD) and UV-Vis detector (SPD-10AV). The concentration of the

nucleotides was calculated on the basis of a standard curve of purified nucleotides and the data was corrected with lyophilized tissue weight.

4-2-5. Statistics

Fetal data was pooled by litter, which served as the unit of analysis. The data was analyzed using Dunnett's parametric multiple *t*-test to compare each of the Leflunomide-treated groups, the Leflunomide plus uridine-treated group with the control group, and the Leflunomide plus uridine-treated group with the Leflunomide-treated group. The *t*-test was used for the analysis of the concentration of nucleotides to compare the compound-treated (Leflunomide and/or uridine) groups with the control group, and the Leflunomide plus uridine-treated group with the Leflunomide-treated group. All pairwise statistical tests were two-sided. A difference was regarded as statistically significant at a value of $p < 0.05$.

4-3. Results

4-3-1. Effects on fetal viability, intrauterine growth, and teratogenicity

In the Leflunomide-treated group, fetal viability, the mean numbers of live fetuses, and fetal body weights were significantly lower than the control group (Table 4-1). Administration of uridine at 0.5, 4, 9, and 24 h (four times) and at 0.5 and 4 h (two times) after treatment with Leflunomide prevented fetal death and reduced intrauterine growth. The number of live fetuses recovered to the level of the control group in both uridine-treated groups. Fetal body weight was

Table 4-1: Embryo toxicity

	Control	Leflunomide (Leflunomide: 70 mg/kg)	Leflunomide+Uridine (Leflunomide: 70 mg/kg) (Uridine: 1000 mg/kg/time, 4 doses)	Leflunomide+Uridine (Leflunomide: 70 mg/kg) (Uridine: 1000 mg/kg/time, 2 doses)
No. of dams	7	7	6	7
No. of implantations	13.9 ± 1.6	13.0 ± 2.2	13.8 ± 2.4	14.1 ± 1.2
Post-implantation loss (%)				
Total	9.0 ± 7.2	32.2 ± 28.7 *	5.5 ± 6.6 ††	7.0 ± 8.1 †
Resorption	9.0 ± 7.2	32.2 ± 28.7 *	4.5 ± 6.9 ††	6.0 ± 6.2 ††
Maceration	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 2.7
Dead	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 2.6	0.0 ± 0.0
Number of live fetuses	12.6 ± 1.3	8.9 ± 4.4 *	13.0 ± 2.0 †	13.1 ± 1.6 ††
Fetal body weight (g)	1.109 ± 0.041	0.782 ± 0.102 **	0.920 ± 0.129 *	0.829 ± 0.094 **

(mean ± SD)
Significantly different from Control group *: $p<0.05$, **: $p<0.01$
Significantly different from Leflunomide-treated group †: $p<0.05$, ††: $p<0.01$

also slightly higher in the four- and two-times groups than in the Leflunomide-treated group.

Fetal external malformations are summarized in Table 4-2. All fetuses treated with Leflunomide showed multiple malformations over the entire body. Cleft palate, hemimelia (hindlimb), bowed hindlimb, pes varus, ectrodactyly, hematoma, short tail, and kinked tail were observed at significantly high incidences. The incidences of the malformations of limbs and digits also tended to be significantly high compared with the control group. In both groups treated with Leflunomide and uridine, the incidences of fetuses with external malformations were slightly lower than the Leflunomide group. In the four-times group, the incidences of cleft palate, hemimelia (hindlimb), bowed hindlimb, ectrodactyly, hematoma, short tail, and kinked tail decreased significantly. Even in the two-times group, the incidence of limb malformations was reduced significantly and incidences of ectrodactyly, hematoma, and tail deformities decreased. However, digit anomalies were seen at high incidences in both the four- and two-times groups, except for ectrodactyly and hematoma.

The results of the skeletal examinations are shown in Table 4-3. Skeletal and visceral examinations of the two-times co-administered uridine group were not conducted since the effects of uridine on skeletal and visceral malformations caused by Leflunomide could be thoroughly evaluated with the four-times co-administered group based on external examination. In the Leflunomide-treated group, the incidence of fetuses with skeletal anomalies was 100%, and multiple malformations were observed. Absent palatines, and deformities of the skull bone, cervical to lumbar vertebrae, forelimb, ischium/pubis, and hindlimb were seen at significantly higher frequencies than in the control group. Administration of uridine effectively inhibited these skeletal malformations. The incidence of fetuses with malformations decreased to 58.8 ± 37.1%; this was significantly lower than in the Leflunomide-treated group. Each malformation noted above also decreased significantly and the others tended to be lower than in the Leflunomide-treated group.

Table 4-2: External malformation of fetuses

	Control		Leflunomide (Leflunomide: 70 mg/kg)		Leflunomide+Uridine (Leflunomide: 70 mg/kg) (Uridine: 1000 mg/kg/time, 4 doses)		Leflunomide+Uridine (Leflunomide: 70 mg/kg) (Uridine: 1000 mg/kg/time, 2 doses)	
No. of dams	7		7		6		7	
No. of examined fetuses	88		62		78		92	
Number of fetuses with malformations (Incidence of fetuses with malformations)	4	(4.3±8.2)	62	(100) **	50	(67.3±44.7) **	78	(86.7±35.3) **
Craniofacial region								
Mandibular.micrognathia	0	(0.0)	13 / 3	(23.2±39.5)	0	(0.0)	1 / 1	(1.0±2.7)
Cleft palate	3 / 2	(3.2±5.8)	60 / 7	(98.0±3.4)	28 / 5	(37.2±27.8) ††	59 / 6	(66.1±33.6) **
Open eye	1 / 1	(1.0±2.7)	0	(0.0)	0	(0.0)	0	(0.0)
Limb								
Anoamly of limb	0	(0.0)	57 / 7	(92.6±12.6) **	17 / 3	(24.2±31.7) ††	33 / 5	(37.1±32.1) * ††
Phocomelia, hindlimb	0	(0.0)	9 / 2	(16.2±37.3)	0	(0.0)	1 / 1	(1.0±2.5)
Hemimelia, forelimb	0	(0.0)	7 / 1	(14.3±37.8)	0	(0.0)	0	(0.0)
Hemimelia, hindlimb	0	(0.0)	44 / 6	(72.6±40.5) **	0	(0.0) ††	4 / 1	(4.8±12.6) ††
Bowed hindlimb	0	(0.0)	28 / 7	(50.2±30.4) **	5 / 2	(7.2±13.4) ††	15 / 5	(17.8±21.6) †
Pes varus	0	(0.0)	14 / 6	(22.8±20.3) *	12 / 3	(16.7±20.7)	15 / 5	(16.3±15.1)
Digit								
Anomaly of digit	0	(0.0)	61 / 7	(96.4±9.5) **	39 / 5	(53.1±43.2) ** †	75 / 6	(83.3±37.3) **
Ectrodactyly (forepaw)	0	(0.0)	52 / 7	(85.9±11.1) **	15 / 4	(20.6±17.7) ††	47 / 6	(52.6±32.1) **
Ectrodactyly (hindpaw)	0	(0.0)	27 / 7	(51.0±38.1) **	4 / 3	(5.3±6.0) †	19 / 4	(21.7±27.6)
Brachydactyly (forepaw)	0	(0.0)	4 / 1	(4.1±10.8)	0	(0.0)	3 / 1	(3.9±10.3)
Brachydactyly (hindpaw)	0	(0.0)	3 / 1	(3.1±8.1)	0	(0.0)	1 / 1	(1.2±3.2)
Polydactyly (forepaw)	0	(0.0)	3 / 2	(3.0±5.6)	4 / 2	(5.6±10.1) *	5 / 3	(6.0±9.3) **
Polydactyly (hindpaw)	0	(0.0)	13 / 3	(14.8±22.4)	21 / 4	(28.6±24.1) *	28 / 6	(31.8±23.9) **
Syndactyly (forepaw)	0	(0.0)	17 / 4	(19.0±21.8)	11 / 4	(14.7±17.5)	32 / 6	(36.1±21.6) **
Syndactyly (hindpaw)	0	(0.0)	14 / 4	(20.8±22.4)	13 / 3	(19.2±31.6)	18 / 5	(20.7±14.4)
Hematoma	0	(0.0)	33 / 6	(57.2±31.8) **	1 / 1	(1.4±3.4) ††	28 / 5	(29.9±24.2) *
Tail								
Short tail	0	(0.0)	44 / 6	(81.3±36.7) **	17 / 2	(20.6±32.7) †	48 / 5	(54.3±43.3) *
Kinked tail	0	(0.0)	38 / 7	(63.3±28.5) **	16 / 3	(19.5±27.8) ††	39 / 7	(43.2±25.1) **
Others								
Anal atresia	0	(0.0)	4 / 2	(6.4±10.8)	0	(0.0)	2 / 2	(2.5±4.3)
Short lower trunk	0	(0.0)	7 / 1	(14.3±37.8)	0	(0.0)	0	(0.0)

Values: Number of fetuses with malformation/Number of dams with abnormal fetuses (%, Mean±SD: Incidences with malformation)
Significantly different from Control group *: $p<0.05$ **: $p<0.01$
Significantly different from Leflunomide-treated group †: $p<0.05$ ††: $p<0.01$

Table 4-3: Skeletal malformations in fetuses

	Control		Leflunomide (Leflunomide: 70 mg/kg)		Leflunomide+Uridine (Leflunomide: 70 mg/kg) (Uridine: 1000 mg/kg/time, 4 doses)	
No. of dams	7		7		6	
No. of examined fetuses	46		32		40	
Number of fetuses with malformations (Incidence of fetuses with malformations)	4	(8.8±11.7)	32	(100) **	22	(58.8±37.1) ** ††
Skull						
Fused basioccipital	0	(0.0)	15 / 5	(45.9±36.6) **	7 / 5	(19.5±14.7)
Absent zygomatic	0	(0.0)	11 / 4	(31.1±40.6) *	0	(0.0) †
Small maxilla	0	(0.0)	7 / 3	(22.0±37.4)	0	(0.0)
Small mandible	0	(0.0)	7 / 3	(22.0±37.4)	1 / 1	(3.3±8.2)
Absent palatine	1 / 1	(2.0±5.4)	31 / 7	(98.0±5.4) **	17 / 5	(44.7±30.2) ** ††
Vertebra and chest						
Malformations of sternebra	1 / 1	(2.0±5.4)	19 / 6	(59.3±37.7) **	1 / 1	(3.3±8.2) ††
Fused sternebra	1 / 1	(2.0±5.4)	2 / 2	(4.9±8.5)	1 / 1	(3.3±8.2)
Misshapen sternebra	0	(0.0)	0	(0.0)	0	(0.0)
Misaligned sternebra	0	(0.0)	17 / 6	(54.4±39.6) **	0	(0.0) ††
Malformations of ribs	0	(0.0)	7 / 4	(24.9±27.3) *	0	(0.0) †
Fused rib	0	(0.0)	7 / 4	(24.9±27.3) *	0	(0.0) †
Discontinuous rib	0	(0.0)	3 / 2	(9.3±16.4)	0	(0.0)
Malformations of cervical vertebra	1 / 1	(2.4±6.3)	22 / 5	(57.6±42.1) **	0	(0.0) ††
Absent cervical arch	0	(0.0)	0	(0.0)	0	(0.0)
Fused cervical arch	0	(0.0)	22 / 5	(57.6±42.1) **	0	(0.0) ††
Misshapen cervical arch	1 / 1	(2.4±6.3)	3 / 2	(10.0±19.2)	0	(0.0)
Supernumerary cervical vertebrae	0	(0.0)	1 / 1	(2.9±7.6)	0	(0.0)
Malformations of thoracic vertebrae	0	(0.0)	27 / 7	(83.9±18.7) **	1 / 1	(2.8±6.8) ††
Fused thoracic arch	0	(0.0)	18 / 6	(53.6±35.2) **	0	(0.0) ††
Misshapen thoracic arch	0	(0.0)	25 / 7	(76.7±20.9) **	0	(0.0) ††
Misaligned thoracic arch	0	(0.0)	1 / 1	(2.9±7.6)	0	(0.0)
Fused thoracic centrum	0	(0.0)	8 / 4	(23.4±27.8) *	1 / 1	(2.8±6.8)
Fused thoracic centrum/arch	0	(0.0)	13 / 6	(45.5±30.1) **	0	(0.0) ††
Fused thoracic arch and rib	0	(0.0)	5 / 2	(18.6±32.9)	0	(0.0)
Malformations of lumber vertebrae	1 / 1	(2.4±6.3)	18 / 4	(49.4±46.7) *	3 / 2	(8.9±14.4)
Fused lumbar centrum	0	(0.0)	4 / 3	(12.1±16.3) *	0	(0.0)
Fused lumbar centrum/arch	0	(0.0)	3 / 2	(8.6±15.7)	0	(0.0)
Fused lumbar arch and supernumerary rib	0	(0.0)	1 / 1	(2.9±7.6)	0	(0.0)
Misaligned lumbar vertebrae	0	(0.0)	4 / 3	(14.3±37.8)	0	(0.0)
Absent lumbar vertebrae	1 / 1	(2.4±6.3)	12 / 3	(29.4±37.6)	3 / 2	(8.9±14.4)
Malformations of sacral vertebrae	0	(0.0)	1 / 1	(2.9±7.6)	0	(0.0)
Fused sacral centrum	0	(0.0)	1 / 1	(2.9±7.6)	0	(0.0)
Malformations of caudal vertebrae	0	(0.0)	9 / 2	(28.6±48.8)	0	(0.0)
Absent caudal vertebrae	0	(0.0)	9 / 2	(28.6±48.8)	0	(0.0)
Limb						
Malformations of forelimb	0	(0.0)	13 / 4	(35.9±40.9) *	1 / 1	(3.3±8.2)
Misshapen scapula	0	(0.0)	4 / 1	(11.4±30.2)	0	(0.0)
Fused radius bone	0	(0.0)	4 / 2	(9.7±17.3)	0	(0.0)
Bent radius bone	0	(0.0)	2 / 2	(5.6±10.1)	0	(0.0)
Small radius bone	0	(0.0)	4 / 4	(10.5±10.5) **	0	(0.0) †
Absent radiu bone	0	(0.0)	2 / 1	(7.1±18.9)	0	(0.0)
Small ulna	0	(0.0)	4 / 4	(10.5±10.5) **	0	(0.0) †
Absent ulna	0	(0.0)	3 / 2	(9.2±18.8)	0	(0.0)
Fused metacarpal	0	(0.0)	1 / 1	(2.0±5.4)	0	(0.0)
Misshapen metacarpal	0	(0.0)	0	(0.0)	1 / 1	(3.3±8.2)
Malformaitons of ilium	0	(0.0)	7 / 2	(22.9±40.7)	1 / 1	(2.8±6.8)
Malpositioned ilium	0	(0.0)	3 / 1	(8.6±22.7)	0	(0.0)
Small ilium	0	(0.0)	2 / 1	(7.1±18.9)	1 / 1	(2.8±6.8)
Absent ilium	0	(0.0)	2 / 1	(7.1±18.9)	0	(0.0)
Malformations of ischium/pubis	0	(0.0)	16 / 6	(60.6±42.0) **	2 / 1	(5.6±13.6) ††
Absent ischium	0	(0.0)	9 / 4	(32.1±35.8) *	1 / 1	(2.8±6.8)
Small ischium	0	(0.0)	7 / 5	(28.5±34.5) *	1 / 1	(2.8±6.8) †
Absent pubis	0	(0.0)	3 / 1	(10.7±28.4)	1 / 1	(2.8±6.8)
Small pubis	0	(0.0)	2 / 2	(6.4±11.1)	0	(0.0)

Table 4-3: Continued

	Control		Leflunomide (Leflunomide: 70 mg/kg)		Leflunomide+Uridine (Leflunomide: 70 mg/kg) (Uridine: 1000 mg/kg/time, 4 doses)	
No. of dams	7		7		6	
No. of examined fetuses	46		32		40	
Number of fetuses with malformations (Incidence of fetuses with malformations)	4	(8.8±11.7)	32	(100) **	22	(58.8±37.1) ** ††
Malformations of hindlimb	0	(0.0)	23 / 7	(76.5±29.4) **	7 / 2	(20.6±34.7) ††
Absent femur	0	(0.0)	3 / 1	(10.7±28.4)	1 / 1	(2.8±6.8)
Absent fibula	0	(0.0)	18 / 6	(58.1±35.3) **	4 / 1	(11.1±27.2) ††
Short fibula	0	(0.0)	4 / 4	(15.6±18.4) *	0	(0.0) †
Absent tibia	0	(0.0)	9 / 3	(36.4±46.1)	1 / 1	(2.8±6.8)
Bent tibia	0	(0.0)	6 / 4	(17.0±21.6)	3 / 2	(8.9±14.4)
Short tibia	0	(0.0)	6 / 4	(17.0±21.6) *	2 / 2	(6.1±9.5)
Fused metatarsal	0	(0.0)	0	(0.0)	1 / 1	(3.3±8.2)

Values: Number of fetuses with malformation/Number of dams with abnormal fetuses (%, Mean±SD: Incidences with malformation)
Significantly different from Control group *: $p<0.05$ **: $p<0.01$
Significantly different from Leflunomide-treated group †: $p<0.05$ ††: $p<0.01$

The incidence of fetuses with visceral malformations in the groups treated with Leflunomide was significantly higher than that in the control group (Table 4-4), which was 63.3 ± 25.2%. Membranous ventricular septum defects, overriding aorta, and diaphragmatic hernia were seen at significantly high incidences. As minor malformations, hydrocephaly, absent rhinencephalon, misshapen retina, right-sided aortic arch, persistent truncus arteriosus, small thymus, absent accessory lobe of lung, hydronephrosis, and retrocaval ureter were observed in a small number of fetuses. In the four-times co-administered uridine group, the incidence of fetuses with malformations was significantly lower than that of the Leflunomide-treated group, and each malformation (membranous ventricular septum defect, overriding aorta, and diaphragmatic hernia) decreased significantly. Minor malformations also disappeared or decreased.

4-3-2. Effects on levels of nucleotides

As shown in Fig. 4-1, the concentrations of UTP and CTP in the uridine-treated group were the same as those in the control group, and decreased significantly in the Leflunomide-treated group, dropping to 1.0% and 10.0%, respectively. UTP and CTP levels did not recover to the control level by co-administration of uridine after Leflunomide treatment, remaining at 66.7% and 53.9%, respectively, which were significantly higher than in the Leflunomide-treated group (p <0.01). Purine nucleotides (ATP and GTP) in the Leflunomide- and Leflunomide plus uridine-treated groups decreased slightly by 75 to 78%, but they were not altered markedly in the uridine-treated group.

4-4. Discussion

Leflunomide has been reported to cause teratogenicity in rats and rabbits (Sanofi-Aventis,

Table 4-4: Visceral malformations in fetuses

	Control	Leflunomide (Leflunomide: 70 mg/kg)	Leflunomide+Uridine (Leflunomide: 70 mg/kg) (Uridine: 1000 mg/kg/time, 4 doses)
No. of dams	7	7	6
No. of examined fetuses	42	30	38
Number of fetuses with malformations (Incidence of fetuses with malformations)	0 (0.0)	18 (63.3±25.2) **	3 (7.6±12.3) ††
Hydrocephaly	0 (0.0)	4 / 2 (16.1±37.3)	0 (0.0)
Absent rhinencephalon	0 (0.0)	3 / 1 (14.3±37.8)	0 (0.0)
Misshapen retina	0 (0.0)	1 / 1 (1.8±4.7)	0 (0.0)
Malformations of heart and great vessels	0 (0.0)	10 / 5 (41.7±36.3) **	1 / 1 (2.8±6.8) †
Membranous ventricular septum defect	0 (0.0)	10 / 5 (41.7±36.3) **	1 / 1 (2.8±6.8) †
Overriding aorta	0 (0.0)	8 / 5 (38.1±36.6) **	1 / 1 (2.8±6.8) †
Right-sided aortic arch	0 (0.0)	1 / 1 (7.1±18.9)	0 (0.0)
Persistent truncus arteriosus	0 (0.0)	1 / 1 (7.1±18.9)	1 / 1 (2.8±6.8)
Retroesophageal subclabian	0 (0.0)	0 (0.0)	1 / 1 (2.8±6.8)
Misshapen thyroid gland	0 (0.0)	0 (0.0)	1 / 1 (2.8±6.8)
Small thymus	0 (0.0)	1 / 1 (4.8±12.6)	0 (0.0)
Absent submaxillary glands	0 (0.0)	2 / 1 (9.5±25.2)	0 (0.0)
Absent accessory lobe of lung	0 (0.0)	2 / 2 (5.6±10.1)	0 (0.0)
Diaphragmatic hernia	0 (0.0)	13 / 6 (45.2±30.1) **	0 (0.0) ††
Hydronephrosis	0 (0.0)	1 / 1 (1.8±4.7)	2 / 2 (5.6±13.6)
Retrocaval ureter	0 (0.0)	4 / 2 (21.4±39.3)	0 (0.0)

Values: Number of fetuses with malformation/Number of dams with abnormal fetuses (%, Mean±SD: Incidences with malformation)
Significantly different from Control group **: $p<0.01$
Significantly different from Leflunomide-treated group †: $p<0.05$ ††: $p<0.01$

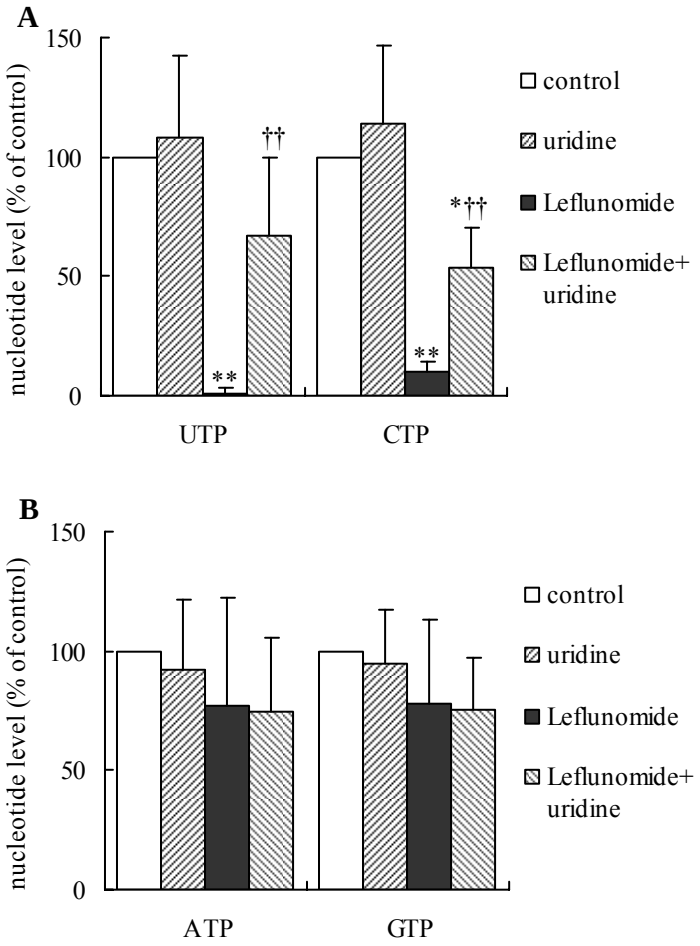


Figure 4-1.

Decrease in nucleotide levels in embryonic tissue by Leflunomide exposure and the effects of exogenous uridine on each nucleotide level.

Uridine was dosed 0.5, 4, and 9 h after Leflunomide or carboxymethyl cellulose administration, and embryonic tissue samples were collected 10 h after Leflunomide or CMC administration. Each nucleotide level is shown as a percentage of control. The number of samples per group was 11 in the control and 10 in other groups. **A**, Pyrimidine nucleotide levels (UTP, CTP). **B**, Purine nucleotide levels (ATP, GTP).

Significantly different from Control group *: $p < 0.05$, **: $p < 0.01$

Significantly different from Leflunomide-treated group ††: $p < 0.01$

2003), and mice. Leflunomide inhibits the enzymatic activity of protein tyrosine kinases and of DHODH, which is involved in pyrimidine nucleotide *de novo* synthesis. The potency of A77 1726 as a DHODH inhibitor is 100- to 1000-fold greater than that reported for its inhibition of protein tyrosine kinases (Davis *et al*, 1996, Xu, *et al*, 1995). DHODH inhibition by Leflunomide may be responsible for the embryo toxic and teratogenic effects (Brent, 2001), but the involvement of DHODH inhibition in the teratogenicity had not yet been confirmed.

Exogenous uridine markedly prevents embryo lethality, reduced intrauterine growth, and severe teratogenicity caused by Leflunomide in mice, and the alleviative effect of uridine on embryo toxicity and teratogenicity by Leflunomide depends on the frequency of uridine dosing. In a previous study, the concentration of A77 1726 in the blood of pregnant mice was measured after 30 mg/kg Leflunomide was administered. The mean value of the concentration of A77 1726 in plasma 24 h after Leflunomide administration was 48.9% of the value of C_{max} (CHAPTER 2). However, a report on the kinetics of uridine in blood showed extremely rapid clearance of a single injection of uridine. The kinetics of degradation of uridine in blood was observed by tracer injection of [5-^{3}H] uridine, and by 2 min after a single injection, the distribution of total radioactivity was nearly complete, and the concentration of [5-^{3}H] uridine in blood continued to decrease with a $T_{1/2}$ of ~ 3 min (Moyer *et al*, 1981). This report and previously results support our results, in which uridine reduced the effects of Leflunomide depending on the administration frequency, since the blood concentration of uridine was maintained at a higher level for a longer time by uridine administration given four times rather than twice. These results and the current study indicate that DHODH inhibition is related to the teratogenicity and embryo toxicity of Leflunomide.

However, the malformations in the digit region remained even after uridine co-administration for four times. We suggest a possibility for why these malformations remained. It is the inhibition of protein tyrosine kinases, which is the second pharmacological effect of

Leflunomide. Some researchers have demonstrated that restriction of *de novo* pyrimidine nucleotide synthesis occurs secondary to inhibition of *in vitro* proliferation of cultured CTTL-4 cells as this is reversed by uridine rescue, except at high concentrations of A77 1726 (Rückemann *et al*, 1998). A77 1726 inhibits the IL-2-driven proliferation of CTTL-4. At up to 12.5 μM of A77 1726, the addition of uridine at 50 μM completely reverses the inhibition caused by A77 1726. At higher concentrations of A77 1726, uridine does not completely reverse inhibition (Elder *et al*, 1997). Furthermore, unpublished data from Elder and coworkers indicates that the inability to reverse the effects of higher concentrations of A77 1726 is not due to insufficient uridine, since uridine at 100 μM produces the same level of proliferation as 50 μM uridine. The concentration of A77 1726 in serum from pregnant mice is 385 μM at 2 h after administration of Leflunomide at dose of 30 mg/kg (CHAPTER 2). The level of exposure to mouse embryos through the blood-placenta barrier, however, is not known, and pregnant mice were administered 70 mg/kg in the current study, a dose at which inhibition of protein tyrosine kinases in embryonic tissue was expected to occur. From these data, we suggest that inhibition of protein tyrosine kinases might be related to the incomplete prevention of teratogenicity in digits.

Measurement of nucleotide concentrations demonstrated that UTP and CTP decreased significantly (UTP: 99% drop, CTP: 90% drop) in fetuses treated with Leflunomide, while co-administration with uridine led to their partial recovery. Purine nucleotides were not markedly affected by treatment with Leflunomide with or without uridine. This result was not contradictory to the result of the teratogenicity experiment.

There are no reports that refer to the linkage between the inhibition of pyrimidine nucleotide *de novo* synthesis and teratogenicity in mice. Bredinin blocks the pathway from inosine 5'-monophosphate to xanthosine 5'-monophosphate or from xanthosine 5'-monophosphate to guanosine 5'-monophosphate in purine nucleotide *de novo* synthesis. This compound also has

embryo lethality and severe teratogenic actions in mice (Kobayashi *et al*, 1974) and rats (Okamoto *et al*, 1978). Furthermore, it has been reported that bredinin has a cytotoxic effect on mouse embryonic cells *in vitro*. Co-administration of guanosine reduces the teratogenic and embryo lethal effects of bredinin on rat fetuses, and an *in vitro* study showed a dose-dependent protective effect of guanosine against cytotoxicity in mouse embryonic cells. These studies indicate that bredinin might induce cytotoxicity by inhibition of *de novo* synthesis of purine nucleotides in fetal cells and result in teratogenic and fetal lethal actions (Okamoto *et al*, 1980). In current study, any cytotoxicity assay for Leflunomide was not conducted, although, as with inhibition of purine *de novo* synthesis, pyrimidine *de novo* synthesis inhibition can be attributed to teratogenicity *via* the anti-proliferative activity of Leflunomide on embryonic cells.

4-5. Conclusion

In conclusion, exogenous uridine led to partial recovery of the reduced pyrimidine nucleotide pool caused by Leflunomide in fetal tissue, and prevented teratogenicity except for digit malformations and embryo lethality, also caused by Leflunomide. These data support the hypothesis that the developmental toxicity of Leflunomide is due to inhibition of pyrimidine nucleotide biosynthesis, although the additional involvement of other mechanisms (*e.g.*, inhibition of protein tyrosine kinase activity) cannot be ruled out at this time because of the incomplete prevention of digit malformations.

CHAPTER 5

THE TERATOGENIC MECHANISM STUDY USING THE GENE EXPRESSION ANALYSIS IN LIMB BUD OF MOUSE EMBRYOS EXPOSED WITH LEFLUNOMIDE

Mechanism study for the limb malformations induced by Leflunomide in mice using the transcriptomic analysis

5-1. Introduction

Leflunomide, an isoxazol derivative, is an immunosuppressive agent (Brent, 2001). It inhibits T-lymphocyte activation and production of IgM and IgG with its actions, *i.e.*, inhibitions of DHODH and protein tyrosine kinases. Some researchers have suggested that the immunosuppressive effects of Leflunomide relate to a profound inhibition of pyrimidine nucleotide *de novo* synthesis (Dimitrova *et al*, 2002, Fox, 1998, Huang *et al*, 2002, Pinschewer *et al*, 2001, Silva *et al*, 1996). Inhibition of T cell activation and modulation of T cell differentiation by Leflunomide are antagonized by the presence of UTP, suggesting that those effects are mediated *via* the inhibition of DHODH (Dimitrova *et al*, 2002). The other action of Leflunomide is the inhibition of protein tyrosine kinases, which are known to play essential roles in signal transduction from cytokine receptors. Since the differentiation and proliferation of T-cells depend on signaling from cytokines, inhibition of intracellular tyrosine phosphorylation by Leflunomide may contribute

to inhibiting the activation of T-cells (Mattar *et al*, 1993, Siemasko *et al*, 1998, Waldman *et al*, 2001). Elder *et al* compared both activities to determine which of the biochemical targets of Leflunomide was responsible for the inhibition of proliferation and cytotoxic activity in murine CTLL cell lines. At low concentrations of A77 1726, pyrimidine biosynthesis is the target, since inhibition of proliferation correlates with a reduction in pyrimidine nucleotide levels and is reversed by uridine which is the precursor substance of pyrimidine nucleotides. At higher concentration of A77 1726 (150 μM), uridine does not reverse the inhibition of CTLL cell proliferation to any significant extent. This second mechanism for inhibiting proliferation is probably the inhibition of protein tyrosine kinases, since higher concentrations of A77 1726 inhibit IL-2-induced tyrosine phosphorylation of Jak1 and Jak3. This report established that both inhibitions of pyrimidine biosynthesis and of protein tyrosine kinases contribute to the effects of A77 1726 on CTLL cell lines (Elder *et al*, 1997).

Leflunomide has been reported to cause multiple malformations in rats (Sanofi-Aventis, 2003), rabbits (Sanofi-Aventis, 2003), and mice. In rats, 10 mg/kg/day of Leflunomide induces multiple malformations, including cranioschisis and exencephaly, when administered to dams during embryonic organogenesis, GDs7-17. Fused and incomplete ossification of the sternebra are seen when rabbits are treated with Leflunomide during fetal organogenesis (GDs6-18) at a dose of 10 mg/kg/day (Sanofi-Aventis, 2003). Described in CHAPTER 2, Leflunomide causes multiple malformations over the entire body of the fetus when administered at a dose of 30 mg/kg/day to pregnant mice on GDs6-15. Characteristic malformations are exencephaly, cleft palate, tail deformity, anomalies of the axial skeleton, and cardiovascular malformations. Mice given a single dose of 70 mg/kg Leflunomide on GD10 showed cleft palate and deformities of the limb, tail, entire skeleton, heart, and great vessels (CHAPTER 4). And it was demonstrated that exogenous uridine reduces the teratogenicity of Leflunomide in mice. Furthermore, Leflunomide decreases the concentration of pyrimidine nucleotides in mouse embryos, but not of purine nucleotides, and

uridine co-administered with Leflunomide restores the level of pyrimidine nucleotides. These results indicated that the inhibitory effect of DHODH activity is related to the teratogenicity of Leflunomide. However, in that study, malformations in the digit region remained even after uridine co-administration, suggesting that another mechanism, *i.e.,* inhibition of protein tyrosine kinases, may be involved in the teratogenicity of Leflunomide (CHAPTER 4).

In the current study, it was investigated the teratogenic mechanisms in the limbs of mouse embryos treated with Leflunomide on GD10. Gene expression profiles for mouse limb buds following Leflunomide treatment were analyzed using oligonucleotide-based DNA microarrays to which more than 40,000 genes are attached. Gene ontology (GO) analysis revealed that the expression of various genes changed, which genes are involved in cholesterol biosynthesis, regulation of transcription, morphogenesis, and cell proliferation and differentiation. But, in this study, it remains unclear what the effects are of altered expression of genes involving in morphogenesis and cell differentiation on abnormal limb patterning in mouse embryos treated with Leflunomide. Most of the genes of the cholesterol biosynthesis pathway decreased by Leflunomide treatment, suggesting that the ability to produce cholesterol may have been reduced. Since cholesterol in the developing limb bud plays an important role to regulate Sonic hedgehog (*Shh*) which is one of the morphogen of the limb (Li *et al*, 2006), the change of concentration of cholesterol in the limb bud may interfere in its normal development. And gene functional analysis and Leflunomide's pharmacological action, *i.e.*, DHODH and protein tyrosine kinases inhibition, suggest that Leflunomide may affect cell proliferation in the limb bud. Thus, we conducted additional experiments to elucidate the effects of Leflunomide on cholesterol biosynthesis and the proliferative ability of mesenchymal cells in limb buds of mouse embryos. We found that the regulation of genes in some distinct functional classes is important for teratogenicity, and these results implicate possible mechanisms involved in limb malformation by Leflunomide.

5-2. Materials and methods

5-2-1. Animals

CD-1 (ICR) mice (Charles River Japan Inc.) were maintained under laboratory conditions of 20 to 26°C, 30 to 70% relative humidity, and a 12-h light/dark photocycle (8:00 light on), and were allowed to access food and tap water *ad libitum* (Certified Rodent Diet: CRF-1, Oriental Yeast Co., Ltd.). The use of animals in this study was approved by the animal care and use committee of Shionogi & Co., Ltd. Ten-week-old females were mated with 11-week-old males overnight on a one-to-one basis. The morning on which a copulation plug was observed was designated as GD0.

5-2-2. Animal treatment and sampling for DNA microarray analysis

Leflunomide (purity 99.5%), purchased from Sigma-Aldrich, was suspended in a 1.0 wt/vol% CMC (Nacalai Tesque) solution. Dams in the Leflunomide-treated group received a single administration of Leflunomide by gastric intubation at a dose of 70 mg/kg in the morning of GD10. The dosage level was selected based on the previous study, which was expected to cause limb deformities in almost all fetuses under this dosing condition (CHAPTER 4). The dosing volume was calculated as 10 mL/kg based on the body weight measured just before administration. The control animals received 1.0 wt/vol% CMC on GD10. At 4 and 24 h after treatment, all animals in the control and Leflunomide-treatment groups (three dams per time point and group) were euthanized with exsanguinations from caudal vena cava under diethyl ether anesthesia, hysterectomized, and their embryos were removed. The fore- and hind limb buds of embryos were rapidly removed and soaked in RNAlater (Ambion, Austin, TX, USA). Limb bud tissues of all embryos in each dam were pooled as a sample (three samples per time point and group), and stored at -80°C until total RNA isolation.

5-2-3. Total RNA isolation

Total RNA from limb bud tissues was isolated using Qiazol Lysis Reagent (Qiagen, Valencia, CA, USA) and further purified using RNeasy Mini Kit (Qiagen) according to the manufacture's standard protocols. The RNA concentration was determined by absorbance at 260 nm, and RNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Equal amounts of total RNA from individual samples were used as templates for the microarray analysis.

5-2-4. DNA microarray analysis

Purified total RNA (5 μg/chip) was used to obtain cRNA and labeled for GeneChip® expression analysis (technical manual provided by Affymetrix, Santa Clara, CA, USA). cRNA were hybridized to a GeneChip® Mouse Genome 420 2.0 Array (Affymetrix), and washed, stained, and scanned according to the manufacturer's instructions. The scanned data images were digitalized using Affymetrix microarray suite version 5.0 software (Affymetrix), and the data were scaled by adjusting the mean signal value as 200. Microarray data analysis was performed by using Spotfire® DecisionSite for Functional Genomics (Spotfire, Göteborg, Sweden) and Microsoft Excel® (Microsoft, Redmond, WA, USA). An arbitrary cut-off value was used to filter genes with very low expression signal values, *i.e.,* genes that were classified into the *Absent* detection call in all 12 of the samples were excluded. The expression signal values of genes were then normalized to a global mean, and genes were selected if they exhibited 3.0-fold up-regulation or 0.3333-fold down-regulation with a *p*-value cutoff of < 0.01 by a two-sided Welch's *t*-test in the Leflunomide-treated group compared with the control group. Finally, a total of 244 probe sets were identified as Leflunomide-responsive genes. Gene ontology (GO) analysis was performed using the DAVID Functional Annotation tool (http://david.abcc.ncifcrf.gov/) to identify overrepresented

gene categories in each gene list (Dennis *et al*, 2003) , and a *p* value of < 0.01 determined by Fisher's exact test was considered statistically significant.

5-2-5 Real-time RT-PCR

To validate the results from the microarray experiment, real-time RT-PCR analysis using TaqMan® Gene expression Assays (Applied Biosystems Inc., Foster City, CA, USA) was conducted. Taqman® Gene Expression Assay IDs and Design Standard Sequences are listed in Table 5-1. Genes were selected for the validation study, which belonged to functional categories detected by GO analysis or reported to function in developing embryos. From each sample used for microarray analysis, 2.0 µg of total RNA was used to generate cDNA using Super Script II (Invitrogen, Carlsbad, CA, USA), and real-time RT-PCR was carried out using a 7500 Fast Real-Time PCR system (Applied Biosystems Inc.), according to the manufacture's instructions. Two technical replicates were run for each gene in each sample in a 96-well format plate. The endogenous control gene (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*) and no-template control were also run in duplicate. Amplification reactions were carried out using the following temperature profile: 50°C for 30 min, 95°C for 15 min, and 40 cycles of melting (95°C, 15 sec) and annealing/extension (60°C,

Table 5-1. Primers used in real time RT-PCR analysis

Gene symbol	Taqman® Assay ID	Design Standard Sequence
Mllt3	Mm00550927_m1	GCCGAAGTCGCAGGGTCAGCTTGAG
Id4	Mm00499701_m1	CGATGGATGGCCAGGTGTGCGGCCG
Mycn	Mm00476449_m1	CTTGAGCGACTCAGATGATGAGGAT
Shh	Mm00436527_m1	GGCTGATGACTCAGAGGTGCAAAGA
Edn1	Mm00438656_m1	CAGAAACAGCTGTCTTGGGAGCCGA
Idi1	Mm00836417_g1	AACATCGACAAAGGATTATTACATC
Sc4mol	Mm00499386_m1	GGACGCCAGGAATAAAAGGTCCCTC
Ldlr	Mm00440169_m1	CGGAGCTGCCTCACAGAAGTCGACA
Fdft1	Mm00815354_s1	GCCACTTGTCAGCGCGTCCTCCAGG
Hmgcs1	Mm00524111_m1	TGTCCTTCACACAGCTCTTTCACCA
Mvd	Mm00507014_m1	GCTATGCCTGCCTAGCCTATACCCT

60 sec). Relative quantification of gene expression was determined using the standard curve method. Values are reported as an average of duplicate analyses normalized to *GAPDH* mRNA levels.

5-2-6. Extraction and quantitation of cholesterol from limb buds of embryos

The concentration of cholesterol was measured in limb buds of mouse embryos exposed to Leflunomide. Pregnant mice were administered 70 mg/kg of Leflunomide in the morning on GD10. The control group received CMC solution. The dams were hysterectomized under diethyl ether anesthesia and their embryos were collected 4, 24, or 48 h after Leflunomide treatment. The concentrations of cholesterol extracted from limb bud tissue were measured using a cholesterol/cholesteryl ester quantitation kit (Calbiochem, EMD Chemicals Inc., San Diego, CA, USA). Limb bud tissues of all embryos in each dam were pooled as a sample (three samples per time point and group). Tissue samples were weighed and briefly homogenized with 200 μL hexane-isopropanol mixture (3:2, vol/vol). The homogenate was immediately centrifuged at 3360 × *g* for 10 min. The organic phase was collected and vacuum-dried. The dried lipid was re-dissolved in 20 μL of isopropanol with 10% Triton® X-100 detergent. Measurement of cholesterol was performed according to the manufacturer's instructions. The data of cholesterol concentration was corrected with wet tissue weight. The *t*-test was used for the analysis of the concentration of cholesterol to compare the compound-treated group with the control group at each time point. All pairwise statistical tests were two-sided. A difference was regarded as statistically significant at a value of $p < 0.05$.

5-2-7. Mitotic figure index in mesenchyme of forelimb buds

The mitotic capacity of mesenchymal cells in forelimb buds of mouse embryos treated with

Leflunomide was evaluated using hematoxylin-eosin-stained sections. The dams were treated orally with Leflunomide at 70 mg/kg or CMC in the morning on GD10. Animals in the control group were given CMC alone. Dams were sacrificed at 4 and 24 h after dosing under diethyl ether anesthesia. Embryos were collected, fixed with 10% neutral buffered formalin, and embedded in paraffin. Serial sagittal sections through a plane perpendicular to the anterior-posterior axis of the forelimbs of embryos were cut at a thickness of 3 μm. The sections were stained with hematoxylin-eosin and mesenchymal cells in the M phase, and especially at prophase to anaphase, were counted in forelimb bud. The cells during these phases were identified with chromosomal condensation. The number of mitotic cells was counted in 6 sections per embryo (four embryos per time point and group), with sections separated by 30-60 μm. Cell numbers from each section were corrected according to the dimensions of the forelimb bud measured with image analysis software (Image-Pro Plus 5.0, Media Cybernetics, Inc., Bethesda, MD, USA). For the statistical analysis of the corrected numbers of mitotic cells, the differences between the control and Leflunomide-treated groups were analyzed using the two-sided *t*-test, with $p < 0.05$ designated as the level of statistical significance.

5-3. Results

5-3-1. Gene expression profiles in limb buds of mouse embryos exposed to Leflunomide

Global gene expression changes in the limb buds of mouse embryos treated with Leflunomide were examined using whole-genome oligonucleotide microarrays. Based on the threshold, at 4 and 24 h after treatment with Leflunomide, 20 and 85 up-regulated probe sets and 106 and 33 down-regulated probe sets were selected, respectively. Finally, a total of 244 probe sets

Table 5-2. Altered GO Categories by Leflunomide exposure[#]

GO biological process	GO term	*p* value	No. of genes in category
Down-regulated genes at 4 h after treatment (106 genes)			
GO:0045449	regulation of transcription	4.34×10^{-3}	15
GO:0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	5.17×10^{-3}	15
GO:0006355	regulation of transcription, DNA-dependent	7.81×10^{-3}	14
GO:0006351	transcription, DNA-dependent	8.60×10^{-3}	14
GO:0032774	RNA biosynthetic process	8.76×10^{-3}	14
Up-regulated genes at 24 h after treatment (85 genes)			
GO:0048754	branching morphogenesis of a tube	2.22×10^{-5}	5
GO:0021924	cell proliferation in the external granule layer	3.84×10^{-4}	3
GO:0021930	granule cell precursor proliferation	3.84×10^{-4}	3
GO:0001569	patterning of blood vessels	2.21×10^{-3}	3
GO:0009887	organ morphogenesis	2.64×10^{-3}	7
GO:0022008	neurogenesis	3.05×10^{-3}	6
GO:0014033	neural crest cell differentiation	3.55×10^{-3}	3
GO:0042475	odontogenesis of dentine-containing teeth	4.9×10^{-3}	3
GO:0014031	mesenchymal cell development	5.70×10^{-3}	3
GO:0030902	hindbrain development	5.70×10^{-3}	3
GO:0042476	odontogenesis	6.0×10^{-3}	3
Down-regulated genes at 24 h after treatment (35 genes)			
GO:0016125	sterol metabolic process	1.82×10^{-7}	6
GO:0016126	sterol biosynthetic process	2.13×10^{-7}	5
GO:0008299	isoprenoid biosynthetic process	4.41×10^{-6}	4
GO:0008202	steroid metabolic process	5.90×10^{-6}	6
GO:0008203	cholesterol metabolic process	5.95×10^{-6}	5
GO:0006694	steroid biosynthetic process	7.04×10^{-6}	5
GO:0006720	isoprenoid metabolic process	3.19×10^{-5}	4
GO:0008610	lipid biosynthetic process	9.16×10^{-4}	5

[#]Predominant GO categories of genes that were up- or down regulated at 4 or 24 h after treatment with Leflunomide, which were up- or down-regulated by ≥3- or ≤0.3333-fold. A p-value of <0.01 determined by Fisher's exact test was considered statistically significant. The analysis was performed using the DAVID Functional Annotation tool.

Table 5-3 Genes with altered expression upon treatment with Leflunomide

Affymetrix ID	RefSeq ID	Gene Symbol	Gene Title	Log_2 ratio Leflunomide/control	
				4 h	24 h
Down-regulated genes at 4 h after treatment					
1429026_at	NM_027658	*Hexim2*	Hexamthylene bis-acetamide inducible 2	-2.26±1.43	0.19±0.28
1429205_at	NM_027326	*Mllt3*	Myeloid/lymphoid or mixed lineage-leukemia translocation to 3 homolog (Drosophila)	-2.01±0.20	0.33±0.13
1455298_at	NM_031166	*Id4*	Inhibitor of DNA binding 4	-1.86±0.80	-0.01±0.39
1439847_s_at	NM_010636	*Klf12*	Kruppel-like factor 12	-1.82±0.30	-0.40±0.23
1434398_at	NM_029891	*Nkrf*	NF-kappaB repressing factor	-1.74±0.24	0.47±0.65
Up-regulated genes at 24 h after treatment					
1427300_at	NM_010713	*Lhx8*	LIM homeobox protein 8	-0.26±2.25	2.83±0.29
1449033_at	NM_008764	*Tnfrsf11b*	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	0.28±0.18	2.59±0.13
1417155_at	NM_008709	*Mycn*	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	-0.10±0.03	1.99±0.19
1436869_at	NM_009170	*Shh*	Sonic hedgehog	-1.15±0.24	1.84±0.56
1451924_a_at	NM_010104	*Edn1*	Endothelin 1	-0.80±1.74	1.69±0.08
1419519_at	NM_010512	*Igf1*	Insulin-like growth factor 1	-0.53±0.36	1.65±0.08
Down-regulated genes at 24 h after treatment					
1451122_at	NM_145360	*Idi1*	Isopentenyl-diphosphate delta isomerase	0.99±0.10	-2.60±0.51
1423078_a_at	NM_025436	*Sc4mol*	Sterol-C4-methyl oxidase-like	0.41±0.16	-2.41±0.78
1421821_at	NM_010700	*Ldlr*	Low density lipoprotein receptor	-0.12±0.17	-2.31±1.00
1448130_at	NM_010191	*Fdft1*	Farnesyl diphosphate farnesyl transferase 1	0.04±0.07	-1.67±0.22
1415993_at	NM_009270	*Sqle*	Squalene epoxidase	-0.28±0.05	-1.65±0.39
1433444_at	NM_145942	*Hmgcs1*	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	0.15±0.06	-1.64±0.29
1417303_at	NM_138656	*Mvd*	Mevalonate (diphospho) decarboxylase	0.51±0.21	-1.59±0.19

were identified as Leflunomide-responsive genes (data not shown). GO analysis revealed that several genes involved in various GO biological processes are coordinately induced or reduced in Leflunomide-treated limb buds (Table 5-2). Genes of interest selected based on their role for each GO function are listed in Table 5-3. These genes are involved in the multiple GO functions or are known to be expressed in developing embryos. Integration of the information with DAVID bioinformatics tools indicated down-regulation of genes at 4 h after treatment that are involved in regulation of transcription. These were mainly Hexamethylene bis-acetamide inducible 2 (*Hexim2*); Myeloid/lymphoid or mixed lineage-leukemia translocation 3 to homolog (*Drosophila*) (*Mllt3*, alias of *AF9*); Inhibitor of DNA binding 4 (*Id4*); Kruppel-like factor 12 (*Klf12*, alias of *AP-2rep*), and NF-kappaB repressing factor (*Nkrf*). At 24 h after treatment, up-regulated genes were involved in morphogenesis, cell proliferation, and cell differentiation. Notable genes included LIM homeobox protein 8 (*Lhx8*); Tumor necrosis factor receptor superfamily, member 11b (*Tnfrsf11b*, alias of *Osteoprotegerin*); V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian) (*Mycn*); *Shh*; Endothelin 1 (*Edn1*); and Insulin-like growth factor 1 (*Igf1*). Down-regulated genes at 24 h after treatment were related to sterol/cholesterol biosynthetic and metabolic processes, including Isopentenyl-diphosphate delta isomerase (*Idi1*); Sterol-C4-methyl oxidase-like (*Sc4mol*); Low density lipoprotein receptor (*Ldlr*); Farnesyl diphosphate farnesyl transferase 1 (*Fdft1*); Squalene epoxidase (*Sqle*); 3-hydroxy-3-methylglutaryl-Coenzeyme A synthase 1 (*Hmgcs1*), and Mevalonate (diphospho) decarboxylase (*Mvd*). No overrepresented gene categories considered to be statistically significant were detected from up-regulated genes at 4 h after treatment.

5-3-2. Validation of selected genes via real-time RT-PCR

The microarray data was validated using real-time RT-PCR for a portion of genes listed in

Figure 5-1.

Validation using Real-time RT-PCR assays for a subset of specifically down-regulated genes at 4 and 24 h and up-regulated genes at 24 h after treatment with Leflunomide in limb buds.

The data shown are relative quantification for each common gene compared with the GAPDH internal control gene in the limb buds of mouse embryos exposed to Leflunomide. Data are expressed as means ± SD (n = 3). a): genes down-regulated at 4 h after treatment, b): genes down-regulated at 24 h after treatment, c): genes up-regulated at 24 h after treatment.

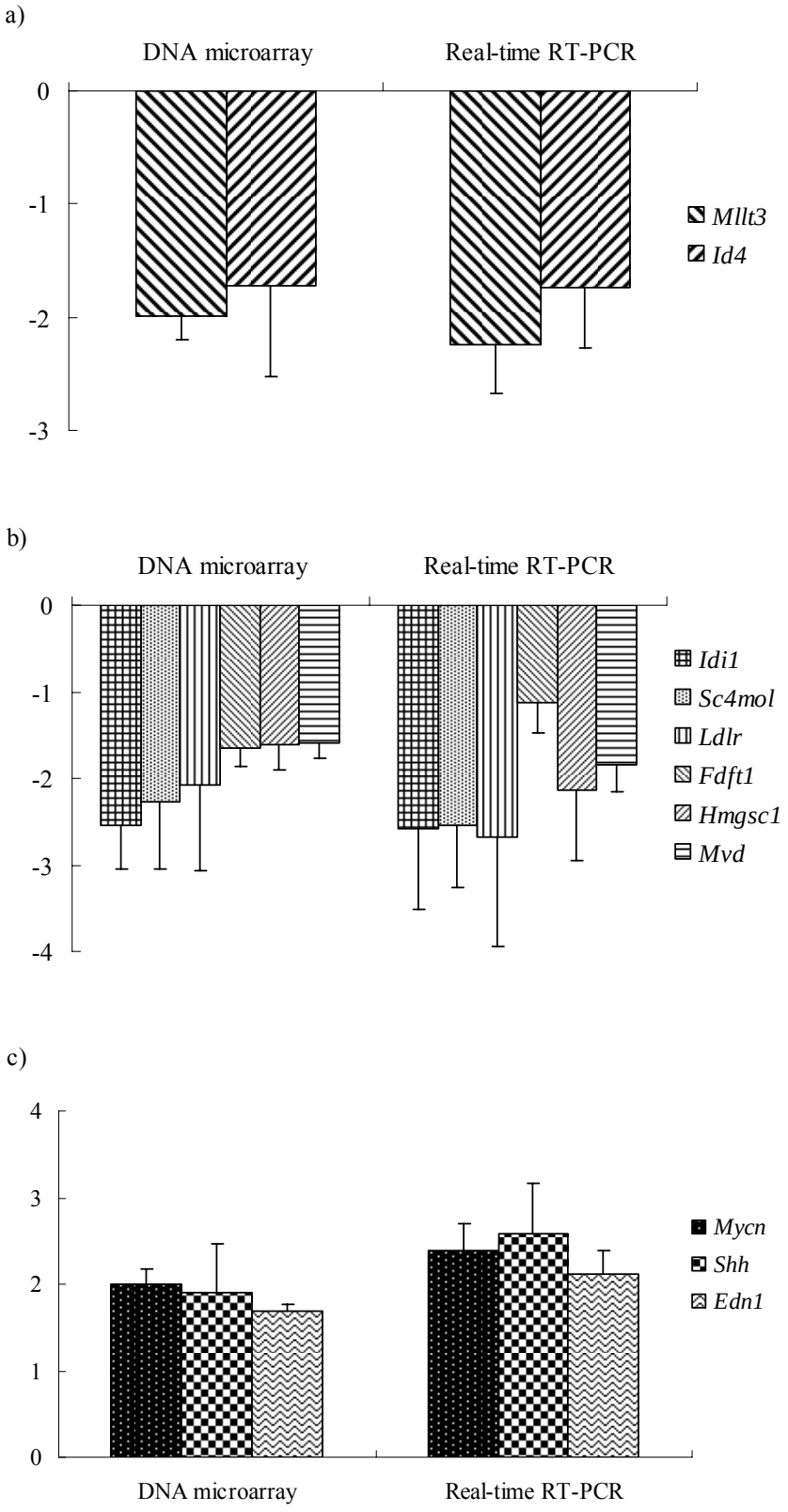

a)
DNA microarray
Real-time RT-PCR
0
-1
-2
-3
Mllt3
Id4
b)
DNA microarray
Real-time RT-PCR
0
-1
-2
-3
-4
Idi1
Sc4mol
Ldlr
Fdft1
Hmgsc1
Mvd
c)
4
3
2
1
0
Mycn
Shh
Edn1
DNA microarray
Real-time RT-PCR

Table 5-3. The genes validated with the Taqman® assay are presented in Table 5-1. The Taqman® assay yielded results that showed quantitative agreement with the microarray results (Fig. 5-1).

5-3-3. The concentration of cholesterol from limb buds of embryos

Since the gene expression profile at 24 h after treatment suggested a decrease in the biosynthesis of cholesterol in limb buds of mouse embryos, cholesterol was quantitated in forelimb bud tissue of mouse embryos at 4, 24, or 48 h after treatment with Leflunomide on GD10. However, while cholesterol tended to decrease slightly at 24 h after treatment with Leflunomide, the concentrations of cholesterol in limb buds did not differ significantly between the control and Leflunomide-treated groups at any time point as shown in Fig. 5-2.

5-3-4. Mitotic figure index in mesenchyme of forelimb buds

To test whether there was a reduction in the number of mitotic cells in forelimb buds of mouse embryos exposed to Leflunomide on GD10, we counted the number of cells at prophase to anaphase representing chromosomal condensation in forelimb buds. The means of mitotic cell numbers per 1 mm^2 of forelimb bud area were 7.59 ± 1.88 and 6.90 ± 2.08 at 4 and 24 h after treatment in the control group, respectively. They significantly dropped into 2.09 ± 0.80 and 1.11 ± 0.26 in the Leflunomide-treated group, respectively (Fig. 5-3). This result indicated that Leflunomide administered on GD10 prevented mesenchymal cells from mitosis in forelimb buds at 4 and 24 h after treatment with Leflunomide.

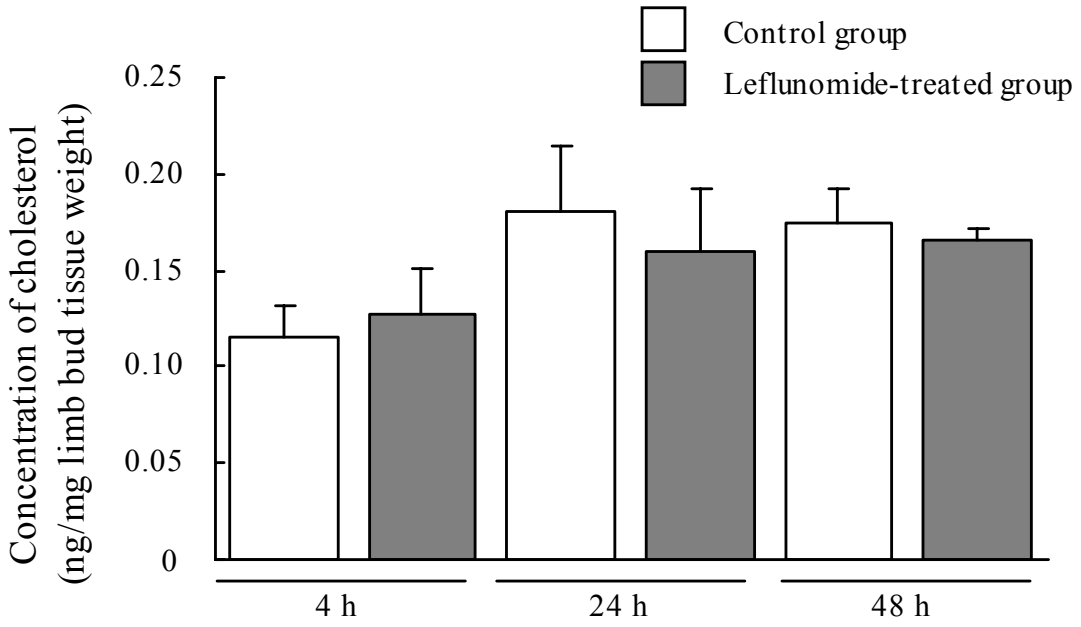


Figure 5-2.

Cholesterol levels in mouse limb bud tissue after Leflunomide exposure.

Limb bud tissue samples were collected 4, 24, or 48 h after Leflunomide or vehicle administration. The data shown are the concentrations of cholesterol as means ± SD (n = 3).

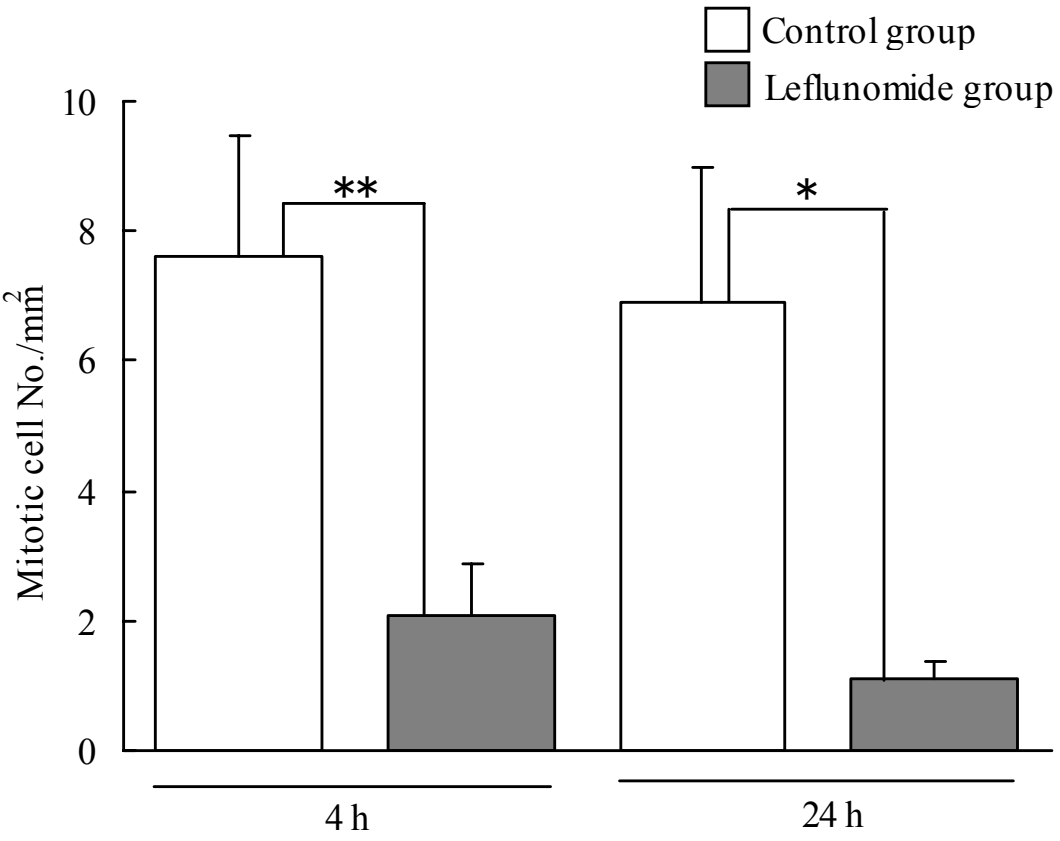


Figure 5-3.

Quantification of mitotic mesenchymal cells in forelimb buds of mouse embryos exposed to Leflunomide.

Incidence of proliferating mesenchymal cells in the forelimb buds of embryos at 4 and 24 h after treatment with Leflunomide or vehicle on GD10. The data shown are the means ± SD of the number of mitotic cells representing chromatin condensation corrected with the dimensions of the forelimb bud (n = 4). *, ** significantly different between Leflunomide- and vehicle-exposed embryos in the forelimb bud (p <0.05 and 0.01, respectively).

5-4. Discussion

Leflunomide is teratogenic in rats, rabbits (Sanofi-Aventis, 2003), and mice. This compound inhibits DHODH and tyrosine kinase activities. The potency of A77 1726 as a DHODH inhibitor is 100- to 1000-fold greater than that reported for its inhibition of protein tyrosine kinases (Xu *et al*, 1995, Davis *et al*, 1996). Described in CHAPTER 4, it was demonstrated previously that DHODH inhibition relates to the teratogenicity of Leflunomide in mice. But at the same time, the additional involvement of other mechanisms, *i.e.*, inhibition of protein tyrosine kinase activity, could not be ruled out because of the incomplete prevention of limb malformations after co-administration of uridine. In the current study, the teratogenic mechanisms were investigated using oligonucleotide-based DNA microarrays to which more than 40,000 genes are attached, and two additional experiments.

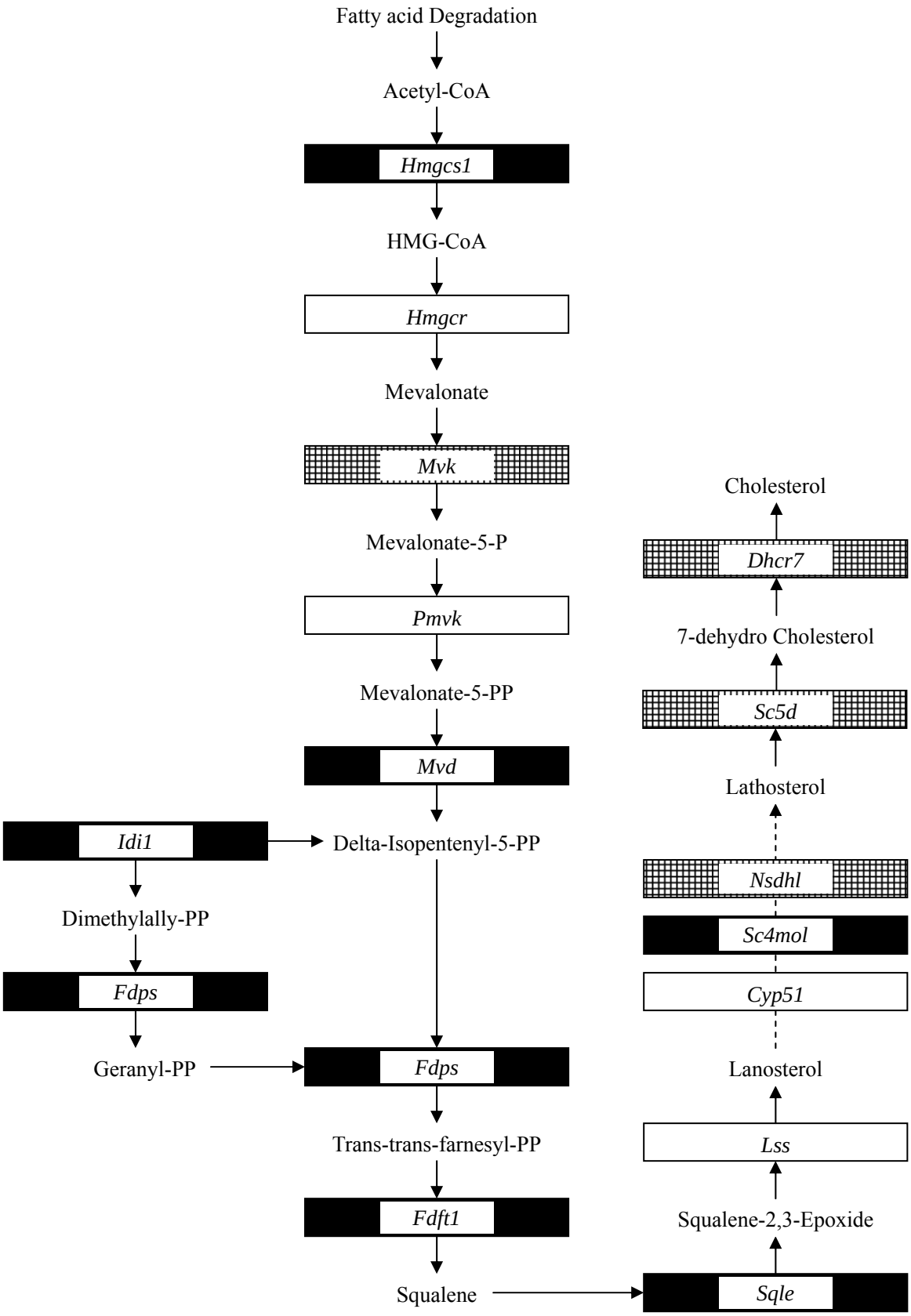
Microarray analysis identified a large number of differentially expressed genes in the limb buds of mouse embryos following Leflunomide treatment. GO analysis using the DAVID Functional Annotation tool revealed that the genes which were changed their expression involves cholesterol biosynthesis, regulation of transcription, morphogenesis, and cell proliferation and differentiation. As of this report, it remains unclear what the relationship is between the up-regulated genes classified as functioning in morphogenesis and cell differentiation, and limb malformations caused by Leflunomide. Thus, it was discussed about cholesterol biosynthesis, regulation of transcription, and cell proliferation.

There are no reports demonstrating Leflunomide effects on cholesterol biosynthesis directly or indirectly. But gene expression analysis revealed that Leflunomide caused down-regulation of gene expression of enzymes in the cholesterol biosynthesis pathway. In addition to seven genes

Figure 5-4.

Schematic presentation of altered expression of genes involving cholesterol biosynthesis.

This scheme represents the down-regulated genes in the cholesterol biosynthesis pathway at 24 h after treatment with Leflunomide. The data source of this pathway is GenMAPP2.0 (Gene Map Annotator and pathway profiler), which is a free, open-source bioinformatics software tool (Gladstone Institutes, University of California, CA, USA). Filled rectangles: seven genes down-regulated to below one-third; meshed rectangles: four genes decreased to below 0.5-fold compared with the control group; opened rectangles: genes not changed.


Fatty acid Degradation
Acetyl-CoA
Hmgcs1
HMG-CoA
Hmgcr
Mevalonate
Mvk
Mevalonate-5-P
Pmvk
Mevalonate-5-PP
Mvd
Idi1
Delta-Isopentenyl-5-PP
Dimethylally-PP
Fdps
Geranyl-PP
Fdps
Trans-trans-farnesyl-PP
Fdft1
Squalene
Sqle
Squalene-2,3-Epoxide
Lss
Lanosterol
Cyp51
Sc4mol
Nsdhl
Lathosterol
Sc5d
7-dehydro Cholesterol
Dhcr7
Cholesterol

down-regulated to below one-third (Table 5-3), 4 genes decreased to below 0.5-fold in the pathway of cholesterol biosynthesis (Fig. 5-4). These 4 genes are Mevalonate kinase (*Mvk*); NAD(P) dependent steroid dehydrogenase-like (*Nsdhl*); Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (*S. cerevisae*) (*Sc5d*), and 7-dehydrocholesterol reductase (*Dhcr7*) (the expression data for these genes are not shown). Expression of 11 of the 15 genes involved in the cholesterol/sterol biosynthesis pathway markedly decreased. Furthermore, Sterol regulatory element binding factor 2 (*Srebf2*), which codes a transcription factor that induces the expression of genes in the cholesterol synthetic pathway and contributes to efficient cholesterol synthesis (Amemiya-Kudo *et al*, 2002, Sakakura *et al*, 2001), decreased to below 0.5-fold. These microarray data strongly suggested a decrease in the biosynthesis of cholesterol in limb buds of mouse embryos treated with Leflunomide. A decrease in the cholesterol level in limb bud tissue and/or maternal blood induces patterning defects of limbs (Barbu *et al*, 1988, Gofflot *et al*, 2003). Cholesterol may regulate the range and shape of the Shh morphogen gradient across the limb bud during early development, and Shh produced in the zone of polarizing activity is the major determinant of anteroposterior development of the limb (Li *et al*, 2006). If the biosynthesis of cholesterol is reduced in limb buds following a change of gene expression, this suggests that the down-regulation of genes involved in cholesterol biosynthesis may relate to the teratogenicity of Leflunomide. But, no differences in the concentrations of cholesterol in the limb buds were seen between the control and Leflunomide-treated groups at 4, 24, or 48 h after treatment, it is inconsistence with gene expression change. AY 9944, a cholesterol biosynthesis inhibitor, causes fetal malformations, but exogenous cholesterol prevents its teratogenicity (Barbu *et al*, 1988). And RSH/SLOS mice, which have a mutation in 7-dehydrocholesterol reductase, show no limb malformations (Wassif *et al*, 2001). These reports indicate that maternal cholesterol can cross the placenta and is distributed into the developing limb bud when cholesterol biosynthesis is inhibited in embryos. Based on these reports,

this contrariety between the concentration of cholesterol in the limb buds and the gene expression profile may also be due to placental transfer of maternal cholesterol during critical periods of development. We conclude, therefore, that even though gene expression of cholesterol biosynthesis was down-regulated, it does not relate to the limb malformations caused by Leflunomide.

We next focused on the gene set down-regulated at 4 h after treatment which was classified into regulation of transcription (Table 5-3). *Mllt3* (alias of *AF9*) is required for controlling pattern formation in mice, and is highly expressed in the developing skeletal system of limb buds, prevertebrae, rib anlagen, and developing jaw, nose, and skull (Collins et at, 2002). *Klf12* (alias of *AP-2rep*) mediates positive and negative regulation of *AP-2* expression which is associated with embryonic differentiation of neuroectodermal, urogenital, and ectodermal tissues (Imhof *et al*, 1999). The *Id* gene family, including *Id4*, is implicated in the control of differentiation and cell cycle progression. The mechanism by which Id proteins exert this control is to antagonize the activities of basic helix-loop-helix proteins which are involved in many developmental processes (Bedford *et al*, 2005, Jen *et al*, 1997). The role of *Hexim2* in embryonic development, however, is unclear. This gene controls global transcription as well as cell growth and differentiation, which inhibits general and disease-specific transcriptional elongation by phosphorylation of RNA polymerase II (Li *et al*, 2005). The down-regulation of these transcription regulating factors in the limb buds of embryos suggests that they may contribute to limb malformations caused by Leflunomide. Leflunomide has been reported to inhibit the tyrosine phosphorylation of various receptors, including receptors of platelet-derived growth factor, fibroblast growth factor, and epidermal growth factor (Mattar *et al*, 1993, Si *et al*, 2008, Steeghs *et al*, 2007, Xu *et al*, 1999), and their subsequent pathways (Siemasko *et al*, 1998, Si *et al*, 2008) in cultured cells or in mice. As described above, not only DHODH inhibition, but also protein tyrosine kinase inhibition may relate to the teratogenicity of Leflunomide. In the current study, however, we did not examine tyrosine

phosphorylation in limb buds exposed to Leflunomide. It is speculated that the altered gene expression of regulators of transcription may relate with the inhibition of tyrosine phosphorylation of these growth factors' receptors in embryonic limb buds, which may be related to Leflunomide's teratogenicity in mice.

The genes up-regulated at 24 h after treatment with Leflunomide are concerned with morphogenesis, cell proliferation, and differentiation. *Lhx8* plays critical roles in the control of pattern formation and cell type specification, including lip, palate, and tooth (Inoue *et al*, 2006, Shibaguchi *et al*, 2003, Zhao *et al*, 1999). Inoue *et al* suggested that *Lhx8* contributes to epithelial mesenchymal interactions in facial morphogenesis (Inoue *et al*, 2006). *Tnfrsf11b* (alias of *Osteoprotegerin*) works cooperatively with receptor activator of nuclear factor-κB ligand (*RANKL*), and the *Tnfrsf11b/RANKL* pathway regulates the activation/inactivation and proliferation of osteoblasts and osteoclasts (Kostenuik, 2005, Lacey *et al*, 1998). Ota *et al* reported that *Mycn* is required for multiple aspects of limb development. The multiple functions of *Mycn* include regulating the size of precartilaginous condensations in the limb bud at early development, and are needed for digit individualization and joint formation at later stages of limb formation (Ota *et al*, 2007). *Edn1* and *Igf1* also have functions relating to cell proliferation and anti-apoptotic activity in embryonic cells (Abe *et al*, 2007, Velazquez *et al*, 2009). *Shh* is one of the most important genes for limb bud development (Robert and Lallemand, 2006). Since these genes have various GO biological processes, the functions of the genes up-regulated at 24 h are highly redundant with respect to each other (Table 5-2). In the current study, concerning cell proliferation, we examined the mitotic figure index in the mesenchyme of forelimb buds. However, while the genes up-regulated at 24 h after treatment function to activate cell proliferation, a significant decrease in mitotic mesenchymal cells in the limb bud tissues in the Leflunomide-treated group was observed at both 4 and 24 h after treatment. The mitotic cell number decreased in the compound dosing group,

dropping to 27.5 and 16.1% compared with the control group at 4 and 24 h, respectively. Although the difference between the gene expression analysis and mitotic figure index in the mesenchyme is unclear at present, we suggest that the altered gene expression at 24 h may be a compensatory change from the severe inhibitory effects of mitosis in mesenchymal cells in the limb bud.

5-5. Conclusion

Based on the down-regulated gene expression of regulators of transcription, it is speculated that Leflunomide may cause the inhibition of tyrosine phosphorylation in the limb buds of mouse embryos, and that this may relate to the inhibition of mesenchymal cell proliferation. Bredinin (Mizoribin), an inhibitor of purine nucleotide *de novo* synthesis, prevents the proliferation of mouse embryonic cells in *in vitro* experiments, indicating that the inhibition of purine nucleotide biosynthesis may induce cytotoxicity (Kawai *et al*, 1993, Okamoto *et al*, 1980). It was demonstrated that the inhibition of pyrimidine nucleotides relates to limb malformation (CHAPTER 4). These reports suggest that Leflunomide may also prevent the proliferation of mesenchymal cells in limb buds via the inhibition of DHODH. In conclusion, the evidence suggests that both DHODH inhibition and tyrosine kinase inhibition of Leflunomide may interfere with the normal proliferation of mesenchymal cells in limb buds of mouse embryos, and that the damaged mesenchyme may develop into limb malformations.

CHAPTER 6

SUMMARY

6-1. Introduction

Immunosuppressant Leflunomide, which has inhibitory effects on DHODH and protein tyrosine kinases, causes the teratogenicity in rats and rabbits. This compound is assigned an FDA pregnancy category X based on its pharmacological mechanisms of action in human. Based on comparing between these mechanisms, it is suspected that inhibition of DHODH may be involved in the teratogenicity of Leflunomide in animals. But until now, little information is available for suggestion of the mechanism of its teratogenicity. In the course of the work on this dissertation, I examined the teratogenic profile in mice circumstantially and conducted the mechanism study using the biochemical and global gene expression analysis techniques.

6-2. The effects of Leflunomide on embryo/fetal development in mice administered during organogenesis

As the first experiments to examine the teratogenicity of Leflunomide in mice, pregnant mice were treated orally with Leflunomide at a dose of 10, 30 or 70 mg/kg/day during their organogenesis (GD6 to 15), and embryo toxicity and teratogenicity were assessed in detail, and

system exposure of A77 1726 in maternal blood was estimated when dosed at 30 mg/kg/day. At 70 mg/kg, all embryos were resorbed. At 30 mg/kg, Leflunomide reduced fetal viability, intrauterine growth, and increased the incidence of multiple external, skeletal and visceral malformations. Characteristic external malformations were neural tube defects, cleft palate and tail deformities. Limb malformations were observed in a small number of fetuses. Skeletal examinations revealed malformations of cervical to sacral vertebrae, ribs and sternebrae. In the viscerae, the main anomalies were membranous ventricular septum defect and persistent truncus arteriosus. In conclusion, the results of this study indicate that Leflunomide administered at 30 mg/kg during organogenesis can induce embryo lethality, intrauterine growth arrest and severe teratogenicity, including craniofacial malformations and deformities of axial skeleton, heart and great vessels in mice.

6-3. The study of the critical periods for the teratogenicity of Leflunomide in mice

The second study was conducted to determine the critical periods for teratogenicity in mice in attempt to gain details about the teratogenicity of Leflunomide seen in CHAPTER 1. A single dose 50 mg/kg Leflunomide was administered to pregnant mice on one of GD6 to 11. Characteristic external malformations were craniofacial defects following dosing on GD7, cleft palate on GD9, cleft palate and limb and tail deformities on GD10, and limb deformities on GD11. Skeletal examination revealed cervical to caudal vertebral malformations after treatment on GD7, GD8, GD9 or GD10. In the viscera, cardiovascular deformities were observed in the GD7 and GD9 Leflunomide treated groups. These data suggest that Leflunomide may inhibit broadly *de*

novo synthesis of pyrimidine nucleotide and/or tyrosine phosphorylation on various organs at fetal organogenesis. In conclusion, it is demonstrated that multiple malformations were seen in various organs and the most malformations observed in this study appeared to be a developmental stage-specific response to Leflunomide treatment.

6-4. The evaluation of relationship between DHODH inhibition and teratogenicity of Leflunomide in mice

In this study, pregnant mice were co-administered Leflunomide and uridine, a precursor substance of pyrimidine nucleotides, and it was examined whether or not a decreased level of intracellular pyrimidine nucleotides with inhibition of DHODH is related to the teratogenicity of Leflunomide. Then we examined the alteration of the nucleotide level in fetal tissue by Leflunomide and the effect of co-administered uridine. We administered 70 mg/kg Leflunomide with or without uridine to pregnant mice on GD10, and used the vehicle of Leflunomide as a control. Leflunomide caused multiple malformations in all fetuses, but co-administration with uridine inhibited most of its teratogenicity except for digit malformations. Leflunomide decreased the concentration of pyrimidine nucleotides, not purine nucleotides, while uridine co-administered with Leflunomide partially restored the level of pyrimidine nucleotides. These results indicate that the inhibitory effect of DHODH activity is related to the teratogenicity of Leflunomide and they also suggest the possibility that the additional involvement of other mechanisms (*e.g.*, inhibition of protein tyrosine kinases) may contribute to the teratogenicity in mice.

6-5. The teratogenic mechanism study using the gene expression analysis in limb bud of mouse embryos exposed to Leflunomide

Described above, we demonstrated that DHODH inhibition relates to the teratogenicity of Leflunomide, but the results also suggested the involvement of other mechanisms. In the current study, we attempted to elucidate teratogenic mechanisms other than DHODH inhibition in the limb buds of mouse embryos exposed to Leflunomide. Pregnant mice received a single administration of 70 mg/kg Leflunomide or vehicle alone on GD10 and were sacrificed 4 or 24 h later. DNA microarray analysis of embryonic limb buds implied that the inhibition of tyrosine kinases may contribute to teratogenicity in the limb. Although genes involving in cholesterol biosynthesis were down-regulated by Leflunomide treatment, this change suggests not to be related with teratogenicity, since Leflunomide did not effect on the concentrations of cholesterol in limb bud. Additionally, histoplanimeterical study showed that Leflunomide inhibited the proliferation of mesenchymal cells of limb buds. These data suggest that inhibition of DHODH and tyrosine kinase may be responsible for the teratogenicity in limbs of mice caused by Leflunomide treatment *via* inhibition of mesenchymal cell proliferation in limb buds.

6-6. Conclusion

The studies presented here revealed that Leflunomide is teratogenic in mice, and it interferes the normal development on entire body of mouse embryo, not in specific organs. And uridine coadministration experiments demonstrated that DHODH inhibition mainly contributes to its teratogenicity. The global gene expression analysis in limb bud of mouse embryos treated with

Leflunomide implied that protein tyrosine kinase inhibition, the other pharmacological action of this compound may also relate with the teratogenicity and both DHODH inhibition and protein tyrosine kinase inhibition may induce the abnormal morphogenesis of limb *via* inhibition of mesenchymal cell proliferation in limb bud. Although the teratogenic mechanisms of Leflunomide is not completely uncovered, the present studies suggest that compounds which have the effects of DHODH inhibition and/or protein tyrosine kinase inhibition can cause the teratogenicity.

Since data present in this dissertation is concerned about only Leflunomide, these can not broadly accommodate various compounds. However, an abundance data obtained from investigations of teratogenic mechanisms in various compounds may develop in the valuable data base in future. I expect that the accomplishment from studies presented here contributes to produce the safer medicines more effectively.

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