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An Evaluation of the Human Relevance of S-1563-induced Liver Tumours in Rats Based on Mode of Action

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I. Summary

S-1563 is a newly synthesized pyrethroid insecticide produced by Sumitomo Chemical Co., Ltd. (Tokyo, Japan), and it is expected to be a high performance household insecticide. Short term tests for genotoxic potential have demonstrated that S-1563 is not a genotoxic agent. However, S-1563 was shown to increase the incidence of liver tumours in the rat. We have identified a mode of action (MOA) by which S-1563 produces liver tumours in the rat and have assessed the relevance of the rat liver tumours for human cancer risk. This was developed by employing the International Programme on Chemical Safety (IPCS) framework for establishing a MOA by which a chemical can produce tumours in rodents and then assessing the relevance of the animal MOA for human risk.

Based on the findings of a number of *in vivo* rat studies and *in vitro* rat hepatocyte studies, the proposed MOA for S-1563-induced rat liver tumour formation involves activation of the constitutive androstane receptor (CAR), which results in a pleiotropic response including the induction of cytochrome P450 (CYP) CYP2B subfamily enzymes and stimulation of liver hypertrophy and cell proliferation (i.e., a mitogenic effect). Prolonged treatment results in the formation of altered hepatic foci and liver tumours. While CAR activation, increased cell proliferation and altered hepatic foci comprise key events in the MOA, CYP2B induction and hypertrophy comprise associative events in that they are markers for CAR activation. This MOA is similar to that of certain non-genotoxic agents which are known as CAR activators.

To assess the relevance of the proposed MOA for rat liver tumour formation for humans, experiments were performed with cultured rat and human hepatocytes. Treatment with S-1563 increased CYP2B1/2 mRNA levels in cultured Wistar rat hepatocytes and CYP2B6 mRNA levels in cultured human hepatocytes. However, while S-1563 produced a stimulation of replicative DNA synthesis in rat hepatocytes, no stimulation was observed in human hepatocytes at any concentration tested. The functional viability of the human hepatocyte preparations to a mitogenic agent was confirmed in human hepatocytes treated with hepatocyte growth factor (a known mitogenic agent) where a significant stimulation of replicative DNA synthesis was observed. These results demonstrate that while some of the key and associative events (including CAR activation and CYP enzyme induction) in the proposed MOA for S-1563-induced rat liver tumour formation could occur in human liver, human hepatocytes are refractory to the mitogenic effects of S-1563. Hence, it is therefore concluded that the proposed MOA for S-1563-induced rat liver tumour formation is not qualitatively plausible for humans. Our conclusion from this assessment with the CAR

activator S-1563 is in agreement with literature data on other CAR activators (especially, a close structural analogue, metofluthrin). Moreover, the MOA for rodent liver tumour formation by CAR activators has many similarities to the MOA for rodent liver tumour formation by peroxisome proliferator-activated receptor alpha (PPARα) activators. The MOA for liver tumour production by PPARα activators in rodent liver is considered not relevant for humans as agreed by the European Chemicals Agency (ECHA) in "Guidance on the Application of the CLP Criteria, Guidance to Regulation (EC) No 1272/2008 on Classification, Labelling and Packaging (CLP) of Substances and Mixtures, Version 2.0" (2012).

II. Introduction

S-1563 is a newly synthesized pyrethroid insecticide produced by Sumitomo Chemical Co., Ltd. (Tokyo, Japan), and it is expected to be a high performance household insecticide. Short term tests for genotoxic potential have demonstrated that S-1563 is not a genotoxic agent. However, S-1563 was shown to increase the incidence of liver tumours in the rat (see below). The purpose of this document is to identify a mode of action (MOA) by which S-1563 produces liver tumours in the rat and to assess the relevance of the rat liver tumours for human cancer risk.

In recent years, a framework for analysing the modes of action (MOAs) by which chemicals produce tumours in rodents and relevance of such tumour data for human risk has been developed through the International Life Sciences Institute (ILSI) (Cohen *et al*., 2004; Meek *et al.,* 2003) and the International Programme on Chemical Safety (IPCS) (Boobis *et al.,* 2006). In order to identify a MOA for S-1563-induced rat liver tumour formation, a number of investigative studies have been performed (Okuda, 2012a-g, 2013; Yamada, 2012a-c). The data obtained from these investigative studies have been evaluated according to the 2006 IPCS framework for analysing the relevance of a cancer MOA observed in experimental animals for humans (Boobis *et al*., 2006). Key and associative events for the postulated MOA have been identified using a weight of evidence approach based on the modified Bradford Hill criteria and the human relevance of the animal MOA has then been evaluated.

III. Carcinogenicity

The carcinogenicity of S-1563 has been studied in male and female rats and mice in standard bioassays under the guidelines of Good Laboratory Practice and to test protocols of the US.EPA (US. Environmental Protection Agency), OECD (Organization for Economic Cooperation and Development), and JMHLW (Japan Ministry of Health, Labour and Welfare).

Male and female HanBrl:WIST rats were fed 0 (control), 200, 500, 1500 or 3000 ppm S-1563 in the diet for two years (average chemical intakes: 9.5, 23, 73 and 154 mg/kg/day for males; 11.1, 28, 88 and 182 mg/kg/day for females, respectively) (Rached, 2012a).

A summary of the results of this bioassay is presented in Table 1. The incidences of the total number of animals with hepatocellular adenomas and/or carcinomas of the 0, 200, 500, 1500 and 3000 ppm groups were 2, 0, 4, 12and 33% for males, and 0, 0, 2, 2 and 10% for females, respectively. Treatment with 3000 ppm S-1563 significantly increased the incidence of hepatocellular adenoma in both sexes and of hepatocellular

carcinoma in male rats. The combined incidence of hepatocellular adenoma and carcinoma was significantly increased in male and female rats given 3000 ppm S-1563, with a non statistically significant increase being observed in male rats given 1500 ppm S-1563. The historical background incidences for liver tumours in the laboratory conducting this bioassay were 0-8.0% and 0-10.2% for hepatocellular adenoma in males and females, respectively; 0-2.8% and 0-2.0% for hepatocellular carcinoma in males and females, respectively; and 0-10.0% and 0-12.0% for combined adenoma and carcinoma in males and females, respectively (Annex I). Therefore, the incidences of hepatocellular adenoma, carcinoma, and combined in males given 1500 and 3000 ppm S-1563 were equivalent to or higher than the maximum incidence of the historical background; and the combined incidence of female rats given 3000 ppm S-1563 was within the historical background incidence, while incidence of carcinoma was equivalent to the maximum incidence of the historical background. Overall, treatment with S-1563 in rats for 2 years produced hepatocellular tumours in males at 1500 and 3000 ppm (73 and 154 mg/kg/day) and in females at 3000 ppm (182 mg/kg/day). The no tumourigenic dose levels (no observed effect levels (NOEL) for tumours) in male and female rats were established at 500 ppm (23 mg/kg/day) and 1500 ppm (88 mg/kg/day), respectively. The treatment of male and female rats with S-1563 for two years did not produce tumours in any other tissue.

Table 1. Summary of 2-year bioassay in rat with S-1563

	Males					Females				
Dose levels (ppm)	**0**	**200**	**500**	**1500**	**3000**	**0**	**200**	**500**	**1500**	**3000**
No. of animals examined	**51**	**51**	**51**	**51**	**51**	**51**	**51**	**51**	**51**	**51**
Number of Survivors	**37**	**40**	**38**	**40**	**40**	**37**	**28**	**32**	**34**	**37**
Survival rate (%)	**(73)**	**(78)**	**(75)**	**(78)**	**(78)**	**(73)**	**(55)**	**(63)**	**(67)**	**(73)**
Body weight change (% of control)	**-**	**-1**	**-6**	**-11***	**-21****	**-**	**5**	**-5**	**-13 ****	**-19****
Body weight gain change (% of control)	**-**	**1**	**-6**	**-12****	**-24****	**-**	**11**	**-3**	**-16****	**-22****
Hepatocellular adenoma	**1/51**	**0/51**	**2/51**	**4/51**	**8/51****	**0/51**	**0/51**	**1/51**	**1/51**	**4/51***
(%)	**2**	**0**	**4**	**8**	**16**	**0**	**0**	**2**	**2**	**8**
Hepatocellular carcinoma	**0/51**	**0/51**	**0/51**	**4/51**	**9/51****	**0/51**	**0/51**	**0/51**	**0/51**	**1/51**
(%)	**0**	**0**	**0**	**8**	**18**	**0**	**0**	**0**	**0**	**2**
Combined adenoma & carcinoma	**1/51**	**0/51**	**2/51**	**6/51**	**17/51****	**0/51**	**0/51**	**1/51**	**1/51**	**5/51***
(%)	**2**	**0**	**4**	**12**	**33**	**0**	**0**	**2**	**2**	**10**

Note: Statistically significant; *p<0.05, **p<0.01. Highlight represents alteration with toxicological significance.
Historical background incidence from 20 studies conducted Harlan Laboratory: the data sets were updated as shown in Annex I.
Hepatocellular adenoma; for male; average 2.54%; range 0.0-8.0%; for female; average 2.80%; range, 0.0-10.2%
Hepatocellular carcinoma; for male; average 0.47%; range, 0.0-2.80%; for female; average 0.32%; range, 0.0-2.0%
Combined hepatocellular adenoma & carcinoma; for male; average 3.01%; range, 0.0-10.0%; for female; average 3.12%; range, 0.0-12.0%.

Body weight gain was reduced by 12 and 16% in male and female rats, respectively, at the 1500 ppm dose level and by 24 and 22% in male and female rats, respectively, at the 3000 ppm dose level. The reduction in body weight gain in this study demonstrates that the maximum tolerated dose (MTD) was achieved and hence this study is adequate for assessing the carcinogenicity of S-1563 in the rat

In contrast, S-1563 was not carcinogenic in the liver or any other tissue in male and female CD-1 mice when administered for 78 weeks at dietary levels of 600, 2500 and 5500 ppm (average chemical intakes: 72, 308 and 639 mg/kg/day for males; and 99, 427 and 853 mg/kg/day for females, respectively) (Rached, 2012b).

S-1563 was not genotoxic in a battery of *in vitro* and *in vivo* assays.

IV. Postulated Mode of Action for Induction of Liver Tumours in Rats

Metofluthrin, a close structural analogue to S-1563, also increased hepatocellular tumours in rats. The mode of action (MOA) for tumour formation by metofluthrin in rats is known to be similar to hepatic CYP2B enzyme inducers which are constitutive androstane receptor (CAR) activators (Deguchi *et al.,* 2009; Hirose *et al.,* 2009;

Yamada *et al.*, 2009). Therefore, the MOA for S-1563-induced rat liver tumour formation is postulated to involve activation of CAR, which results in a pleiotropic response including the stimulation of the cytochrome P450 (CYP) CYP2B subfamily enzymes, liver hypertrophy and increased cell proliferation. Prolonged treatment results in the formation of altered hepatic foci and liver tumours. This MOA is similar to that of certain other non-genotoxic agents which are CAR activators (Carmichael *et al.,* 2011; Holsapple *et al*., 2006; Osimitz and Lake, 2009).

Key Events

The key and associative events in the postulated MOA for S-1563-induced rat liver tumour formation are shown in Table 2 and Fig. 4.

Table 2. Key and associative events in the S-1563 mode of action of rat liver tumour formation

KEY EVENTS	ASSOCIATIVE EVENTS
Definition: an empirically observable causal precursor step to the adverse outcome that is itself a necessary element of the MOA.	*Definition: a biological process that is not a causal necessary key event for the MOA, but is a reliable indicator or marker for a key event.*
▪ Activation of CAR	▪ Induction of hepatic CYP2B enzymes
▪ Increased cell proliferation	▪ Liver hypertrophy
▪ Development of altered hepatic foci	

Activation of CAR, increased cell proliferation and the development of altered hepatic foci are considered to be key events in the MOA for tumour formation as they constitute necessary steps in the MOA. The induction of CYP2B enzymes and liver hypertrophy (both morphological changes and increases in liver weight) may be considered associative events and as such represent reliable markers of CAR activation.

A number of investigative studies were performed to obtain data on the proposed key and associative events (Okuda, 2012a-g, 2013; Yamada, 2012a-c). These various studies are described below and are summarised in (Annex II) for male rats, in (Annex III) for female rats, in (Annex IV) for male mice, and in (Annex V) for female mice. In one MOA study (Okuda, 2012d), rats were also given sodium phenobarbital (PB), which is known to activate CAR in the rat and to produce a variety of hepatic effects including CYP2B enzyme induction and increased cell proliferation (IARC, 2001; Lake, 2009; Ueda *et al*., 2002; Whysner *et al*., 1996; Yoshinari *et al*., 2001). PB served as a positive control to confirm the potential responsiveness of the animals used in these

MOA studies to a known CAR activator (Okuda, 2012d). PB was also employed for *in vitro* studies with rat and human hepatocytes as a positive control to confirm the potential responsiveness of hepatocytes used in these MOA studies to a known CAR activator (Okuda, 2012f, 2013).

A. Activation of the nuclear constitutive androstane receptor (CAR)

CYP2B enzyme induction in rodent liver involves activation of nuclear receptors, particularly CAR (Deguchi *et al.*, 2009; Ueda *et al*., 2002; Wei *et al*., 2000; Yamamoto *et al*., 2004; Yoshinari *et al*., 2001). To probe the role of CAR in the MOA for S-1563-induced rat liver tumour formation, the RNA interference (RNAi) technique was employed. The RNAi technique enables sequence-specific gene silencing using short-interfering RNA (siRNA) (Caplen *et al*., 2001; Elbashir *et al*., 2001; Fire *et al*., 1998). Use was made of CAR-siRNA to reduce CAR mRNA levels in order to examine the effect of S-1563 on CYP2B1/2 mRNA induction in rat hepatocytes (Okuda, 2012f), employing a similar experimental design to that previously used in the metofluthrin study (Deguchi *et al.*, 2009). Z-CMCA, one of the major metabolites of S-1563 in the rat (Mikata, 2011), was also examined. PB was employed as a positive control for these studies. The treatment of rat hepatocytes with CAR-siRNA significantly reduced CAR mRNA in the presence of either PB (Figs. 1A and 2A), S-1563 (Fig. 1C) or Z-CMCA (Fig. 2C), resulting in a significant reduction in the magnitude of induction of CYP2B1/2 mRNA levels by each compound (Figs. 1B and 1D, Figs. 2B and 2D). These findings demonstrate that S-1563 and Z-CMCA induce CYP2B1/2 through CAR in rat hepatocytes and hence are CAR activators.

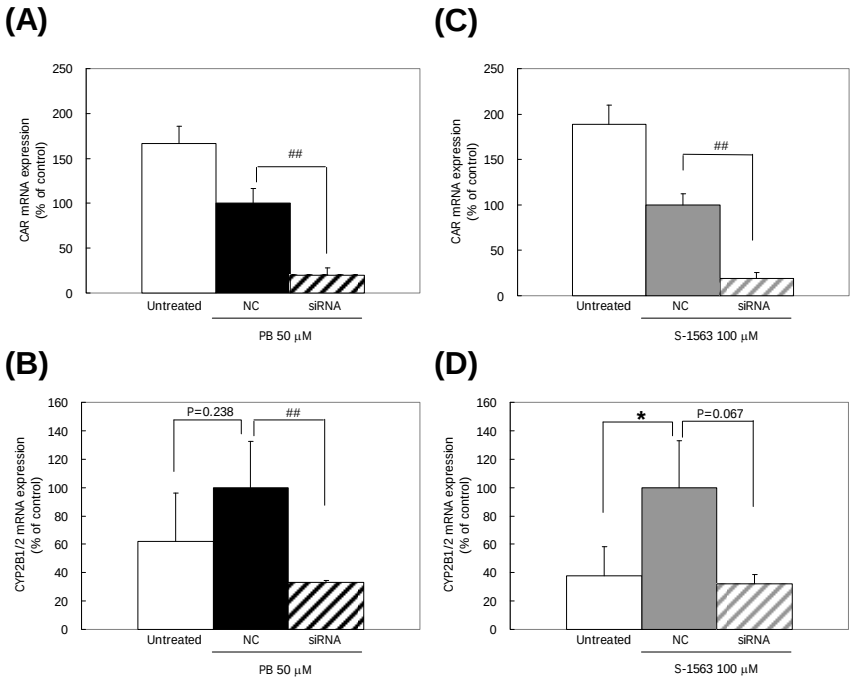

Fig. 1. Relative CAR mRNA (A, C) and CYP2B1/2 (B, D) expression levels in cultured hepatocytes treated with PB 50 µM (A, B) and S-1563 100 µM (C, D). NC means siRNA(Cont) as negative control. PB+siRNA(Cont) level (A, B) and S-1563 +siRNA(Cont) level (C, D) are shown as 100 %. Mean ±SD, N=3. (A, B,) ## p<0.01 vs. PB+siRNA(Cont) , (C, D) *p<0.05 vs. Untreated, ## p<0.01 vs. S-1563+siRNA(Cont).

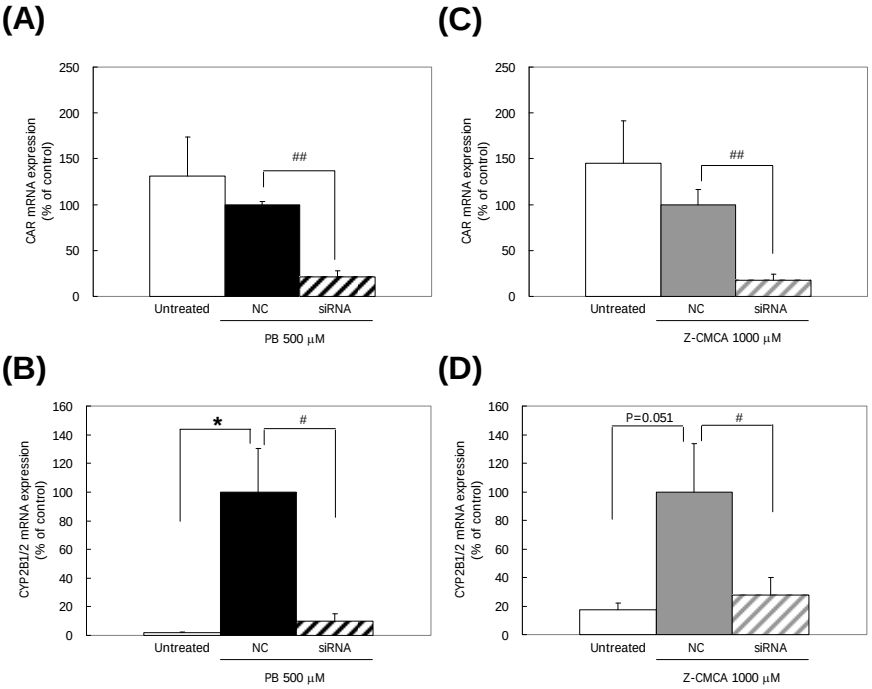

Fig. 2. Relative CAR mRNA (A, C) and CYP2B1/2 (B, D) expression levels in cultured hepatocytes treated with PB 500 µM (A, B) and Z-CMCA 1000 µM (C, D). NC means siRNA(Cont) as negative control. PB+siRNA(Cont) level (A, B) and Z-CMCA+siRNA(Cont) level (C, D) are shown as 100 %. Mean ±SD, N=3. (A, B) ## p<0.01 vs. PB+siRNA(Cont), *p<0.05 vs. Untreated, (C, D)## p<0.01 vs. Z-CMCA +siRNA(Cont).

B. CYP2B induction

S-1563 MOA studies (Okuda, 2012b,d,e) utilized the 200, 500, 1500 and 3000 ppm bioassay dose levels and additional dose levels including 6000 and 10000 ppm as greater than bioassay dose levels (see Annexes II and III). In these studies, levels of CYP2B1/2 mRNA and the CYP2B enzyme marker 7-pentoxyresorufin O-dependentylase (PROD) were determined (Deguchi *et al.*, 2009).

In males, CYP2B activities were induced greater than 2-fold (considered as toxicologically significant) after 7-day treatment at S-1563 dose levels of 3000 ppm and higher (Annex II). A small increase in CYP2B activity to 1.4-fold control was observed in male rats given 1500 ppm S-1563 for 7 days. The treatment of male rats with 3000 ppm S-1563 for 7 and 14 days resulted in large 16- or 18- fold increases in CYP2B1/2 mRNA levels, suggesting that CYP2B mRNA levels would also have been significantly increased at lower doses of S-1563. In females, increases in CYP2B activity were observed at S-1563 dose levels of 1500 and 3000 ppm after 7 days and at a dose level of 3000 ppm after 14 days (Annex III).

Another investigation also demonstrated that S-1563 (and also Z-CMCA, a major metabolite of S-1563 in rats) could induce CYP2B1/2 mRNA in rat cultured hepatocytes (Okuda, 2012f, 2013).

C. Hypertrophy

Treatment with S-1563 has been shown to result in significant increases in absolute and relative liver weights (Annexes II and III). The increases in liver weight were associated with a diffuse hepatocellular hypertrophy which was observed throughout the liver lobule**.** In addition, examination by transmission electron microscopy of liver sections from male and/or female rats given 3000 ppm S-1563 for 1 or 2 weeks (Okuda, 2012b) and 6000 ppm S-1563 for 3 months (Sommer, 2011a) revealed increased smooth endoplasmic reticulum, characteristic of enzyme inducers (Ghadially, 1997).

D. Cell proliferation

The effect of S-1563 on replicative DNA synthesis was studied by employing osmotic mini pumps to continuously infuse 5-bromo-2'-deoxyuridine (BrdU) over 7 days periods. Liver sections were stained with an anti-BrdU antibody and the hepatocyte labeling index (i.e. the percentage of hepatocyte nuclei undergoing replicative DNA synthesis) was assessed by microscopic examination of the liver sections. This is a sensitive measure of cell proliferation as the DNA precursor is continuously administered to the animals throughout the treatment period. The

treatment of male and female rats with 1500 and 3000 ppm S-1563 for 7 days resulted in significant increases in replicative DNA synthesis (Okuda, 2012d,e). Significant increases in replicative DNA synthesis were also observed when male and female rats were given 3000 ppm S-1563 for 14 days, with cell proliferation being assessed by BrdU incorporation over the last 7 days of treatment. However, the magnitude of the increase in replicative DNA synthesis after 14 days was less in both sexes than after 7 days of S-1563 treatment (Okuda, 2012c).

Another investigation also demonstrated that S-1563 (but not Z-CMCA) could increase replicative DNA synthesis in rat cultured hepatocytes (Okuda, 2013).

E. Altered hepatic foci

The treatment of male and female rats with S-1563 for two years resulted in liver tumour formation (Rached, 2012a). In addition to the formation of liver tumours, the chronic treatment of male and female rats with 3000 ppm S-1563 also resulted in significant increases in eosinophilic hepatocellular foci.

Concordance of Dose-Response Relationships

The effects of S-1563 on CYP2B enzyme induction, hypertrophy, cell proliferation and formation of altered hepatic foci were dose-dependent (Annexes II and III). MOA studies were performed at S-1563 doses used in the two year bioassay (Rached, 2012a) as well as at other dose levels that were greater than the bioassay dose levels, including 6000 and 10000 ppm in male rats.

As shown in Fig. 3, after 7 days treatment hepatic CYP2B enzyme activities were statistically significantly induced at 3000 ppm in males (Fig. 3D), and at 1500 and 3000 ppm in females (Fig. 3H). In other 7- and 14-day studies, treatment with 3000 ppm S-1563 produced a statistically significant increase in CYP2B1/2 mRNA level in male rats (Okuda, 2012a).

While the short term MOA studies of 7 and 14 days duration did not reveal significant effects on hepatocellular hypertrophy at a S-1563 dose level of 1500 ppm, increases in hepatocyte hypertrophy were observed at 1500 ppm and above in both male and female rats after treatment for 52 weeks or longer (Annexes II and III).

The treatment of male and female rats with 1500 and 3000 ppm S-1563 for 7 days also resulted in significant increases in relative liver weight (Figs. 3C and 3G). Significant increases in relative liver weight in male and female rats given 1500 ppm and higher dose levels of S-1563 were observed after treatment for longer periods

(Annexes II and III).

As shown in Figs. 3B and 3F, the treatment of male and female rats with S-1563 at 1500 and 3000 ppm for 7 days produced significant increases in replicative DNA synthesis.

The incidence of total animals with hepatocellular adenomas and/or carcinomas was increased in male rats given 1500 and 3000 ppm S-1563, and in female rats given 3000 ppm S-1563 (Figs. 3A and 3E, Rached, 2012a). At high dose levels, treatment with S-1563 in both sexes resulted in increased incidences of eosinophilic hepatocellular foci (Annexes II and III).

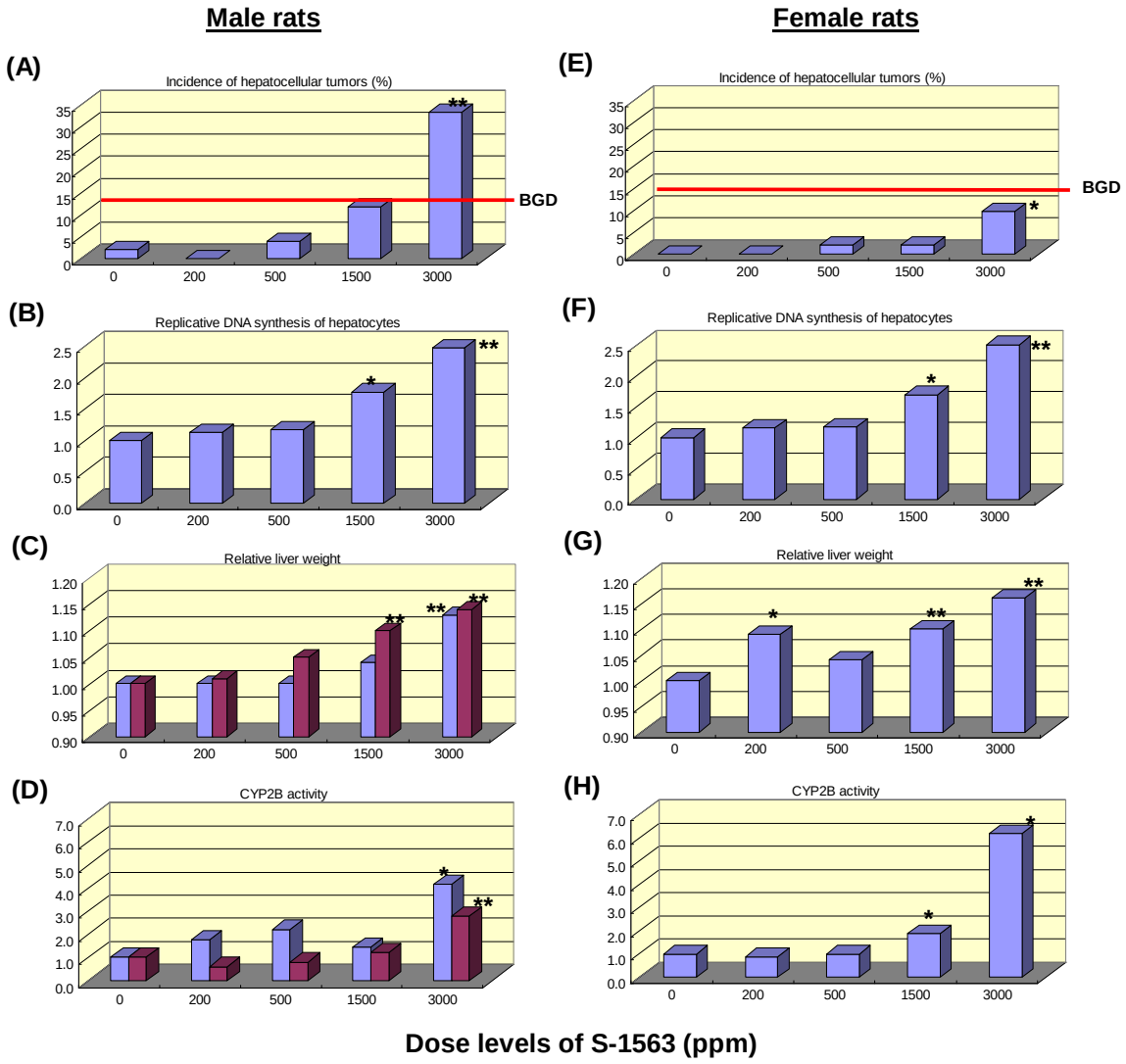

Fig. 3. Concordance of dose-response relationships of tumour appearance (A, E) and key/associative events in S-1563 MOA of rat hepatocellular tumour formation (B-D, and F-H). Date of B-D and F-H are adapted from the 7-day treatment MOA studies (B, Okuda, 2012d; C, left bar, Okuda, 2012b; C, right bar, Okuda, 2012d; D, left bar, Okuda, 2012b; D, right bar, Okuda, 2012d, F-H, Okuda, 2012e). *p<0.05, **p<0.01. BGD: upper limit of historical background data.

Temporal Association

If a key event (or events) is an essential element for carcinogenesis, it must precede the appearance of the tumours. Data are available for the effect of treatment of male and female rats with S-1563 at various time points ranging from 7 days to two years. The treatment of male and female rats with S-1563 for 7 days resulted in an induction of CYP2B enzymes. Cell proliferation, assessed as the hepatocyte labeling index, was increased in male and female rats given carcinogenic dose levels of S-1563 for 7 days (Okuda, 2012d,e). S-1563 produced increases in liver hypertrophy (assessed both by morphology and as increases in liver weight) at a number of early time points and also at later time points. Examination of liver sections from the S-1563 two year bioassay (Rached, 2012a) revealed increases in altered cell foci at the carcinogenic dose levels of 3000 ppm in male and female rats. Three out of 51 male rats a given 1500 ppm S-1563 also had altered cell foci. As altered foci are the precursor lesions for subsequent tumour formation in rodent liver (Williams, 1997), it is considered that the liver foci developed before the appearance of liver tumours. Overall, there is a logical temporal sequence for all key and associative events in S-1563-induced liver tumour formation, in which all key and associative events precede tumour formation.

Strength, Consistency, and Specificity of Association of Key Events and Tumour Response

The treatment of male and female rats with S-1563 results in a pleiotropic response. The effects on the hepatic key and associative events were observed at carcinogenic bioassay dose levels. Increases or trends to increased liver weights and/or hepatocellular hypertrophy were observed in short term (1 and 2 week) (Okuda, 2012a-e,g), sub-acute (13 week) (Sommer, 2011a) and chronic (52 week) (Sommer, 2011b) studies. The induction of CYP2B enzymes and increased replicative DNA synthesis were observed after 7 days of treatment, whereas altered hepatic foci and liver tumours were only observed after chronic treatment.

The effects of S-1563 on liver weight, hepatocellular hypertrophy, and CYP2B enzyme induction after 7 days of treatment were shown to be reversible after 7 days of cessation of treatment (Okuda, 2012b). Therefore, effects of short term treatment with S-1563 on the liver are reversible, which is consistent with the known hepatic effects of other CAR activators.

Biological Plausibility and Coherence

The liver is the most common target tissue affected in rodent cancer bioassays

(Gold *et al*., 2001). This may be due to the fact that the liver is the major site of metabolic activation of chemicals, and furthermore, the liver is the first organ exposed to the chemical following absorption from the gastrointestinal tract (if administered orally, as in the case of the bioassays performed with S-1563). In addition, there is a relatively high background incidence of liver tumours in several strains of mice and rats. The background incidences of combined hepatocellular adenoma/carcinoma in the strain of rats used in the studies with S-1563 at the laboratory where the studies were performed were 0-10.0% for males and 0-12.0% for females (Annex I).

The proposed MOA for liver tumour formation by S-1563 involves activation of CAR which results in increased cell proliferation, with the associated stimulation of CYP2B enzymes and liver hypertrophy. Prolonged treatment results in the formation of altered hepatic foci and liver tumours. This MOA is similar to that of certain other non -genotoxic agents which are CAR activators (Yamada *et al.,* 2009; Holsapple *et al*., 2006; Lake, 2009; Osimitz and Lake, 2009).

The proposed MOA is plausible as it is consistent with current knowledge of MOAs for rodent liver tumour formation by non-genotoxic agents (Cohen and Arnold, 2011; Cohen *et al*., 2004; Holsapple *et al*., 2006; Klaunig *et al*., 2003; Lake, 2009; Meek *et al*., 2003; Yamada *et al.*, 2009). Many studies have demonstrated that activation of nuclear receptors such as CAR and PPARα in rodent liver leads to a pleiotropic response which includes the stimulation of cell proliferation. The stimulation of cell proliferation is normally determined as the hepatocyte labeling index (i.e. the percentage of hepatocyte nuclei undergoing replicative DNA synthesis). The increased hepatocyte labeling index values in rodent liver by compounds that are activators of CAR and PPARα receptors is normally transient and not sustained, being observed after 7 and perhaps 14 or 28 days of treatment, but generally not at longer time points (Cohen and Arnold, 2011; Deguch *et al*., 2009; Furukawa *et al*., 2000; IARC, 2001; Klaunig *et al*., 2003; Kolaja *et al*., 1996; Lake, 2009; Whysner *et al*., 1996). However, while the hepatocyte labeling index returns to control levels with continued treatment with the compound, the overall number of cell replications per animal is increased (Cohen and Arnold, 2011; Lake, 2009). This is because treatment with such chemicals causes a sustained increase in liver weight which results in an overall increase in the number of hepatocytes per animal. For example, in one study employing a stereological technique, an increase in the total number of hepatocytes per animal was observed in rats treated with PB for 12 weeks (Carthew *et al.,* 1998). Thus, although hepatocyte labeling index values return to control levels after continued S-1563 treatment, the number of cell replications in treated animals will be enhanced due to the increase in the total number

of hepatocytes per animal. The continued stimulation of cell proliferation may lead to tumour formation as a result of critical errors being produced during cell replication and/or to the promotion of spontaneously initiated pre-neoplastic hepatocytes (Schulte-Hermann *et al*., 1983).

As described above, the key events for the MOA for S-1563-induced rat liver tumour formation comprise CAR activation, increased cell proliferation and the development of altered hepatic foci as these constitute necessary steps in the MOA. The induction of CYP2B enzymes and liver hypertrophy comprise associative events and represent reliable markers of CAR activation. The role of CAR in CYP2B enzyme induction by S-1563 was also demonstrated in cultured rat hepatocytes using the RNA interference (RNAi) technique (Okuda, 2012f).

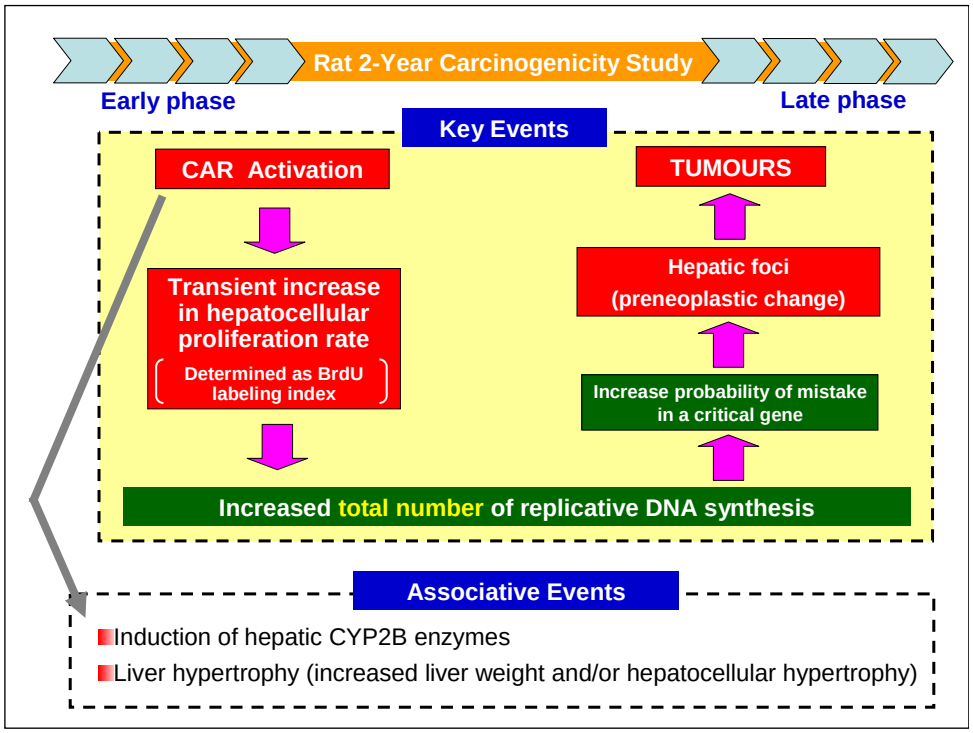

Fig. 4. Schematic representation of key and associative events in the proposed mode of action for S-1563-induced rat hepatocellular tumour formation. The MOA for S-1563-induced rat liver tumour formation is postulated to involve activation of the constitutive androstane receptor (CAR), which results in a pleiotropic response including the stimulation of cytochrome P450 (CYP) CYP2B subfamily enzymes, liver hypertrophy and increased cell proliferation. The stimulation of hepatocyte labeling index values in rodent liver by compounds that are activators of the CAR and the PPARα receptors is normally transient and not sustained, being observed after 7 and perhaps 14 or 28 days of treatment, but generally not at longer time points. However, while the hepatocyte labeling index returns to control levels with continued treatment with the compound, the overall number of cell replications is actually still increased (Cohen and Arnold, 2011; Lake, 2009). This is because treatment with such chemicals causes a sustained increase in liver weight which results in an

overall increase in the number of hepatocytes per animal. Thus, although hepatocyte labeling index values return to control levels after continued S-1563 treatment, the number of cell replications in treated animals will be enhanced due to the increase in the total number of hepatocytes per animal. The continued stimulation of cell proliferation may lead to tumour formation as a result of critical errors being produced during cell replication and/or to the promotion of spontaneously initiated pre-neoplastic hepatocytes (Schulte-Hermann *et al.*, 1983). Prolonged treatment results in the formation of altered hepatic foci and liver tumours.

While S-1563 produced liver tumours in the Wistar rat, no liver tumours were observed in a study with CD-1 mice (Rached, 2012a,b). Non-genotoxic chemicals which are activators of CAR may produce liver tumours in both rats and mice (Lake, 2009). Generally, the mouse appears to be more susceptible than the rat to liver tumour formation by such compounds. However, not all non-genotoxic CAR activators which produce liver tumours in the rat also produce tumours in the mouse. For example, the natural pyrethrins, which are known to produce liver tumours in the rat through activation of CAR, do not produce liver tumours in the mouse (Osimitz and Lake, 2009). Metofluthrin is a close structural analogue to S-1563 and also increased hepatocellular tumours in Wistar rats through activation of CAR but not in CD-1 mice (Yamada *et al.*, 2009). The CD-1 mouse is not a resistant strain to chemically-induced liver tumour formation and is often used to examine the carcinogenicity of non-genotoxic chemicals.

The results of 7- and 14-day treatment studies in male and female CD-1 mice with 600, 2500, and 5500 ppm S-1563 are shown in Annexes II and III (Yamada, 2012a-c). The dietary levels of S-1563 were the same as those used in the S-1563 CD-1 mouse bioassay (Rached, 2012b)**.** Treatment with S-1563 for 7 days produced some increases in liver weight, centrilobular hepatocyte hypertrophy, induction of CYP2B activity and mRNA, a slight induction of CYP4A activity and mRNA, and replicative DNA synthesis in both sexes. However, as shown in Fig. 5 where compound intake is shown in units of mg/kg/day, S-1563 produced increased liver weight (Figs. 5A and 5C) and increased replicative DNA synthesis (Figs. 5B and 5D) in both the rat and mouse. The effects in the mouse at dose levels used in the rat study were equivalent to or less than those in the rat, while the effects in the mouse at higher dose levels (which were not examined in the rat study) were more marked than those in the rat at the highest dose level studied. However, at longer treatment times, the effect of S-1563 on relative liver weight was more marked in the rat than in the mouse. Fig. 6 shows that the increases in relative liver weight were more marked in male and female rats than in male and female mice after 13 weeks and at the end of the bioassays. These findings suggest that while the key events for for liver tumour formation by S-1563 can occur in both the rat and

mouse at early time points, at later time points the effects are more marked in the rat. The apparent species difference between the rat and mouse in the effect of S-1563 on liver hypertrophy may be attributable to a species difference in the metabolism and/or disposition of this compound. In addition, differences in the treament period (i.e. 104 weeks in the rat study, 78 weeks in the mouse) may also be relevant to the interspecies difference observed.

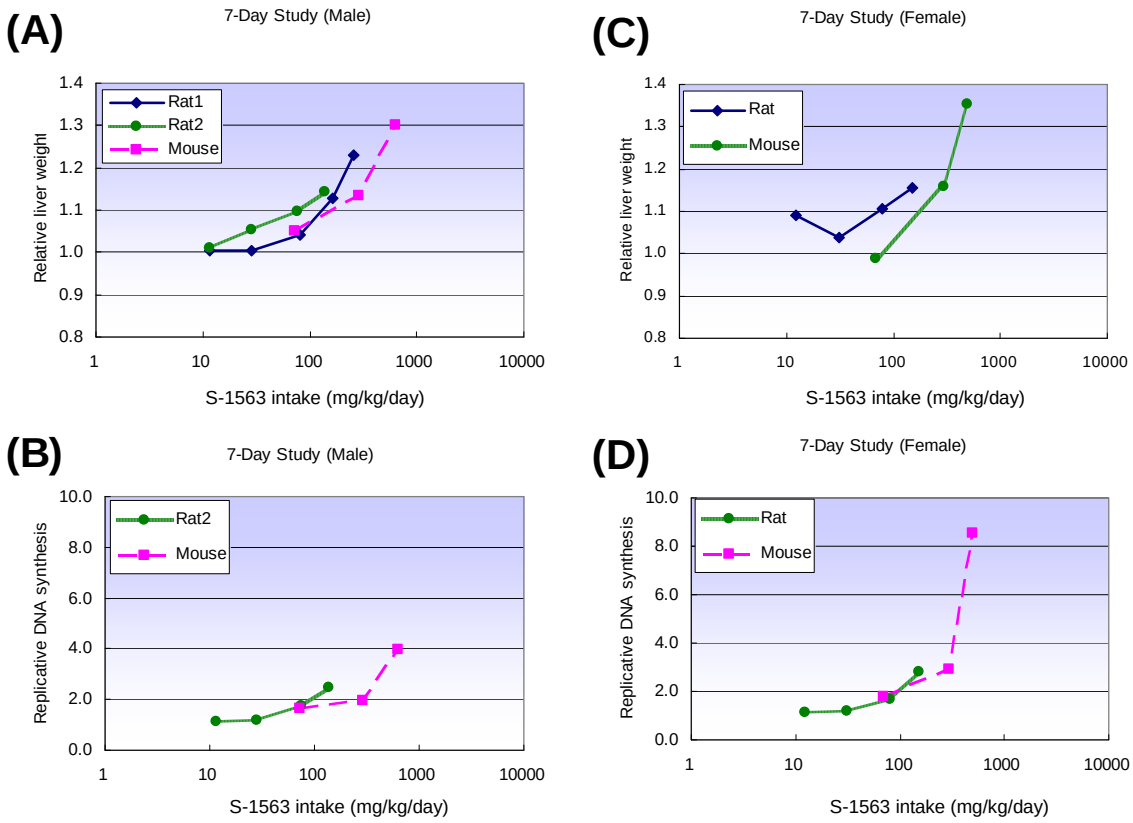

Fig. 5. Effect of S-1563 on relative liver weight (A, C) and replicative DNA synthesis (B, D) after treatment with S-1563 for 7 days in males (A, B) and females (C, D). Data are adapted from MOA studies in male rat (Rat 1: Okuda, 2012b; and Rat 2: Okuda, 2012d), female rat (Okuda, 2012e), male mouse (Yamada, 2012b), and female mouse (Yamada, 2012c). Values present group mean. Control is shown as 1. Dose levels were 0, 200, 500, 1500, and 3000 (additionally 6000 ppm only in the Rat 1 study) for rat; and 0, 600, 2500, and 5500 ppm for mouse; they are bioassay dose levels.

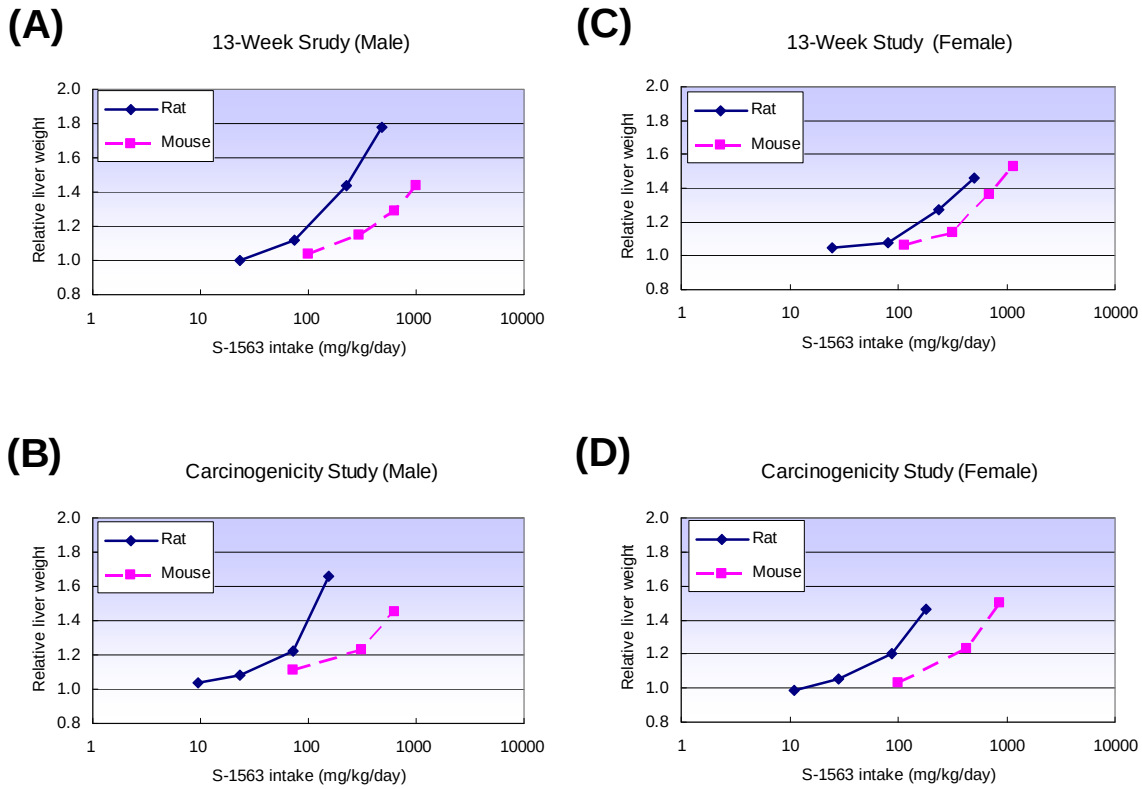

Fig. 6. Effect of S-1563 on relative liver weight after treatment for 13-week studies (A, C) or carcinogenicity studies (B, D) in rats and mice. Data are adapted from subacute toxicity studies in rats (Sommer, 2011a) and mice (Sommer, 2011c), 104week carcinogenicity study in rats (Rached, 2012a), and 78-week carcinogenicity study in mice (Rached, 2012b). Values present group mean. Control is shown as 1. Dose levels were 0, 300, 1000, 3000, and 6000 for rat 13-week study; 0, 200, 500, 1500, and 3000 ppm for rat 104-week carcinogenicity study; 0, 700, 2000, 4500, and 7000 ppm for mouse 13-week study; and 0, 600, 2500, and 5500 ppm for mouse 78-week carcinogenicity study.

Some CAR activators, such as phenobarbital, have effects on thyroid in rats due to increased catabolism of thyroid hormones (triiodothyronine,T3; and thyroxine, T4) driven by increased hepatic UDP-glucuronosyltransferase (UGT) activity, leading to increased thyroid stimulating hormone (TSH) (Capen, 1997; Dellarco, *et al.*, 2006). It is well accepted that TSH stimulation is necessary to induce increased thyroid weight and thyroid follicular cell hypertrophy (Capen, 1997; Dellarco, *et al.*, 2006). The induction of hepatic UGT by phenobarbital is known to be mediated by CAR (Holsapple, *et al.*, 2006; Qatanani, *et al.*, 2005; Ueda, *et al.*, 2002; Wei, *et al.*, 2000; Yamamoto, *et al.*, 2004). After 7- and 14-day treatments with S-1563, hepatic UGT activity was significantly increased (Okuda, 2012c), serum T4 (but not T3) levels were slightly but statistically significantly decreased, and serum TSH levels were slightly increased but without statistical significance (Okuda, 2012g). It was reported that small increases in serum TSH can be sufficient to stimulate thyroid follicular cell proliferation (Hood *et*

al., 1999), so these slight TSH increases without statistical significance seem to be a biologically meaningful change. Furthermore, thyroid follicular cell hypertrophy was also significantly increased. Therefore, the effects of S-1563 on rat thyroid are considered secondary to perturbation of the hypothalamus-pituitary-thyroid axis by a similar mode of action as phenobarbital. However, the magnitude of the alterations produced is not sufficient to result in formation of thyroid follicular cell tumours after prolonged S-1563 administration.

Other Modes of Action

Liver tumours can be produced in rodents by both genotoxic and non-genotoxic agents (Cohen and Arnold, 2011; Williams, 1997). S-1563 is clearly not genotoxic, being negative in a variety of *in vivo* and *in vitro* genotoxicity assays (Ames test, micronucleus test, and *in vitro* chromosomal aberration test). Liver tumours can be produced in rodents by various non-genotoxic MOAs including cytotoxicity, activation of CAR, activation of PPARα, porphyria and hormonal perturbation (Holsapple *et al*., 2006; Klaunig *et al*., 2003; Meek *et al*., 2003; Cohen, 2010). In the general toxicity studies, utilizing both histopathology and electron microscopy techniques, there was no evidence of hepatocellular toxicity (e.g. necrosis, fatty liver), peroxisome proliferation, porphyria, increased iron deposition or any evidence of hormonal perturbations (Sommer, 2011a-c; Rached, 2012a,b). In addition, as shown in Fig. 7, unlike effects on CAR, gene expression profiling analysis studies demonstrated no marked alterations in either PPARα, aryl hydrocarbon receptor (AhR) or pregnane x receptor (PXR) signaling (Okuda, 2012a). Overall, it is considered that the hepatic effects of S-1563 in the rat and subsequent liver tumour formation are primarily mediated by activation of CAR.

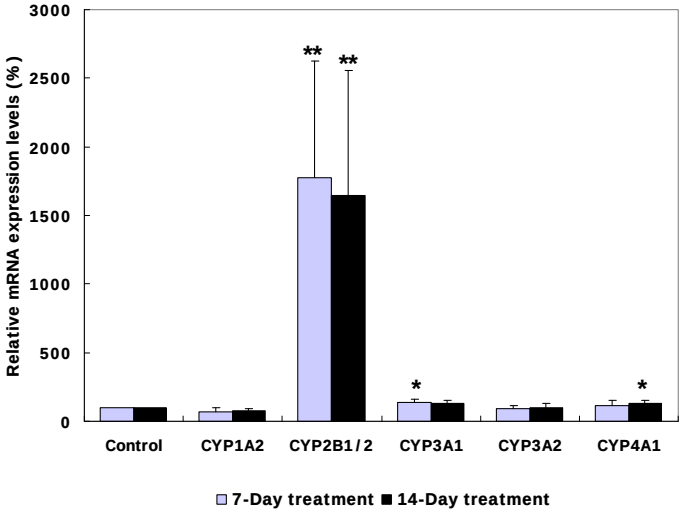

Fig. 7. Hepatic mRNA expression levels of CYP1A2, 2B1/2, 3A1, 3A2, and 4A1 in liver. mRNA expressions of CYP1A2, 3A1, 3A2, and 4A1 were determined in liver from male rats given at 3000 ppm S-1563 for 7 days to confirm lack of contribution of other nuclear receptors for liver tumour induction by S-1563; namely, CYP1A2 for AhR, CYP2B1/2 for CAR, CYP3A1and 3A2 for PXR, and CYP4A1 for PPARα (Lake, 2009). Results are presented as group mean values±SD, N=6. Control value is shown by 100 %. * and ** indicate statistically significant differences from the control (*:$p<0.05$, **: $p<0.01$).

Uncertainties, Inconsistencies, and Data Gaps

The CAR-dependency for the stimulation of cell proliferation by S-1563 has not been established. However, we do not believe that this evaluation is essential as the critical role of CAR in the proposed MOA has previously been demonstrated (Wei *et al.,* 2000; Yamamoto *et al.*, 2004). We thus consider that this data gap does not alter the overall postulated MOA for S-1563-induced rat liver tumours.

No data have been obtained for the role, if any, of effects on apoptosis in the MOA for S-1563-induced rat liver tumour formation. Again, it is not considered that this represents a significant data gap.

The treatment of male rats with 1500 ppm S-1563 did not result in a significant increase in CYP2B enzyme activity (Okuda, 2012b,d), hence the dose response between tumour formation and CYP2B induction was not completely matched. However, there were large 16-18 fold increases in CYP2B1/2 mRNA levels in male rats given 3000 ppm S-1563 for 7 and 14 days, which suggest that some increase in CYP2B1/2 mRNA levels would have been observed in male rats treated with 1500 ppm S-1563. Indeed, in another investigation the treatment of male rats with 500, 1500, 3000 and 6000 ppm S-1563 for 7 days resulted in dose-related increases in CYP2B mRNA levels to 6-, 26-, 97- and 162-fold control, respectively (data not shown). It should be noted there was only a marginal increase in hepatocellular tumour incidence at this dose level, with combined incidences of hepatocellular adenoma and carcinoma being 1/51, 6/51, and 17/51 for control, 1500, and 3000 ppm, respectively. The tumour incidence data suggests that the potency for CAR activation by S-1563 would be less at a dose level of 1500 ppm than at 3000 ppm. Indeed the present data demonstrate that the effects on hepatocellular proliferation, CYP2B enzyme induction and liver hypertrophy were less marked in male rats given 1500 than 3000 ppm S-1563.

Assessment of Postulated Mode of Action

The present data are considered adequate with a high degree of confidence to explain the development of liver tumours in rats following chronic administration of S-1563. As described above, the key and associative events in the postulated MOA for S-1563-induced liver tumour formation have been established, with a strong dose response and temporal consistency. The postulated MOA is similar to that of certain other non-genotoxic agents which are CAR activators including that of a close structural analogue metofluthrin (Yamada *et al.,* 2009; Holsapple *et al.*, 2006; Osimitz and Lake, 2009). Alternative modes of action for S-1563-indcued rat liver tumour formation have been excluded.

V. Human Applicability of the Proposed Mode of Action

In terms of the human relevance of an animal carcinogenic MOA there are three questions to consider (Boobis *et al*., 2006) before reaching a conclusion. These are:

1. Is the weight of evidence sufficient to establish a mode of action (MOA) in animals?
2. Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between animals and humans?
3. Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between animals and humans?

The three questions and the overall conclusion for the human relevance of the animal MOA for liver tumour formation are considered below:

1. Is the weight of evidence sufficient to establish a mode of action (MOA) in animals?

As described above a plausible MOA for S-1563-induced rat liver tumour formation has been established and hence the answer to question 1 is yes.

2. Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between animals and humans?

In assessing the relevance of animal MOA data to humans, a concordance table has been suggested as being of considerable value (Boobis *et al.,* 2006; Meek *et al*., 2003). Such a table is presented below (Table 3). This includes not only the data for the effects of S-1563 in the rat, but also the available data for humans.

Table 3. Comparison of key and associative events for S-1563 hepatocellular tumour formation in rats and humans

Key (K) and Associative (A) Event	Evidence in Rats	Evidence in Humans
Activation of CAR (K)	Inferred from CAR-siRNA studies and from induction of CYP2B enzymes	Probable at high doses (Inferred from induction of CYP2B enzymes *in vitro* in cultured hepatocytes)
Induction of CYP2B (A) [as a marker for CAR activation]	Direct experimental evidence *in vivo* and *in vitro* in cultured hepatocytes	Probable at high doses (Direct experimental evidence *in vitro* in cultured hepatocytes)
Hypertrophy (A)	Direct experimental evidence *in vivo*	Possible at very high doses #
Increased hepatocellular proliferation (K)	Direct experimental evidence *in vivo* and *in vitro* in cultured hepatocytes	Not predicted (Not observed in cultured hepatocytes)
Altered hepatic foci (K)	Direct experimental evidence *in vivo*	Not predicted
Liver tumours	Yes	Not predicted

#: based on studies in human subjects given anticonvulsant drugs

A number of studies have shown that CAR is present in human liver and that this receptor can be activated by drugs and other compounds (Moore *et al.,* 2003). Hence, it is probable that at high doses S-1563 could activate CAR in human liver. As described above, the induction of CYP2B enzymes serves as a marker for CAR activation. In addition to *in vivo* studies, compounds can also be examined for potential to induce CYP enzymes *in vitro* by conducting studies using primary hepatocyte cultures. The effect of S-1563 and a major metabolite Z-CMCA on CYP2B enzyme induction has been studied in cultured rat and human hepatocytes (Fig.8; Okuda, 2013). PB was included as a positive control in these studies as it is known to induce CYP2B mRNA levels in both rat and human hepatocytes. Treatment with 50 ~ 500 μM S-1563 increased CYP2B1/2 mRNA levels in male Wistar rat hepatocytes and CYP2B6 mRNA levels in human hepatocytes. 500 and 1000 μM Z-CMCA increased CYP2B1/2 mRNA levels in male Wistar rat hepatocytes but not increase CYP2B6 mRNA levels in human hepatocytes. Thus, at high doses S-1563 has the potential to activate CAR and induce CYP2B enzymes in human liver.

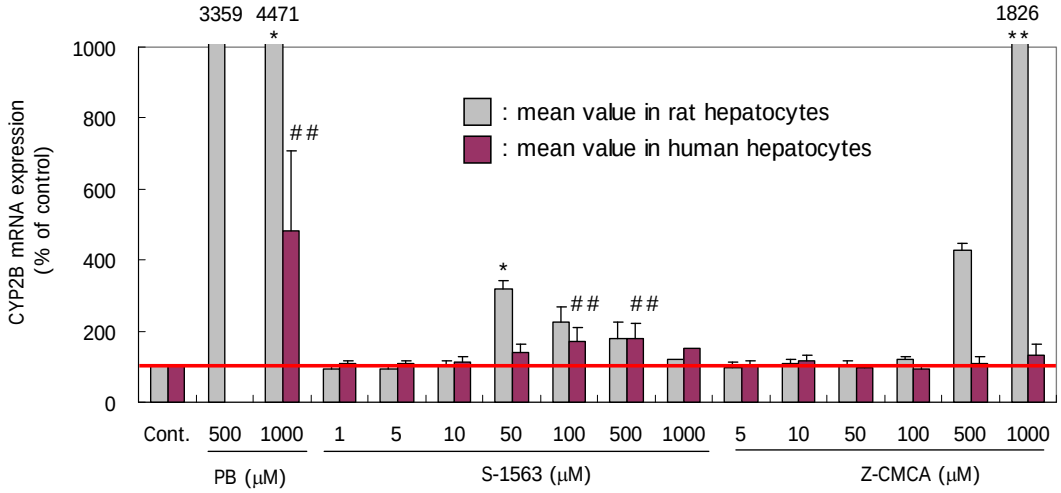

Fig. 8. Effect of PB, S-1563 and Z-CMCA on CYP2B mRNA expression in cultured rat and human hepatocytes. Rat and human hepatocytes were treated with PB (500 and 1000 μM), S-1563 (1-1000 μM) and Z-CMCA (5-1000 μM) for 48 hours and rat CYP2B1/2 and human CYP2B6 mRNA levels were determined by quantitative real-time PCR. Results are presented as mean ± SD (n=2-5 in rat, n=1-7 in human) and each value of rat and human hepatocytes is presented in the report (Okuda, 2013). Values significantly different from control (DMSO only treated) are: * $p<0.05$ and ** $p<0.01$ in rat hepatocytes; and # $p<0.05$ and ## $p<0.01$ in human hepatocytes.

Studies in human subjects given anticonvulsant drugs (which induce hepatic CYP enzymes) have shown that prolonged treatment with high doses can increase liver size in humans, which is associated with liver hypertrophy and increased smooth endoplasmic reticulum (Aiges *et al.,* 1980; Pirttiaho *et al.,* 1978). Thus, by comparison with the effects of such anticonvulsant drugs, at high doses S-1563 has the potential to produce hypertrophy in human liver.

While the potential for compounds to increase cell proliferation in rodent liver can be examined by performing *in vivo* studies as described above, compounds can also be tested for mitogenic potential *in vitro* by conducting studies using primary hepatocyte cultures. For example, a number of compounds that can activate either CAR or PPARα in rodent liver can induce replicative DNA synthesis in cultured rat and mouse hepatocytes (Hasmall and Roberts, 1999; Hirose *et al.*, 2009; Klaunig *et al*., 2003; Lake, 2009; Parzefall *et al*., 1991). As the stimulation of replicative DNA synthesis is a critical key event in the proposed MOA for S-1563-induced rat liver tumour formation, the effect of S-1563 on replicative DNA synthesis has been studied in cultured rat and human hepatocytes (Fig.9; Okuda, 2013). Replicative DNA synthesis was determined by incorporation of BrdU over the last 24 hour of a 48 hour treatment period. To serve as positive controls for induction of replicative DNA synthesis, rat and human hepatocytes were treated with hepatocyte growth factor (HGF) and also with PB. PB is

known to induce replicative DNA synthesis in rat but not in human hepatocytes, whereas HGF is a known mitogenic agent which has previously been shown to increase replicative DNA synthesis in cultured rodent and human hepatocytes (Hirose *et al.*, 2009; Lake, 2009).

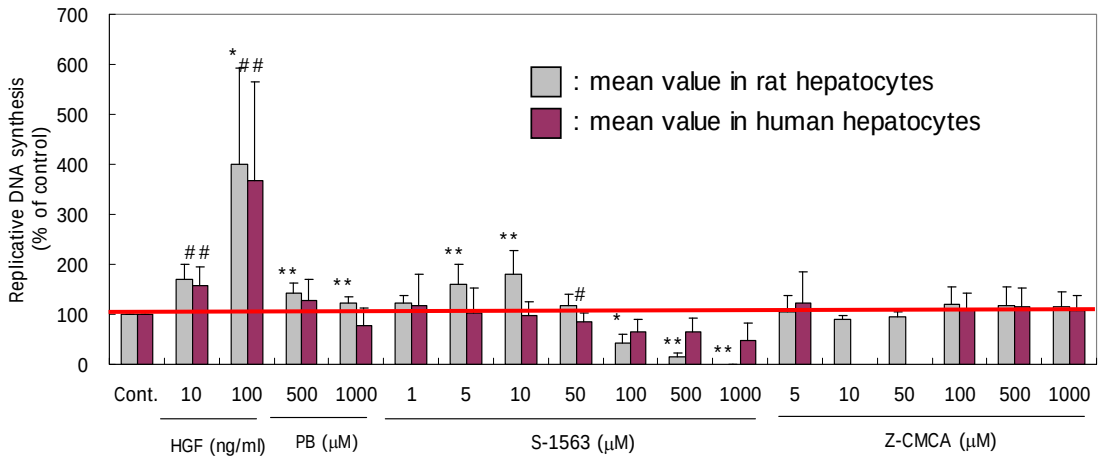

Fig. 9. Effect of HGF, PB, S-1563 and Z-CMCA on replicative DNA synthesis in cultured rat and human hepatocytes. Rat and human hepatocytes were treated with HGF (10 and 1000 μM), PB (500 and 1000 μM), S-1563 (1-1000 μM) and Z-CMCA (5-1000 μM) for 48 hours and replicative DNA synthesis was determined by BrdU incorporation over the last 24 hours of culture. Results are presented as mean ± SD (n=3-8 in rat, n=5-10 in human) and each value of rat and human hepatocytes is presented in the report (Okuda, 2013). Values significantly different from control (DMSO only treated) are: * $p<0.05$ and ** $p<0.01$ in rat hepatocytes; and # $p<0.05$ and ## $p<0.01$ in human hepatocytes.

Using primary hepatocyte cultures a dose-dependent increase in replicative DNA synthesis following treatment with HGF were observed in rat and in human hepatocytes. Replicative DNA synthesis was increased in ten preparations of human hepatocytes treated with HGF which is consistent with previous finding (Hirose *et al.*, 2009). Treatment with 5 and 10 μM S-1563 and 500 and 1000 μM PB resulted in increases in replicative DNA synthesis in rat hepatocytes. In contrast, the treatment of human hepatocytes with 1-1000 μM S-1563, 5-1000 μM Z-CMCA, and 500 and 1000 μM PB had no effect on replicative DNA synthesis. These results demonstrate that while S-1563 induced CYP2B enzymes in both rat and human hepatocytes, S-1563 only induced replicative DNA synthesis in rat and not in human hepatocytes. At higher concentrations (especially 100 μM and higher), S-1563 decreased replicative DNA synthesis in both rat and human hepatocytes (Okuda, 2013). However, the inhibition of DNA synthesis observed at high concentrations *in vitro* does not appear to occur *in vivo,* as no hepatotoxic effects were observed in rat studies at doses up to the maximum

tolerated dose (MTD).

Table 3 summarises the available rat and human data for the key and associative events in the proposed MOA for S-1563-induced rat liver tumour formation. As described above, S-1563 could activate CAR in human liver and produce both CYP2B enzyme induction and liver hypertrophy. However, a key species difference is that while S-1563 is clearly a mitogenic agent in the rat, S-1563 does not stimulate replicative DNA synthesis in cultured human hepatocytes. Overall, while some of the key (activation of CAR) and associative (CYP2B enzyme induction and hepatocellular hypertrophy) events in the MOA for S-1563-induced rat liver tumour formation could occur in human liver, the available experimental data demonstrate that human hepatocytes appear to be refractory to the mitogenic effects of S-1563. Therefore, it is concluded that the proposed MOA for S-1563-induced rat liver tumour formation is not plausible for humans.

The data obtained in these studies with the CAR activator S-1563 is in agreement with literature data on other CAR activators and with compounds that can activate PPARα in rodent liver. Studies with other CAR activators and PPARα activators, including some hypolipidaemic drugs, have shown that these compounds stimulate replicative DNA synthesis in cultured rodent hepatocytes, but not in cultured human hepatocytes (Hirose *et al.,* 2009; Klaunig *et al*., 2003; Lake, 2009; Parzefall *et al*., 1991). S-1563 thus produces similar effects on replicative DNA synthesis in rat and human liver to those produced by a number of other non-genotoxic agents which can produce liver tumours in rodents. The key species difference between rodents and humans appears to be that human hepatocytes are refractory to the mitogenic effects of such agents.

3. Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between animals and humans?

As examination of the available data demonstrates that the MOA for S-1563-induced rat liver tumour formation is qualitatively not plausible for humans, there is no need to consider quantitative differences in either kinetic or dynamic factors between rats and humans. However, it should be noted that likely human chronic exposure to S-1563 would be orders of magnitude lower than the S-1563 dose levels required to produce liver tumours in the rat. Thus, not only is there a qualitative difference between the rat and human in the response of the liver cells to the CAR activators regarding induction of tumours, but also a marked quantitative difference in

the level of exposure. Thus, based on quantitative considerations, the confidence in a lack of effect in humans at expected exposures is even stronger than that based only on qualitative considerations.

4. Conclusion: Statement of confidence, analysis, and implications

Based on the data described above, there is strong evidence to support a plausible MOA for S-1563-induced liver tumour formation in male and female rats. This MOA involves activation of CAR resulting in increased cell proliferation and the development of altered hepatic foci. The induction of CYP2B enzymes and liver hypertrophy comprise associative events and represent reliable markers of CAR activation. In studies with cultured hepatocytes S-1563 was found to induce CYP2B enzymes in both rat and human hepatocytes. However, in marked contrast to the effect in rat hepatocytes, S-1563 did not stimulate replicative DNA synthesis in human hepatocytes. These data demonstrate an important species difference in that a critical key event in the MOA for rat liver tumour formation does not operate in human hepatocytes. Therefore, the data strongly support the conclusion that qualitatively this MOA would not occur in humans following exposure to S-1563. Consequently, S-1563 is of low risk for humans.

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VII. Annexes

I. Historical Control Data on Liver Tumours in 104-Weeks Studies in RccHanTM:WIST, Wistar Hannover Rats
Compiled from 104 weeks Bioassays performed at Harlan Laboratories Ltd. Itingen/Switzerland

Study identification

Study Number	ID Number	Data of Performance	Study Type	Age/Delivery (weeks)	Body Weight: Delivery (g)		Pathologist
					M	F	
5321	2	12.1981 – 12.1983	104-Weeks Feeding	4	79 – 128	48 – 101	JMA
6390	3	01.1982 – 09.1985	104-Weeks Feeding	4	75 – 129	56 – 97	RUD
17820	6	03.1983 – 05.1985	104-Weeks Feeding	4	70 – 105	48 – 76	HHW
24300	8	12.1984 – 12.1986	104-Weeks Feeding	4	73 –104	55 – 70	JMA
46980	14	09.1985 – 10.1987	104-Weeks Feeding	4	65 – 92	52 – 72	BSC
319623	32	04.1992 – 05.1994	104-Weeks Feeding	4	51 – 98	46 – 75	JMA
350010	33	05.1993 – 05.1995	104-Weeks Feeding	4	76 – 103	54 – 85	HHW
379304	34	06.1995 – 08.1997	104-Weeks Feeding	4	64 – 92	45 – 70	JMA
369731	35	04.1994 – 05.1996	104-Weeks Feeding	5	108 – 161	93 – 131	HJC
756584	39	03.2000 – 01.2003	104-Weeks Feeding	5	110 (±20%)	95 (±20%)	WEK
838855	40	06.2002 – 06.2004	104-Weeks Feeding	5	100 (±20%)	80 (±20%)	JMA
847154	41	01.2003 – 01.2005	104-Weeks Feeding	5	130 (±20%)	100 (±20%)	WEK
846244	42	11.2002 – 11.2004	104-Weeks Feeding	5	110 (±20%)	95 (±20%)	WEK
851465	43	01.2004 – 01.2006	104-Weeks Feeding	4	59.1-86.3	52.7-78.5	WEK
849397	44	02.2004 – 02.2006	104-Weeks Feeding	5	110 (±20%)	95 (±20%)	WEK
842886	45	04.2002 – 06.2006	104-Weeks Feeding	4	60.9 – 88.6	54.0 – 82.3	KHE
850398	48	10.2003 – 11.2005	104-Weeks Feeding	5	100 (±20%)	80 (±20%)	HJC
848780	50	05.2003 – 09.2006	104-Weeks Feeding	5	77 – 110	65 - 92	WEK
A08897	51	May 2006 – May 2008	104-Weeks Feeding	5	110 (±20%)	95 (±20%)	WEK
A38531	53	04.2006 – 02.2009	104-Weeks Feeding	4	75 (±20%)	60 (±20%)	KHE

Pathologists:

BSC	Dr. med. vet. B. Schlotke	KHE	Dr. rer. nat. K. Heider
GPZ	Dr. med. vet. G. Pappritz	RUD	Prof. Dr. med. vet. R. Rudolph
HHW	Dr. med. vet. H. Westen	WILL	Dr. med. vet. J. Th. Wilson
HJC	Dr. med. vet. H. J. Chevalier	WEK	Dr. rer. nat. K. Weber
JAW	Dr. med. vet. J. Walberg	WRJ	Dr. med. vet. J. Wright
JMA	Dr. med. vet. J. Armstrong		

Incidence of hepatocellular tumour

Male Study ID	2	3	6	8	14	32	33	34	35	39	40	41	42*	43	44	45	48	50	51	53		Total n	Total %	Mean %	MIN %	MAX %
Numbers of rats examined	100	100	50	100	60	50	100	100	50	79	50	50	50	112	100	100	107	50	50	100		1558				
Hepatocellular adenoma	1	0	1	2	0	2	6	4	1	0	1	4	1	2	5	2	1	3	1	0		37	2.37	2.54	0.00	8.00
% of adenoma	1	0	2	2	0	4	6	4	2	0	2	8	2	1.79	5	2	0.93	6	2	0						
Hepatocellular carcinoma	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	3	1	0	0		7	0.26	0.47	0.00	2.80
% of carcinoma	0	0	0	0	1.67	0	0	0	0	0	0	2	0	0	1	0	2.8	2	0	0						
Combined hepatocellular adenoma + carcinoma	1	0	1	2	1	2	6	4	1	0	1	5	1	2	6	2	4	4	1	0		44	2.82	3.01	0.00	10.00
% of adenoma + carcinoma	1	0	2	2	1.67	4	6	4	2	0	2	10	2	1.79	6	2	3.74	8	2	0						

Female Study ID	2	3	6	8	14	32	33	34	35	39	40	41	42*	43	44	45	48	50	51	53		Total n	Total %	Mean %	MIN %	MAX %
Numbers of rats examined	100	100	50	100	59	50	100	98	50	62	50	50	49	111	100	100	108	50	50	100		1537				
Hepatocellular adenoma	4	0	1	0	0	5	1	10	1	5	3	1	1	1	2	0	2	0	2	0		39	2.54	2.80	0.00	10.20
% of adenoma	4	0	2	0	0	10	1	10.2	2	8.06	6	2	2.04	0.9	2	0	1.85	0	4	0						
Hepatocellular carcinoma	0	0	0	0	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0		4	0.26	0.32	0.00	2.00
% of carcinoma	0	0	0	0	1.69	2	0	0	0	1.61	0	0	0	0	1	0	0	0	0	0						
Combined hepatocellular adenoma + carcinoma	4	0	1	0	1	6	1	10	1	6	3	1	1	1	3	0	2	0	2	0		43	2.80	3.12	0.00	12.00
% of adenoma + carcinoma	4	0	2	0	1.69	12	1	10.2	2	9.68	6	2	2.04	0.9	3	0	1.85	0	4	0						

*: Metofluthrin

II. Summary of key and associative events in male rats treated with S-1563

		Males Dose (ppm)										Treatment period	Ref.
		0	200	300	500	1000	1500	3000	6000	10000	PB 1000		
Neoplastic findings													
Hepatocellular tumors	Adenoma	1/51 (2.0%)	0/51 (0.0%)		2/51 (3.9%)		4/51 (7.8%)	8/51 (15.7%)*				104 weeks	Rached (2012a)
	Carcinoma	0/51 (0.0%)	0/51 (0.0%)		0/51 (0.0%)		4/51 (8.0%)	9/51 (17.6%)**					
	Combined	1/51 (2.0%)	0/51 (0.0%)		2/51 (3.9%)		6/51 (11.8%)	17/51 (33.3%)**					
Preneoplastic findings													
Hepatic foci	Eosinophilic cell foci	0/51	2/51		3/51		3/51	20/51**				104 weeks	Rached (2012a)
Liver weight													
Relative	mean ± SD	2.8		2.81		3.14		4.04**	4.99**			13 weeks	Sommer (2011a)
	vs. control, fold	1.00		1.00		1.12		1.44	1.78				
	mean ± SD	2.43	2.5		2.66		3.14**	3.42**				52 weeks	Sommer (2011b)
	vs. control, fold	1.00	1.03		1.09		1.29	1.41					
	mean ± SD	2.27	2.37		2.46		2.78**	3.76**				104 weeks	Rached (2012a)
	vs. control, fold	1.00	1.04		1.08		1.22	1.66					
Absolute	mean ± SD	11.52±0.896						11.80±1.199				7 days	Okuda (2012a)
	vs. control, fold	1.00						1.02					
	mean ± SD	11.43±0.778						13.22±0.686**				14 days	
	vs. control, fold	1.00						1.16					
	mean ± SD	11.72 ± 0.743	11.53±0.793		11.33±0.864		11.59±0.516	12.79±0.646*	13.02±1.212**			7 days	Okuda (2012b)
	vs. control, fold	1.00	0.98		0.97		0.99	1.09	1.11				
	mean ± SD	12.17 ± 0.886						14.12±0.674**	15.58±1.698**			14 days	
	vs. control, fold	1.00						1.16	1.28				
	mean ± SD	11.59 ± 0.621						12.06±1.4				7-day treatment + 7-day recovery	
	vs. control, fold	1.00						1.04					
	mean ± SD	10.97 ± 0.509						12.29±0.705**	14.23±1.662**			7 days	Okuda (2012c)
	vs. control, fold	1.00						1.12	1.30				
	mean ± SD	11.35 ± 0.597						14.05±1.443**	16.00±1.567**			14 days	
	vs. control, fold	1.00						1.24	1.41				
	mean ± SD	11.18 ± 0.824	11.29±0.964		11.78±0.698		12.03±0.684	12.23±0.685*			14.09±1.077**	7 days	Okuda (2012d)
	vs. control, fold	1.00	1.01		1.05		1.08	1.09			1.26		
	mean ± SD	11.54±0.832						13.23±0.946**		15.24±1.473**		7 days	Okuda (2012g)
	vs. control, fold	1.00						1.15		1.32			
	mean ± SD	11.78±0.932						14.70±0.552**		18.34±1.375**		14 days	
	vs. control, fold	1.00						1.25		1.56			
Relative	mean ± SD	3.57±0.197						3.95±0.212**				7 days	Okuda (2012a)
	vs. control, fold	1.00						1.11					
	mean ± SD	3.42±0.164						4.09±0.164**				14 days	
	vs. control, fold	1.00						1.20					
	mean ± SD	3.46 ± 0.129	3.47±0.2		3.47±0.186		3.6±0.147	3.90±0.216**	4.26±0.256**			7 days	Okuda (2012b)
	vs. control, fold	1.00	1.00		1.00		1.04	1.13	1.23				
	mean ± SD	3.43 ± 0.12						4.16±0.126**	4.62±0.33**			14 days	
	vs. control, fold	1.00						1.21	1.35				
	mean ± SD	3.34 ± 0.114						3.51±0.264				7-day treatment + 7-day recovery	
	vs. control, fold	1.00						1.05					
	mean ± SD	3.25 ± 0.112						3.80±0.203**	4.43±0.342**			7 days	Okuda (2012c)
	vs. control, fold	1.00						1.17	1.36				
	mean ± SD	3.23 ± 0.103						4.06±0.324**	4.74±0.288**			14 days	
	vs. control, fold	1.00						1.26	1.47				
	mean ± SD	3.36 ± 0.188	3.39±0.233		3.54±0.126		3.69±0.095**	3.84±0.130**			4.15±0.269**	7 days	Okuda (2012d)
	vs. control, fold	1.00	1.01		1.05		1.10	1.14			1.24		
	mean ± SD	3.56±0.162						4.24±0.208**		5.14±0.340**		7 days	Okuda (2012g)
	vs. control, fold	1.00						1.19		1.44			
	mean ± SD	3.45±0.188						4.37±0.105**		5.72±0.400**		14 days	
	vs. control, fold	1.00						1.27		1.66			

Statistical significance (*p<0.05, ** p<0.01). Highlighted data represent alterations with toxicological significance.

PB; sodium phenobarbital.

II. Summary of key and associative events in male rats treated with S-1563 (continued)

		Males Dose (ppm)									Treatment period	Ref.
		0	200	300	500	1000	1500	3000	6000	10000	PB 1000		
Cell proloferation													
BrdU labeling index	mean ± SD	6.41±5.60						6.76±4.87				7 days	Okuda (2012a)
	vs. control, fold	1.00						1.05					
	mean ± SD	3.31±2.05						6.10±4.64				14 days	
	vs. control, fold	1.00						1.84					
	mean ± SD	1.21 ± 0.44						6.41±4.35**	20.85±14.34**			7 days	Okuda (2012c)
	vs. control, fold	1.00						5.30	17.20				
	mean ± SD	0.77 ± 0.43						1.46±0.99*	3.46±2.22**			14 days	
	vs. control, fold	1.00						1.90	4.49				
	mean ± SD	1.90 ± 0.92	2.14±0.55		2.22±0.68		3.37±1.34*	4.69±2.51**			7.42±2.46**	7 days	Okuda (2012d)
	vs. control, fold	1.00	1.13		1.17		1.77	2.47			3.91		
Enzyme activities													
CYP1A2 mRNA	mean ± SD	100±40.62						72±24.43				7 days	Okuda (2012a)
	vs. control, fold	1.00						0.72					
	mean ± SD	100±50.97						79±14.50				14 days	
	vs. control, fold	1.00						0.79					
CYP2B1/2 mRNA	mean ± SD	100±12.86						1778±848.60**				7 days	Okuda (2012a)
	vs. control, fold	1.00						17.78					
	mean ± SD	100±16.44						1644±915.37**				14 days	
	vs. control, fold	1.00						16.40					
CYP2B activity	mean ± SD	18 ± 4.8	32±17.2		40±27.3		26±13.1	75±20.8*	194±52.9*			7 days	Okuda (2012b)
	vs. control, fold	1.00	1.78		2.22		1.44	4.17	10.78				
	mean ± SD	25 ± 4.4						32±15.6				7-day treatment + 7-day recovery	
	vs. control, fold	1.00						1.28					
	mean ± SD	5 ± 1.8	3±1.8		4±1.2		6±0.6	14±5.9**			65±14.2**	7 days	Okuda (2012d)
	vs. control, fold	1.00	0.60		0.80		1.20	2.80			13.00		
CYP3A1mRNA	mean ± SD	100±28.99						137±20.88*				7 days	Okuda (2012a)
	vs. control, fold	1.00						1.37					
	mean ± SD	100±23.30						128±24.01				14 days	
	vs. control, fold	1.00						1.28					
CYP3A2 mRNA	mean ± SD	100±37.95						93±22.14				7 days	Okuda (2012a)
	vs. control, fold	1.00						0.93					
	mean ± SD	100±49.10						97±31.52				14 days	
	vs. control, fold	1.00						0.97					
CYP4A1 mRNA	mean ± SD	100±13.95						117±33.19				7 days	Okuda (2012a)
	vs. control, fold	1.00						1.17					
	mean ± SD	100±20.00						130±20.84*				14 days	
	vs. control, fold	1.00						1.30					
CYP4A activity	mean ± SD	162 ± 51.6						211±54.2	291±88.7			7 days	Okuda (2012b)
	vs. control, fold	1.00						1.30	1.80				

Statistical significance (*p<0.05, ** p<0.01).

Highlighted data represent alterations with toxicological significance.

PB; sodium phenobarbital.

II. Summary of key and associative events in male rats treated with S-1563 (continued)

		Males Dose (ppm)										Time	Ref. No.
		0	200	300	500	1000	1500	3000	6000	10000	PB 1000		
Liver histopathology													
Light micoscopy	Hepatocellular hypertrophy	0/12		0/12		0/12		9/12**	12/12**		ND	13 weeks	Sommer (2011a)
		6/21	1/21*		5/21		12/21	17/21**	ND		ND	52 weeks	Sommer (2011b)
		1/51	1/51		0/51		5/51	14/51**	ND		ND	104 weeks	Rached (2012a)
		1/10						3/10				7 days	Okuda (2012a)
		3/10						4/10				14 days	
		0/10	0/10		0/10		0/10	6/10**	10/10**			7 days	Okuda (2012b)
		0/10						4/10*	10/10**			14 days	
		0/10						0/10				7-day treatment + 7-day recovery	
		0/10						4/10*	10/10**			7 days	Okuda (2012c)
		0/10						8/10**	10/10**			14 days	
		0/10	0/10		0/10		0/10	2/10			10/10**	7 days	Okuda (2012d)
		0/10						4/10 *		10/10 **		7 days	Okuda (2012g)
		0/10						10/10 **		10/10 **		14 days	
Electron microscopy	Proliferation of SER	0/2							2/2			13 weeks	Sommer (2011a)
		0/2						0/2				7 days	Okuda (2012b)
		0/2						0/2				14 days	

Statistical significance (*p<0.05, ** p<0.01).

Highlighted data represent alterations with toxicological significance.

PB; sodium phenobarbital.

II. Summary of key and associative events in male rats treated with S-1563 (continued)

		Males Dose (ppm) 0	200	300	500	1000	1500	3000	6000	10000	PB 1000	Treatment period	Ref.
Histopathology													
Thyroid	Follicular cell hypertrophy	0/21	0/21		1/21		0/21	7/21**				52 weeks	Sommer (2011b)
		2/51	1/51		0/51		0/51	6/50				104 weeks	Rached (2012a)
		1/10						0/10	2/10			7 days	Okuda (2012c)
		2/10						2/10	6/10			14 days	
		0/10	0/10		0/10		0/10	0/10			3/10	7 days	Okuda (2012d)
		1/10						4/10		7/10 **		7 days	Okuda (2012g)
		1/10						7/10 **		7/10 **		14 days	
Thyroid weight													
Absolute	mean ± SD	20 ± 2.8						19±3.0	19±2.6			7 days	Okuda (2012c)
	vs. control, fold	1.00						0.95	0.95				
	mean ± SD	20 ± 3.4						19±2.2	21±3.1			14 days	
	vs. control, fold	1.00						0.95	1.05				
	mean ± SD	20 ± 2.6						18±2.9			22±2.0*	7 days	Okuda (2012d)
	vs. control, fold	1.00						0.90			1.10		
Relative	mean ± SD	5.9 ± 0.90						6.0±0.95	5.9±1.04			7 days	Okuda (2012c)
	vs. control, fold	1.00						1.02	1.00				
	mean ± SD	5.6 ± 0.87						5.6±0.67	6.4±0.89			14 days	
	vs. control, fold	1.00						1.00	1.14				
	mean ± SD	6.0 ± 0.67						5.7±0.80			6.6±0.57*	7 days	Okuda (2012d)
	vs. control, fold	1.00						0.95			1.10		
Enzyme activities													
UGT activity (pmol/min/mg S9 protein)	mean ± SD	0.41 ± 0.050						0.50±0.044**	0.51±0.009**			7 days	Okuda (2012c)
	vs. control, fold	1.00						1.22	1.24				
	mean ± SD	0.38 ± 0.045						0.47±0.030*	0.49±0.061**			14 days	
	vs. control, fold	1.00						1.24	1.29				
TSH level (ng/mL)	mean ± SD	7.0 ± 3.28						6.9±2.58	7.1±2.42			7 days	Okuda (2012c)
	vs. control, fold	1.00						0.99	1.01				
	mean ± SD	6.0 ± 1.55						6.1±1.60	6.5±1.71			14 days	
	vs. control, fold	1.00						1.02	1.08				
	mean ± SD	5.3±1.37						6.6±4.08		8.3 [a]		7 days	Okuda (2012g)
	vs. control, fold	1.00						1.25		1.57			
	mean ± SD	5.5 [a]						5.8±1.09		6.9±2.22		14 days	
	vs. control, fold	1.00						1.05		1.25			
T3 level (ng/mL)	mean ± SD	0.5 ± 0.12						0.6±0.16	0.6±0.09			7 days	Okuda (2012c)
	vs. control, fold	1.00						1.20	1.20				
	mean ± SD	0.4 ± 0.10						0.5±0.11	0.5±0.12			14 days	
	vs. control, fold	1.00						1.25	1.25				
	mean ± SD	0.5±0.13						0.5±0.12		0.6±0.07		7 days	Okuda (2012g)
	vs. control, fold	1.00						1.00		1.20			
	mean ± SD	0.5±0.05						0.6±0.45		0.5±0.13		14 days	
	vs. control, fold	1.00						1.20		1.00			
T4 level (μg/dL)	mean ± SD	4.47 ± 0.613						4.80±0.707	4.42±0.795			7 days	Okuda (2012c)
	vs. control, fold	1.00						1.07	0.99				
	mean ± SD	3.75 ± 0.616						3.77±0.678	4.03±0.855			14 days	
	vs. control, fold	1.00						1.01	1.07				
	mean ± SD	5.44±1.177						4.44±0.831*		3.95±0.642**		7 days	Okuda (2012g)
	vs. control, fold	1.00						0.82		0.73			
	mean ± SD	5.16±0.601						4.73±1.097		4.32±0.805		14 days	
	vs. control, fold	1.00						0.92		0.84			

Statistical significance (*p<0.05, ** p<0.01). Highlighted data represent alterations with toxicological significance.

PB; sodium phenobarbital.

III. Summary of key and associative events in female rats treated with S-1563

		Females Dose (ppm)										Treatment period	Ref.
		0	200	300	500	1000	1500	3000	6000	10000	PB 1000		
Neoplastic findings													
Hepatocellular tumors	Adenoma	0/51 (0.0%)	0/51 (0.0%)		1/51 (2.0%)		1/51 (2.0%)	4/51 (7.8%)				104 weeks	Rached (2012a)
	Carcinoma	0/51 (0.0%)	0/51 (0.0%)		0/51 (0.0%)		0/51 (0.0%)	1/51 (2.0%)					
	Combined	0/51 (0.0%)	0/51 (0.0%)		1/51 (2.0%)		1/51 (2.0%)	5/51 (9.8%)*					
Preneoplastic findings													
Hepatic foci	Eosinophilic cell foci	2/51	0/51		2/51		5/51	9/51*					Rached (2012a)
Liver weight													
Relative	mean ± SD	3.05		2.91		3.30		3.87**	4.46**			13 weeks	Sommer (2011a)
	vs. control, fold	1.00		0.95		1.08		1.27	1.46				
	mean ± SD	2.62	2.87		3.09**		3.59**	3.98**				52 weeks	Sommer (2011b)
	vs. control, fold	1.00	1.10		1.20		1.37	1.52					
	mean ± SD	2.32	2.30		2.44		2.79**	3.39**				104 weeks	Rached (2012a)
	vs. control, fold	1.00	0.99		1.05		1.20	1.46					
Absolute	mean ± SD	7.58±0.699	8.21±0.679		7.93±0.933		8.13±0.705	8.43±0.514*				7 days	Okuda (2012e)
	vs. control, fold	1.00	1.08		1.05		1.07	1.11					
	mean ± SD	8.64±0.896						9.10±0.961				14 days	
	vs. control, fold	1.00						1.05					
Relative	mean ± SD	3.44±0.226	3.75±0.380*		3.57±0.223		3.80±0.221**	3.98±0.195**				7 days	
	vs. control, fold	1.00	1.09		1.04		1.10	1.16					
	mean ± SD	3.66±0.405						4.09±0.283*				14 days	
	vs. control, fold	1.00						1.12					
Cell proloferation													
BrdU labeling index	mean ± SD	3.84±1.86	4.41±2.18		4.5±1.68		6.50±1.94*	10.73±6.68**				7 days	Okuda (2012e)
	vs. control, fold	1.00	1.15		1.17		1.69	2.79					
	mean ± SD	1.65±0.99						2.72±1.14*				14 days	
	vs. control, fold	1.00						1.65					
Enzyme activities													
CYP2B activity	mean ± SD	1.7±0.37	1.5±0.23		1.7±0.23		3.2±1.04*	10.5±4.73*				7 days	Okuda (2012e)
	vs. control, fold	1.00	0.88		1.00		1.88	6.18					
	mean ± SD	1.6±0.38						11.0±6.06*				14 days	
	vs. control, fold	1.00						6.88					
CYP4A activity	mean ± SD	162±27.3	131±24.2		132±34.2		131±17.7	142±37.1				7 days	
	vs. control, fold	1.00	0.81		0.81		0.81	0.88					
	mean ± SD	118±27.5						163±42.1				14 days	
	vs. control, fold	1.00						1.38					
Liver histopathology													
Light micoscopy	Hepatocellular Hypertrophy	0/12		0/12		0/12		3/12	7/12**			13 weeks	Sommer (2011a)
		3/21	6/21		14/21**		12/21**	15/21**				52 weeks	Sommer (2011b)
		0/51	0/51		0/51		3/51	10/51**				104 weeks	Rached (2012a)
		0/10	0/10		0/10		0/10	3/10				7 days	Okuda (2012e)
		0/10						0/10				14 days	
Electron microscopy	Proliferation of SER	0/2							2/2			13 weeks	Sommer (2011a)

Statistical significance (*p<0.05, ** p<0.01).

Highlighted data represent alterations with toxicological significance.

PB; sodium phenobarbital.

IV. Summary of key and associative events in male mice treated with S-1563

		Males Dose(ppm) 0	600	700	2000	2500	4500	5500	7000	Treatment period	Ref.
Neoplastic findings											
Hepatocellular tumors	Adenoma	7/52 (13.5%)	7/52 (13.5%)			1/52* (0%)		3/52 (5.8%)		78 weeks	Rached (2012b)
	Carcinoma	3/52 (5.8%)	4/52 (7.7%)			0/52 (0%)		1/52 (1.9%)			
	Combined	10/52 (19.2%)	11/52 (21.2%)			1/52 (1.9%)		4/52 (7.7%)			
Liver weight											
Relative	mean ± SD	5.31		5.53	6.08*		6.83**		7.64**	13 weeks	Sommer (2011c)
	vs. control, fold	1.00		1.04	1.15		1.29		1.44		
	mean ± SD	4.54	5.02			5.59**		6.57**		52 weeks	Rached (2012b)
	vs. control, fold	1.00	1.11			1.23		1.45			
	mean ± SD	4.65	5.37**			5.85**		7.22**		78 weeks	
	vs. control, fold	1.00	1.15			1.26		1.55			
Absolute	mean ± SD	1.93±0.130						2.33±0.174**		7 days	Yamada (2012a)
	vs. control, fold	1.00						1.21			
	mean ± SD	1.99±0.149						2.52±0.243**		14 days	
	vs. control, fold	1.00						1.27			
	mean ± SD	2.17±0.190	2.22±0.122			2.39±0.214*		2.74±0.181**		7 days	Yamada (2012b)
	vs. control, fold	1.00	1.02			1.10		1.26			
	mean ± SD	2.28±0.240						2.23±0.221		7-day treatment + 7-day recovery	
	vs. control, fold	1.00						0.98			
Relative	mean ± SD	5.42±0.243						6.64±0.374**		7 days	Yamada (2012a)
	vs. control, fold	1.00						1.23			
	mean ± SD	5.43±0.240						7.01±0.470**		14 days	
	vs. control, fold	1.00						1.29			
	mean ± SD	5.43±0.227	5.71±0.262			6.16±0.371**		7.06±0.238**		7 days	Yamada (2012b)
	vs. control, fold	1.00	1.05			1.13		1.30			
	mean ± SD	5.77±0.378						5.68±0.346		7-day treatment + 7-day recovery	
	vs. control, fold	1.00						0.98			
Cell proloferation											
BrdU labeling index	mean ± SD	0.49±0.54						3.82±3.25**		7 days	Yamada (2012a)
	vs. control, fold	1.00						7.80			
	mean ± SD	0.68±0.64						1.59±1.18*		14 days	
	vs. control, fold	1.00						2.34			
	mean ± SD	3.06±2.24	4.99±5.00			5.97±3.45		12.19±6.16**		7 days	Yamada (2012b)
	vs. control, fold	1.00	1.63			1.95		3.98			

Statistical significance (*p<0.05, ** p<0.01).

Highlighted data represent alterations with toxicological significance.

IV. Summary of key and associative events in male mice treated with S-1563 (continued)

		Males Dose(ppm) 0	600	700	2000	2500	4500	5500	7000	Treatment period	Ref.
Enzyme activities											
CYP2B10 mRNA	mean ± SD	100±64.78						5361±613.81**		7 days	Yamada (2012a)
	vs. control, fold	1.00						53.61			
	mean ± SD	100±71.98						3222±402.96**		14 days	
	vs. control, fold	1.00						32.22			
CYP2B activity	mean ± SD	3±1.6	12±6.9*			29±11.8*		41±10.4*		7-day treatment	Yamada (2012b)
	vs. control, fold	1.00	4.00			9.67		13.67			
	mean ± SD	5±1.2						7±2.4		7-day treatment + 7-day recovery	
	vs. control, fold	1.00						1.40			
CYP4A10 mRNA	mean ± SD	100±49.61						494±176.57**		7 days	Yamada (2012a)
	vs. control, fold	1.00						4.94			
	mean ± SD	100±41.23						477±277.32*		14 days	
	vs. control, fold	1.00						4.77			
CYP4A activity	mean ± SD	148±68.3	130±50.9			146±38.1		273±67.3**		7 days	Yamada (2012b)
	vs. control, fold	1.00	0.88			0.99		1.84			
	mean ± SD	93±22.7						70±55.7		7-day treatment + 7-day recovery	
	vs. control, fold	1.00						0.75			
Liver histopathology											
Light micoscopy	Hepatocellular Hypertrophy	0/12		0/12	0/12		6/12**		10/12**	13 weeks	Sommer (2011c)
		3/12	0/12			1/12		2/12		52 weeks	Rached (2012b)
		12/52	12/52			35/52**		32/52**		13 weeks	Sommer (2011c)
		1/10						4/10		7 days	Yamada (2012a)
		1/10						7/10**		14 days	
		0/10	0/10			4/10*		9/10**		7 days	Yamada (2012b)
		0/10						0/10		7-day treatment + 7-day recovery	
Electronmicroscopy	Proliferation of SER	0/2						2/4		7 days	

Statistical significance (*p<0.05, ** p<0.01).

Highlighted data represent alterations with toxicological significance.

V. Summary of key and associative events in female mice treated with S-1563

		Females Dose (ppm) 0	600	700	2000	2500	4500	5500	7000	Treatment period	Ref.
Neoplasticl findings											
Hepatocellular tumors	Adenoma	0/52 (0%)	0/52 (0%)			0/52 (0%)		1/52 (1.9%)		78 weeks	Rached (2012b)
	Carcinoma	0/52 (0%)	0/52 (0%)			0/52 (0%)		0/52 (0%)			
	Combined	0/52 (0%)	0/52 (0%)			0/52 (0%)		1/52 (1.9%)			
Liver Weight											
Relative	mean ± SD	5.04		5.33	5.76		6.85**		7.73**	13 weeks	Sommer (2011c)
	vs. control, fold	1.00		1.06	1.14		1.36		1.53		
	mean ± SD	4.61	5.06			5.83**		7.03**		52 weeks	Rached (2012b)
	vs. control, fold	1.00	1.10			1.26		1.52			
	mean ± SD	4.77	4.90			5.89**		7.15**		78 weeks	
	vs. control, fold	1.00	1.03			1.23		1.50			
Absolute	mean ± SD	1.51±0.123	1.67±0.194			1.96±0.156**		2.33±0.427**		7 days	Yamada (2012c)
	vs. control, fold	1.00	1.11			1.30		1.54			
	mean ± SD	1.57±0.073						2.42±0.185**		14 days	
	vs. control, fold	1.00						1.54			
	mean ± SD	1.50±0.155						1.49±0.141		7-day treatment + 7-day recovery	
	vs. control, fold	1.00						0.99			
Relative	mean ± SD	5.63±0.312	5.56±0.609			6.53±0.280**		7.62±0.867**		7 days	
	vs. control, fold	1.00	0.99			1.16		1.35			
	mean ± SD	5.02±0.184						7.70±0.406**		14 days	
	vs. control, fold	1.00						1.53			
	mean ± SD	5.05±0.560						5.26±0.473		7-day treatment + 7-day recovery	
	vs. control, fold	1.00						1.04			
Cell proloferation											
BrdU labeling index	mean ± SD	2.43±3.18	4.29±7.32			7.10±5.10*		20.70±11.61**		7 days	Yamada (2012c)
	vs. control, fold	1.00	1.77			2.92		8.52			
	mean ± SD	3.25±2.43						5.68±5.18		14 days	
	vs. control, fold	1.00						1.75			
	mean ± SD	NE						NE		7-day treatment + 7-day recovery	
	vs. control, fold	-						-			

Statistical significance (*p<0.05, ** p<0.01).

Highlighted data represent alterations with toxicological significance.

V. Summary of key and associative events in female mice treated with S-1563 (continued)

		Females Dose (ppm) 0	600	700	2000	2500	4500	5500	7000	Treatment period	Ref.
Enzyme activities											
CYP2B activity	mean ± SD	35±8.0	72±8.5*			123±6.9*		146±19.4*		7 days	Yamada (2012c)
	vs. control, fold	1.00	2.06			3.51		4.17			
	mean ± SD	32±8.8						146±14.2**		14 days	
	vs. control, fold	1.00						4.56			
	mean ± SD	39±16.8						55±14.8		7-day treatment + 7-day recovery	
	vs. control, fold	1.00						1.41			
CYP4A activity	mean ± SD	212±35.7	282±17.8*			437±32.6**		544±65.0**		7 days	
	vs. control, fold	1.00	1.33			2.06		2.57			
	mean ± SD	208±46.2						565±44.3**		14 days	
	vs. control, fold	1.00						2.72			
	mean ± SD	305±55.2						322±41.2		7-day treatment + 7-day recovery	
	vs. control, fold	1.00						1.06			
Liver histopathology											
Light micoscopy	Hepatocyte Hypertrophy	0/12		0/12	1/12		10/12**		12/12**	13 weeks	Sommer (2011c)
		0/12	1/12			5/12*		9/12**		52 weeks	Rached (2012b)
		4/52	2/52			37/52**		47/52**		78 weeks	Sommer (2011c)
		0/10	0/10			3/10		7/8**		7 days	Yamada (2012c)
		0/10						10/10**		14 days	
		0/6						0/6		7-day treatment + 7-day recovery	
Electronmicroscopy	Hepatocyte, proliferation of SER #	0/2						2/2		7 days	

Statistical significance (*p<0.05, ** p<0.01).

Highlighted data represent alterations with toxicological significance.