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Increase in thyroid follicular cell tumors in nelfinavir-treated rats observed in a 2-year carcinogenicity study is consistent with a rat-specific mechanism of thyroid neoplasia[a]

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The carcinogenic potential of nelfinavir mesylate (nelfinavir) was evaluated in a 2-year oral (gavage) study on Sprague–Dawley rats at dose levels of 0 (control), 0 (vehicle control), 100, 300 and 1000 mg/kg per day. At the end of the treatment, increased incidences of thyroid follicular cell hyperplasia and neoplasms were observed at 300 (males) and 1000 mg/kg per day (both sexes). There were no other treatment-related effects and no tumors at other sites. Results from previous studies indicated a number of effects in the liver and thyroid, as well as metabolic profiles that suggested nelfinavir might cause thyroid hyperplasia/neoplasia secondary to hormone imbalance by altering thyroid hormone disposition. To investigate this hypothesis, the effects of nelfinavir on gene expression in rat hepatocytes and liver slices (*in vitro*), thyroxine plasma clearance, and thyroid gland function were evaluated. Compared to controls, gene expression analyses demonstrated an increased expression of glucuronyltransferase (UDPGT) and CYP450 3A1 in nelfinavir-treated rat hepatocytes and liver slices. In rats treated with nelfinavir (1000 mg/kg per day) for 4 weeks, liver weights and centrilobular hepatocellular hypertrophy were increased and minimal to mild diffuse thyroid follicular cell hypertrophy and follicular cell hyperplasia were evident in the thyroid gland. Thyroid-stimulating hormone (TSH) levels were significantly increased (three-fold), while tri-iodothyronine (T_3)/tetra-iodothyronine (T_4) and reverse T_3(rT_3) levels were unchanged, indicating that a compensated state to maintain homeostasis of T_3/T_4 had been achieved. Plasma ^{125}I-thyroxine clearance was increased and the plasma thyroxine AUC_{0-48} was decreased (24%) compared to control. In conclusion, these data indicate that thyroid neoplasms observed in the nelfinavir-treated rats were secondary to thyroid hormone imbalance. Increased thyroxine clearance contributes to the effects of nelfinavir on thyroid gland function and is probably a result of UDPGT induction that leads to elevated TSH levels in the rat and eventual thyroid neoplasia. These results are consistent with a well-recognized rat-specific mechanism for thyroid neoplasms. *Human & Experimental Toxicology* (2005) **24**, *643–654*

Key words: antiviral; carcinogenicity; HIV; nelfinavir; T_3; T_4; thyroid gland function, thyroid hormone; thyroxine

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Introduction

Nelfinavir mesylate (VIRACEPT®) is a potent and specific inhibitor of human immunodeficiency virus (HIV)-1 protease (Ki 1.7 nM) with documented activity in *in vitro* test systems and *in vivo* with HIV-infected patients. It is highly protein bound (>98%) in humans to albumin and α_1-acid glycoprotein (AAG). The primary pathway of metabolism

is through CYP3A4 and is qualitatively similar in animals and humans. Both induction and inhibition of CYP3A4 occurs following repeated treatment.

In routine non-clinical rodent toxicity studies, nelfinavir was generally well-tolerated up to the maximum feasible dose of 1000 mg/kg per day, which supported the safety of the drug in clinical therapeutic regimens. Developmental and reproductive toxicity studies in rats and rabbits revealed no adverse effects associated with nelfinavir exposure.[1,2] Additionally, nelfinavir is not immunotoxic.[3] There was no evidence for genotoxicity in a comprehensive battery of tests (Ames reverse mutation assay in bacteria, *in vitro* mouse lymphoma forward mutation assay, *in vitro* chromosomal aberrations in human peripheral blood lymphocytes (HPBLs), and the *in vivo* mouse micronucleus test; unpublished observations).

In two 4-week toxicity studies and a 26-week study in rats, thyroid follicular cell hypertrophy indicative of thyroid-stimulating hormone (TSH) stimulation of the thyroid gland was observed in most of the male and female rats treated with nelfinavir at 200 mg/kg per day or higher. Serum thyroid hormones (tri-iodothyronine, T_3; tetraiodothyronine, T_4, and TSH) were measured in one of the 4-week studies and in the 26-week study described above, and although there was a relatively consistent trend for increased serum TSH levels, the increased TSH levels were not always statistically significant. There was no clear trend for changes in serum T_3 and T_4 levels (unpublished observations).

There is considerable experimental evidence indicating that a sustained disruption in thyroid hormone levels in the rat can lead to the secondary development of hypertrophy and hyperplasia in the thyroid follicular cells of this species and, ultimately, to neoplasia.[4] A common mechanism for induction of these lesions involves the hyperstimulation of the thyroid caused by an overproduction of TSH,[4,5] an event that can be precipitated by direct effects on the thyroid (e.g., inhibition of thyroid hormone synthesis or secretion), or by indirect effects (e.g., hepatic microsomal enzyme induction leading to enhanced thyroid hormone clearance and a compensatory increase in TSH).[5–7]

From earlier studies, it has been postulated that the thyroid changes in the rat may be due to microsomal enzyme induction leading to an increased clearance of thyroid hormone and a compensatory increase in pituitary TSH, a relatively commonly observed phenomenon with high dosages of pharmaceutical agents that can cause hepatic metabolic enzyme induction. Thyroid follicular cell hypertrophy may be a consequence of increased levels of hepatic microsomal enzymes.[6,7] Evidence for microsomal enzyme induction by nelfinavir included increased liver weights in rats, decreased plasma nelfinavir levels with repeated dosing, and mild to moderate induction of the metabolism of several CYP450 substrates.

Following an accelerated approval process in 1997, nelfinavir has been marketed worldwide for the treatment of HIV-infected individuals. The carcinogenicity and follow-on studies described herein were performed to complete the comprehensive safety evaluation of this drug, and were completed as Phase 4 commitments to the US Food and Drug Administration (FDA) and/or the Committee for Medicinal Products for Human Use (CHMP).

Materials and methods

Animal welfare statement

All animal husbandry and experimental procedures described in this study were conducted in compliance with the US Department of Agriculture Animal Welfare Act Regulations and the *Guide for the Care and Use of Laboratory Animals*,[8] and were approved by the performing laboratory's Institutional Animal Care and Use Committee prior to initiation of the studies. The numbers of animals used in all studies were the minimum that is consistent with scientific integrity and regulatory acceptability. The studies contained herein do not unnecessarily duplicate any previous work.

GLP compliance statement

The studies described herein were performed in adherence with Good Laboratory Practice (GLP) Regulations except for the *in vitro* gene expression studies.

Carcinogenicity study

Animals Sprague–Dawley Crl:CD(SD)®BR rats were obtained from Charles River Laboratories, Raleigh, NC. Following a 1-week period of acclimation, animals were randomized into the following study subsets: main study (60/sex per group) and toxicokinetics (6/sex per group for the vehicle control and 10/sex per group for the nelfinavir-treated animals). A third subset of rats was treated for 1 or 6 months and evaluated for immunotoxicity and those data are reported elsewhere.[3] The main study included an untreated control group, a vehicle control group (1% carboxymethlycellulose, 400 cps), and three nelfinavir treatment groups at doses of 100, 300 or 1000 mg/kg per day. The

toxicokinetics subset included the vehicle control and the three nelfinavir treatment groups.

Rats were housed individually in wire mesh cages unless poor health (e.g., footpad lesions or masses that limited access to food) necessitated individual housing in polycarbonate caging for variable time periods during the second year of the study. Animals were fed pelleted PMI Certified Rodent Diet (No. 5002) for the duration of the study. Powdered feed (PMI Certified Rodent Diet No. 5002) was provided, if necessary for health reasons. All animals were allowed access to food and water *ad libitum*. Environmental conditions (temperature, relative humidity, air changes, and light) were maintained at appropriate levels for the species. At the initiation of dosing, rats were 6–7 weeks old.

Test article Nelfinavir mesylate (Agouron Pharmaceuticals; white powder 99.7% pure; Figure 1) was prepared in a 1% aqueous carboxymethlycellulose (CMC) vehicle and was administered to rats twice daily as equally divided doses by oral gavage for 104 weeks at 0 (control), 0 (vehicle control), 100, 300, or 1000 mg/kg per day (i.e., 50, 150, or 500 mg/kg per dose). The dosing volume was 5 mL/kg per dose (10 mL/kg per day). This high dose represents the maximum feasible dose due to viscosity of the dosing preparations. Homogeneity, stability, and concentration analyses were performed on the test article formulation at intervals throughout the study and the data were generally within acceptable limits (10% variability), with occasional excursions which did not impact on the study.

Observations and evaluations Animals were observed twice daily for evidence of mortality or moribundity. Animals on the Main Study were observed once daily for the first 2 weeks (approximately 1 hour after the second dose) and any abnormal findings were recorded. Additionally, once weekly a detailed physical exam was performed on each rat. For grossly visible or palpable masses, the following was recorded: time of onset, location, qualitative size, appearance, and progression. Body weights were recorded for all rats at randomization, weekly for Weeks 1–14, once each month thereafter and at study termination. Food consumption was recorded weekly for the main study animals only for Weeks 1–13 and then monthly through Week 104.

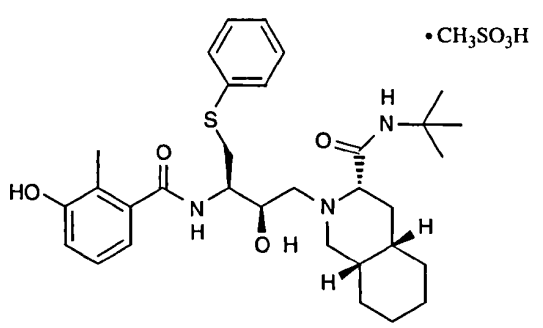

Figure 1 Structure of nelfinavir mesylate.

During Weeks 52 and 78, and at study termination 10 rats/sex per group on the Main Study were bled for evaluation of RBC, WBC, differential blood cell count, blood cell morphology, and thyroid stimulating hormone (TSH) determination. Blood was collected from fasted, unanesthetized animals by jugular venipuncture. EDTA was used in tubes for hematology. Samples for serum TSH were frozen (−70°C) and shipped to Analytics Inc., Gaithersburg, MD, for analysis. Rats were fasted prior to blood collection. On Day 1 and after 6, 12, and 18 months of treatment, blood samples were taken from animals in the Toxicokinetics segment at 8 and 24 hours after the first daily dose. Animals were not fasted prior to blood collection. Following the final blood collection, animals in the Toxicokinetics segment were euthanized, but not necropsied.

All animals in the Main Study that died unexpectedly or were euthanized at an unscheduled interval were weighed and subjected to a complete necropsy. Animals surviving to scheduled euthanasia were fasted overnight, weighed, and euthanized by exsanguination under sodium pentobarbital anesthesia. Complete necropsies were performed and tissues collected for histological evaluation. Tissues from rats in the two control groups and in the high dose group, and all rats in the low and mid doses that died or were euthanized at an unscheduled interval, were examined microscopically. Additionally, the liver, lungs, kidneys, thyroid, and all macroscopic lesions were examined microscopically from all dose groups.

Toxicokinetic analyses Blood was collected from unanesthetized animals by jugular venipuncture into sodium-heparinized tubes. Blood was collected from three (control) or five (treated) rats per sex on Day 1 and during the sixth month of exposure for plasma drug concentration analysis. Samples were collected at 8 and 24 hours after the first daily dose. Timed sampling was designed to represent approximately the C_{max} and C_{min} plasma levels of the drug following bid dosing, as described above. Rats were not fasted prior to blood collection. Samples were centrifuged and plasma collected. Plasma samples were stored at approximately −70°C until shipped to the analytical laboratory (Cedra Corporation, Austin, TX) for evaluation using a Micromass (VG)

Quattro II LC-MS/MS instrument equipped with an HPLC system (Shimadzu LC-600) and a C18 column. The analytical method was validated for quantification of 15–7500 ng/mL nelfinavir in rat plasma. Quantification was performed using a $1/x$ weighted linear least squares regression line generated from plasma calibration samples.

Statistics Statistical evaluation was performed on Main Study animals only. Treated groups were compared to the data for the same sex in the vehicle control (body weight, food consumption, clinical pathology). The vehicle control was also compared to the untreated control. Tests for homogeneity of variance and ANOVA were evaluated at the $P \leq 0.05$ level. Non-homogeneous data were rank-transformed. If transformation did not achieve variance homogeneity, the analyses were performed on the rank-transformed data. Group comparisons were evaluated at the two-tailed $P \leq 0.05$ level.

Adjusted survival data was analysed by the NCI life table package,[9] and included graphical (Kaplan–Meier product limit estimation curves), Cox Tarone binary regression methods for trend and heterogeneity, and Gehan–Breslow non-parametric methods for trend and heterogeneity. Non-neoplastic lesions were evaluated by Cochran–Armitage for trend and Fisher–Irwin exact test for heterogeneity.[10] Incidental tumors (neoplastic lesions) were analysed by Dinse–Lagakos logistic prevalence methods for trend and heterogeneity.[11] Rapidly lethal and palpable tumors were analysed as for survival. When the study pathologist could not assign particular occult neoplasms as the cause of death, the information was in the appropriate analysis in the IARC document.[12]

In vitro *gene expression*

Precision-cut liver slices (250 μm) were prepared from untreated male Sprague–Dawley rats using a Krumdieck tissue slicer. Slices were incubated in roller bottles with 95% O_2/CO_2. Hepatocytes were isolated from untreated Sprague–Dawley rats using a two-step *in situ* perfusion with collagenase digestion. Viability prior to experimentation was >90%. One million cells were seeded into collagen-coated six-well culture plates containing 2 mL of culture medium (serum- and phenol-free Williams' Medium E).

After a 2-hour pre-incubation, isolated rat hepatocytes ($n=3$) and liver slices ($n=2$) were treated for 48 hours with the vehicle (0.1% ethanol), nelfinavir (20 μM), indinavir (20 μM), or phenobarbital (50 μM). Total RNA was isolated and cDNA was synthesized using Superscript Choice System (Gibco BRL). For the isolated hepatocytes, three replicate wells of a six-well plate were pooled to provide sufficient RNA for analysis. Subsequent complementary RNA (cRNA) was subjected to analysis by Affymetrix gene array using the rat toxicology genechip (RT-U34) containing 972 probe sets (840 genes). The experiments were repeated two (liver slices) or three (hepatocytes) times. Only affected genes were presented.

Induction of UDP-glycuronyltransferase was determined by Northern blot analysis using RNA from a single assay in liver slices. Total RNA was fractionated on a 1% denaturing agarose gel and transferred to a nylon membrane. The membrane was hybridized with ^{32}P-labeled UGT 2B (Gene Bank Accession No. M13506) cDNA representing the 3′UTR. The density of the blots was determined by phosphorimaging and the values expressed as mRNA band density normalized to 18S rRNA.

Thyroid gland function and thyroxine clearance

Male Sprague–Dawley Crl:CD(SD)® IGS BR rats were obtained from Charles River Laboratories, Montreal, Canada. Following a period of acclimation, animals were randomized into the following studies: thyroid gland function (20 rats/group) and thyroxine clearance (12 rats/group). Two groups of animals were in each study: vehicle control (1% CMC, 400 cps) and nelfinavir-treated (1000 mg/kg per day).

Rats were housed individually in wire mesh cages. Animals were fed pelleted Harlan Teklad Certified Rodent Diet (No. 8728C). All animals were allowed access to food and water *ad libitum*. Environmental conditions (temperature, relative humidity, air changes, and light) were maintained at appropriate levels for the species.

Nelfinavir was prepared in a 1% aqueous CMC vehicle and was administered to rats twice daily as equally divided doses (approximately 6 hours apart) by oral gavage 7 days/week for 4 weeks with 0 (vehicle control) or 1000 mg/kg per day of nelfinavir. The dosing volume was 5 mL/kg per dose (10 mL/kg per day). Homogeneity, stability, and concentration analyses were performed on the test article formulation on Day 1 and Day 28. Animals were observed twice daily for evidence of mortality or moribundity, and once daily for clinical signs of toxicity. Additionally, once weekly a detailed physical exam was performed on each rat. Body weights were recorded for all rats at randomization, on Day 1 and weekly thereafter. Food consumption was recorded weekly beginning at Week −1.

Thyroid gland function study After 28 days of treatment, blood was collected between 08:00 and 11:00 h alternating between control and treated animals until all rats were euthanized; animals were not fasted for collections. Animals were anesthetized with carbon dioxide/oxygen (70/30) and blood (approximately 8 mL) was collected via the vena cava using a 10-mL syringe and needle. Blood samples were placed in serum separator tubes (no anticoagulant) and kept on ice after collection and allowed to clot. Samples were centrifuged and serum from each sample was harvested, divided into four Nunc tubes/animal (approximately 1 mL each), and stored in a freezer, set to maintain −60 to −80°C. Two sample tubes/animal were retained by Covance and two tubes/animal were shipped on dry ice for hormone analyses. Samples were analysed for T_3 and rT_3 by radioimmunoassay and for T_4 and TSH by a chemiluminescence assay (DPC Immulite).

At scheduled necropsy, livers were weighed and organ-to-body weight percentages were calculated. A liver section was taken from the left lateral lobe and preserved in 10% neutral-buffered formalin. The thyroid glands attached to the trachea were collected and preserved in 10% neutral-buffered formalin. The remaining carcass of each animal was discarded. Liver sections from each animal were embedded in paraffin, sectioned, and stained with hematoxylin and eosin and examined microscopically. The fixed thyroid glands, attached to the trachea, were carefully trimmed, weighed and sectioned for semi-quantitative histopathological evaluation for follicular cell hypertrophy, follicular cell hyperplasia, and follicle size.

Organ weight, food consumption, and body weight data of the treated group were compared statistically to the data from the control. Levene's test,[13] was performed to test for variance homogeneity. Non-homogeneous data were rank transformed to stabilize the variance. If the transformation did not achieve homogeneity of variance, the analyses were still performed on the rank-transformed data. One way ANOVA,[14] techniques were used to analyse the data. If the ANOVA was significant ($P \leq 0.05$), Dunnett's t-test[15] was used to compare the treated with the control group. Group comparisons (Group 2 versus Group 1) were evaluated at the 5% two-tailed probability level. Statistical analyses for the thyroid hormone data were analysed by parametric (Cochran's t-test) and non-parametric (Wilcoxon's test) analyses. Statistical significance was set at $P \leq 0.05$.

Thyroxine (T_4) clearance study After 28 days of treatment, rats were injected IV via the tail vein with [^{125}I]-thyroxine. The volume of radiolabeled thyroxine dose formulation administered to each animal was calculated based on the body weight taken on the day of dose administration. Each animal received a dose of approximately 1 μg/kg of T_4 (37 μCi/kg) in a volume of approximately 2 mL/kg. The actual amount of l-[^{125}I] thyroxine administered was determined by weighing the dose syringe before and after dose administration and the weight and volume was recorded. After the dose was administered, but before the needle was removed from the animal, a gauze pad was placed over the injection site and slight pressure was applied as the needle was removed. The gauze pad was saved for radioanalysis. The apparent success of the intravenous administration of radiolabeled thyroxine was recorded.

Blood (approximately 1 mL) was collected via the jugular vein into sodium-heparinized tubes from the first six animals/group at 2, 8, and 24 hours post-dose with l-[^{125}I] thyroxine and from the second six animals/group at 4, 12, and 48 hours post-dose. Samples were uniquely identified to indicate origin and collection time. After the last blood collection, animals were euthanized with an overdose of halothane anesthesia and carcasses were disposed of appropriately. Blood was stored on wet ice in a chilled Kryorack, or at approximately 5°C until centrifuged. Plasma was harvested and transferred to Nunc vials and duplicate samples (0.1 mL) were analysed for radioactivity. The remaining plasma samples were stored at approximately −20°C until no longer needed. The cellular fraction of the blood was discarded. Samples were analysed for radioactivity content by solid scintillation counting (SSC). All samples were analysed in duplicate if sample size allowed, and were counted for at least 5 min or 1 000 000 counts.

Statistical evaluations of body weight and food consumption data were limited to the calculation of means and standard deviations. Concentrations of radioactivity in plasma were evaluated using a non-parametric analysis, the Wilcoxon–Mann–Whitney test.[16,17]

Results

Carcinogenicity study

Survival and clinical observations Adjusted survival through Week 104 is presented in Figure 2. Survival rates for male rats in the untreated control, vehicle control, and 100, 300 and 1000 mg nelfinavir/kg per day were 32, 28, 24, 30, and 28%, respectively, and for females the survival rates

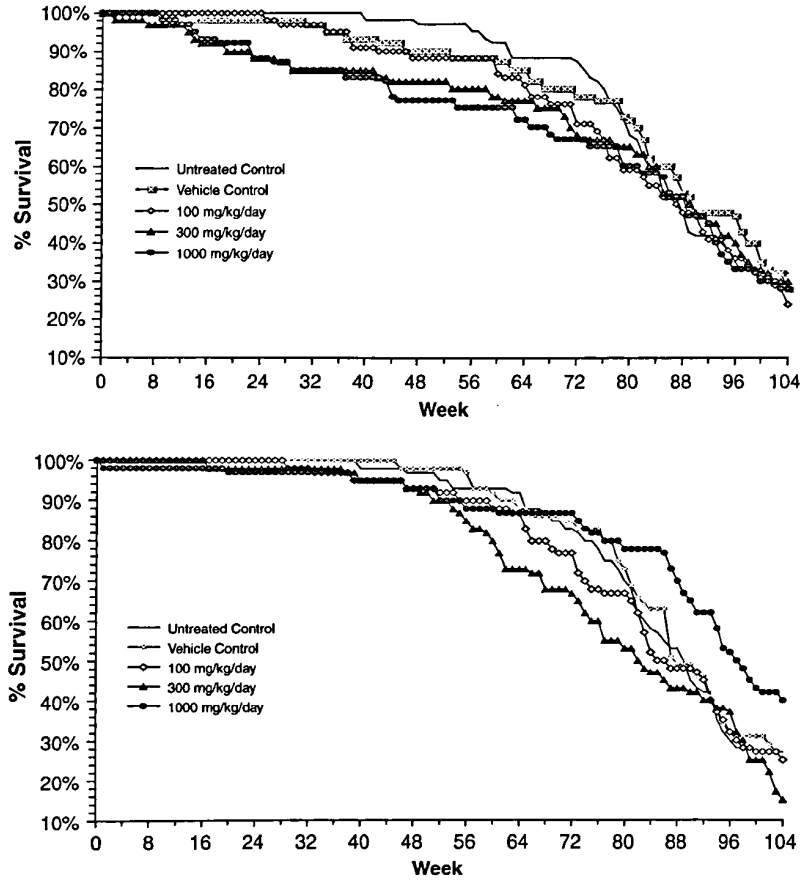

Figure 2 Adjusted survival curves for male rats (top panel) and female rats (bottom panel) in the 2-year carcinogenicity study with nelfinavir.

were 27, 25, 25, 15, and 40%, respectively. There were no apparent nelfinavir-associated adverse effects on adjusted survival or survival rates throughout the study. An increased incidence of slightly abnormal respiratory sounds was observed occasionally in all nelfinavir dosed groups and was considered related to dosing difficulties associated with delivering a test article with increasing viscosity. The 1000 mg/kg per day dose was considered a maximum feasible dose of nelfinavir due to the homogeneity and viscosity of the nelfinavir suspension at an acceptable daily dose volume. There were no other nelfinavir-related clinical signs over the course of the study. Body weights in male rats decreased in the 300 and 1000 mg/kg per day groups during Weeks 22 and 70 (data not shown); however, a dose–response relationship was not evident. There were no significant decreases in mean body weights for female rats, though the body weights for the 1000 mg/kg per day group were consistently lower than the vehicle control during the last year of the study (data not shown). Mean food consumption in nelfinavir-treated groups was generally similar to the untreated controls (data not shown).

Pathology There were no nelfinavir-related changes in the incidence of hematopoietic neoplasia in any of the treated rats (data not shown). Mean TSH levels at Weeks 52, 78, and 105 were similar among the groups. An increased incidence of enlarged liver was observed in male rats (unscheduled deaths) in all treatment groups, though there was no microscopic correlate. At the end of the 104 weeks of treatment, an increased combined incidence of thyroid follicular cell adenomas and follicular cell carcinomas was noted in male (300 and 1000 mg/kg per day) and female (1000 mg/kg per day) rats (Table 1). There were no other nelfinavir-related findings in this study. No other neoplasms were considered related to treatment.

Toxicokinetics Mean plasma nelfinavir concentrations on Day 1 and at Month 18 are presented in Table 2. On Day 1, plasma nelfinavir levels were below the limit of detection by 24 hours after the first daily dose in the low and mid dose groups.

Table 1 Incidence of thyroid follicular cell proliferation in a 2-year carcinogenicity study with nelfinavir

Sex	*Male*					*Female*				
Doses[a]	*NA*	*VH*	*100*	*300*	*1000*	*VH1*	*VH2*	*100*	*300*	*1000*
Scheduled euthanasia										
Number examined	19	17	14	18	17	16	15	15	8	24
Hyperplasia	2	0	1	3	0	0	0	0	0	1
Adenoma	1	0	0	4	4	0	0	0	0	5
Carcinoma	0	0	0	1	1	0	0	0	0	4
Unscheduled deaths										
Number examined	41	43	46	42	43	43	45	45	52	36
Hyperplasia	1	0	1	1	5[b]	0	0	2	2	3[b]
Adenoma	0	0	3	2	3[b]	1	0	1	1	3[b]
Carcinoma	0	1	1	0	2	0	0	0	0	1
All animals										
Number examined	60	60	60	60	60	59	60	60	60	60
Hyperplasia	3	0	2	4	5[b]	0	0	2	2	3[b]
Adenoma	1	0	3	6	7[b]	1	0	1	1	9[b]
Carcinoma	0	1	1	1	3	0	0	0	0	5
Totals										
Adenoma+carcinoma	1	1	4	7	10	1	0	1	1	14

[a]NA: untreated control; VH: vehicle control; all nelfinavir doses are mg/kg per day.
[b]One rat had both hyperplasia and adenoma.

Nelfinavir was detected in the plasma of all high dose rats at this same time point. As expected, at the 18-month evaluation, plasma nelfinavir concentrations were reasonably similar at the 0 (prior to the first morning dose) and 24 hour (prior to the next morning dose) time points in all dose groups. The 8-hour time points in the 100 and 300 mg/kg per day groups showed similar plasma drug exposure, despite the three-fold difference in dose. Plasma nelfinavir concentrations were somewhat increased at the 8-hour time point on Day 1 and at Month 18 at the 100 mg/kg per day dose. This is in contrast to the 300 and 1000 mg/kg per day dose groups, in which the 8-hour evaluation showed a moderate to marked decrease in exposure from Day 1 to Month 18. These data at the higher doses are consistent with autoinduction of nelfinavir metabolism and/or alteration in drug absorption following chronic administration.

In vitro *gene expression*

Table 3 summarizes the genes that were altered in the phenobarbital and nelfinavir-treated hepatocytes and liver slices relative to the vehicle controls. Of the 840 transcripts present on the Affymetrix rat U34 genechip, the only transcripts altered greater than two-fold were related to drug metabolism. Phenobarbital induced CYP 2B1 and 2B2 in excess of 12-fold in hepatocytes and over nine-fold in liver slices, and UDPGT was induced eight-fold in hepatocytes and over 18-fold in liver slices. In hepatocytes, nelfinavir induced CYP 3A1 eight-fold and UDPGT 3.8-fold over the vehicle control, while 11.8-fold and 9.5-fold increases were observed in liver

Table 2 Mean plasma nelfinavir concentrations in unfasted Sprague–Dawley rats

	Mean plasma nelfinavir concentration (ng/mL)[a]					
	100 mg/kg per day		*300 mg/kg per day*		*1000 mg/kg per day*	
Time point	Males	Females	Males	Females	Males	Females
Day 1						
8 hours	2066±580	2598±1184	5348±1474	6172±3977	19400±2526	13070±3181
24 hours	BLD[b]	BLD	BLD	BLD	201±202	745±474
18 months						
0 hours	BLD	BLD	BLD	10.2	83.4	11.9
8 hours	3508±1566	4532±1444	4064±1642	4524±2126	8954±2808	9953±2459
24 hours	BLD	BLD	BLD	6.9	333±267	68.2±40.6

[a]Data are expressed as the mean±standard deviation of the mean. Samples were analysed by LC-MS/MS. No nelfinavir was detected in the plasma of VH control animals (data not shown).
[b]BLD: below detection limit of 15 ng/mL (defined as 0 for calculation of mean±SD).

Table 3 *In vitro* induction of drug metabolizing enzymes by nelfinavir and phenobarbital[a]

Test system	*Treatment*	*Gene transcript*	*Fold induction*[b]
Hepatocytes	Nelfinavir	CYP 3A1	8.0±3.0
	(20 μM)	UDPGT	3.8±1.3
	Phenobarbital	CYP 2B1	13.2±11.5
	(50 μM)	CYP 2B2	12.7±7.6
		UDPGT	8.0±3.0
Liver slices	Nelfinavir	CYP 3A1	11.8±5.2
	(20 μM)	UDPGT	9.5±0.6
	Phenobarbital	CYP 2B1	9.2±2.3
	(50 μM)	CYP 2B2	10.9±5.3
		UDPGT	18.3±5.4

[a]Rat hepatocytes and liver slices were treated with either 20 μM nelfinavir or 50 μM phenobarbital and their mRNA subjected to DNA array analysis using the Affymetrix RT-U34 rat toxicology genechip.
[b]Fold induction of drug treatment versus vehicle control (0.1% ethanol) ± range or standard deviation are shown.

slices for CYP 3A1 and UDPGT, respectively. Confirmation by Northern blot phosphorimage quantitation revealed that UDPGT was increased 20-fold by phenobarbital and 13-fold by nelfinavir (Figure 3). In contrast, UDPGT induction by indinavir (another protease inhibitor) was less than five-fold and difficult to distinguish from control levels that were below the level of detection.

Thyroid gland function

In the 4-week thyroid gland function study, two animals dosed with 1000 mg/kg per day nelfinavir died due to accidental gavage error. No other test article-related adverse clinical signs were observed. No drug-related changes in absolute body weight, body weight gains, or food consumption were noted (data not shown). The serum thyroid stimulating hormone (TSH) levels in rats treated with nelfinavir were significantly elevated (2.65×) compared to the controls (Table 4). No effect of treatment was

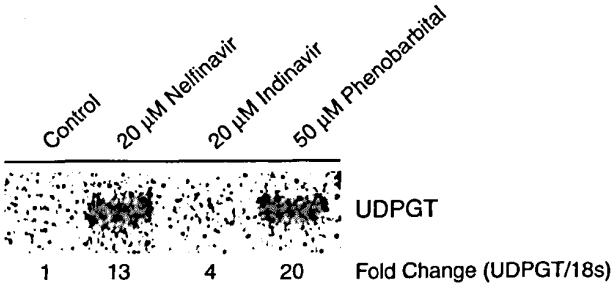

Figure 3 Northern blot analysis of UDP-glycuronyltransferase (UDPGT) in *in vitro*-treated rat liver slices. Isolated rat liver slices were treated for 48 hours with the indicated drug. Control samples were treated with the vehicle (0.1% ethanol). Total RNA was isolated and UDPGT mRNA was probed by Northern blot analysis.

Table 4 Serum thyroid hormone levels in male rats following 4 weeks of treatment with nelfinavir[a]

	T3 (ng/dL)	*rT3 (ng/dL)*	*T4 (μg/dL)*	*TSH (μIU/mL)*
Control	113±5[b]	14.2±0.6	5.9±0.26	0.268±0.03
Nelfinavir	118±6	13.6±0.3	5.4±0.19	0.710±0.09*

[a]Rats (n = 20/group) were treated with the vehicle control (1% CMC) or nelfinavir (1000 mg/kg per day) for 4 weeks. At the end of the exposure period, blood was collected and serum separated and harvested for evaluation of thyroid gland function. Samples from each animal were analysed for the presence of T3 and rT3 by radioimmunoassay, and for T4 and TSH by a chemiluminescence assay.
[b]Data are presented as the mean ± SEM.
*Statistically significant at $P \leq 0.05$.

observed on T_4 (0.91× control), T_3 (1.04× control), or rT_3 (0.96× control).

When compared to the vehicle control, a significant increase in mean absolute liver weight (1.22 × control) and mean liver-to-body weight (1.21 × control) were observed in animals given 1000 mg/kg per day nelfinavir. These changes correlated with an increase in centrilobular hepatocellular hypertrophy (minimal; Table 5) and were consistent with previous observations in rats after repeated dosing with nelfinavir (Pfizer internal reports; data not shown).

Nelfinavir exerted a mild, diffuse hypertrophy in thyroid follicular cells after 4 weeks of treatment as evidenced by the histologic changes observed in the thyroid (Table 5). Follicles in nelfinavir-treated rats were smaller than those in age-matched vehicle

Table 5 Hepatocellular and thyroid follicular cell hypertrophy in male rats following 4 weeks of treatment with nelfinavir[a]

Histopathologic findings	*Control*	*Nelfinavir*
Hepatocellular hypertrophy, minimal[b]	1/20	5/18
Thyroid follicular cell hypertrophy[c]		
Grade 1[d]	20/20	4/18
Grade 2	0/20	10/18
Grade 3	0/20	4/18

[a]Rats (n = 20/group) were treated with the vehicle control (1% CMC) or nelfinavir (1000 mg/kg per day) for 4 weeks. At the end of the exposure period, the livers and thyroid glands were collected and weighed. A section of the left lateral lobe of the liver as well as the thyroid glands were preserved in 10% neutral-buffered formalin. Liver sections were processed for light microscopy. Thyroid glands were sectioned for semi-quantitative histopathology.
[b]Liver weight = 18.7 g (control) and 22.7 g (nelfinavir).
[c]Thyroid weight = 25.1 g (control) and 25.2 g (nelfinavir).
[d]Grade 1: within normal limits for active rat thyroids. Grade 2: minimal diffuse follicular cell hypertrophy; greater than the range observed in age- and sex-matched control rat thyroids. Grade 3: mild diffuse follicular cell hypertrophy; minimal (early), focal or diffuse follicular cell hyperplasia. Thyroid follicles were often smaller with less colloid and frequently had evidence of vacuolation of the eosinophilic colloid. Grade 4: moderate diffuse follicular cell hypertrophy; mild, diffuse follicular cell hyperplasia. Grade 5: severe diffuse follicular cell hypertrophy; moderate diffuse follicular cell hyperplasia.

controls and contained more lightly eosinophilic and finely vacuolated colloid. There were no corresponding changes in the thyroid weight. The histopathologic changes observed were consistent with the observation of a two-fold increase in circulating levels of TSH in nelfinavir-treated rats.

Thyroxine clearance

After 4 weeks of treatment, two animals dosed with 1000 mg/kg per day nelfinavir died due to accidental gavage error. No other test article-related adverse clinical signs were observed. No drug-related changes in body weight or food consumption were noted (data not shown). Animals in the vehicle control and nelfinavir-treated groups received a mean dose of 1.05 ± 0.04 and 1.04 ± 0.04 µg [^{125}I]-thyroxine/kg, respectively. Following 4 weeks of dosing, plasma concentrations of [^{125}I]-thyroxine in the nelfinavir-treated group were less than in the control group at all time points (Figure 4, upper panel). The results of the statistical analysis for the concentration of radioactivity in plasma showed that the difference at each time point was statistically significant (Figure 4, lower panel). The maximum concentration (C_{max}) was observed 2 hours post-dose in both groups (Table 6). The elimination half-life ($t_{1/2}$) was shorter in the nelfinavir-treated group compared to the control group, and the $AUC_{(0-48)}$ was reduced by approximately 24%.

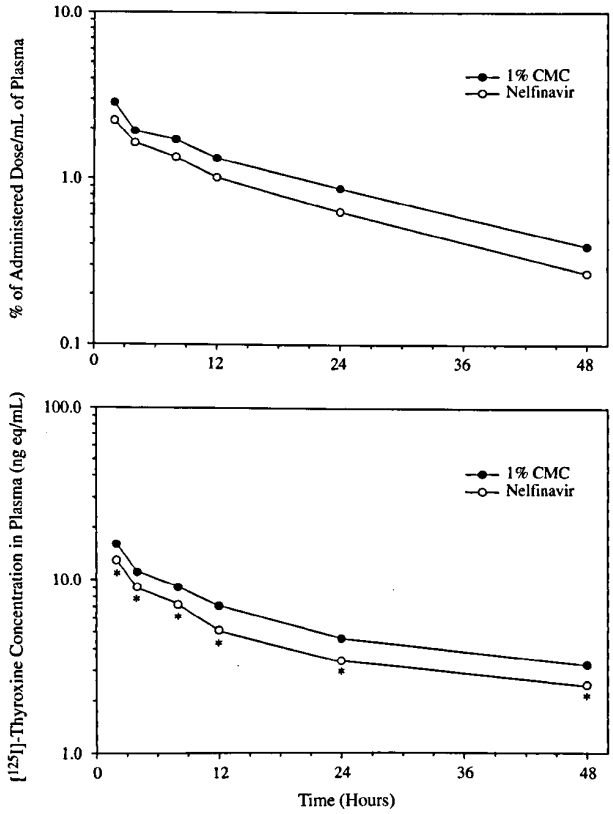

Figure 4 Plasma clearance of [^{125}I]-thyroxine in male rats. Rats ($n = 12$/group) were treated as indicated for 4 weeks. At the end of the exposure period, the remaining animals in both groups were injected intravenously with [^{125}I]-thyroxine. Blood (1 mL) was collected from the first half of the animals in each group at 2, 8 and 24 hours following the administration of the radiolabel. Blood was collected from the remaining animals in each group at 4, 12, and 48 hours following the administration of the radiolabel. Percent of radioactive dose (upper panel) and a corresponding [^{125}I]-thyroxine concentration (ng equivalents/mL; lower panel) were determined. *Statistically significant from 1% CMC at $P \leq 0.05$ as determined by the Wilcoxon–Mann–Whitney test.

Discussion

In the 2-year carcinogenicity study in Sprague–Dawley rats, nelfinavir treatment at dose levels of 0 (untreated control), 0 (vehicle control), 100, 300, and 1000 mg/kg per day resulted in an increase in the combined incidence of thyroid follicular cell adenomas and carcinomas in male rats at the mid-dose of 300 mg/kg per day and male and female rats at the high dose of 1000 mg/kg per day. No tumors were considered related to treatment at sites other than thyroid. The mechanism for inducing follicular cell proliferation that resulted in neoplasms in this 2-year carcinogenicity study was not apparent from the evaluations conducted from the samples collected in the carcinogenicity study. Nelfinavir was previously shown to be negative in both *in vitro* and *in vivo* tests for genotoxicity (unpublished observations), suggesting that the mechanism underlying the follicular cell neoplasms in this study was due to a non-genotoxic mode of action.

One commonly observed mode-of-action for the induction of thyroid follicular cell tumors in rats involves a compound-induced hormonal imbalance in thyroid hormone production or disposition.[6,18,19] In this mechanism, a sustained compensatory increase in the synthesis and secretion of pituitary TSH occurs via a negative feedback system in response to altered thyroid gland function to maintain normal levels of thyroid hormone (T_3 and T_4). Stimulation by TSH results in thyroid follicular cell hypertrophy and hyperplasia, and if sustained in the rat, neoplasia.

There are marked physiological differences in thyroid gland function between rats and humans.[20] Notably, the rodent lacks thyroid binding globulin (TBG), the predominant plasma protein transporting thyroid hormone in human blood, with an affinity for the hormone of three- to five-fold greater than both pre-albumin and albumin (both are present in the rat). Additionally, the plasma half-life of T_4 in the rat is relatively short (12 hours)

Table 6 Mean pharmacokinetic parameters for [^{125}I]-thyroxine in male rats following 4 weeks of treatment with nelfinavir[a]

	T_{max} *(hour)*	C_{max} *(ng/mL)*	$t_{1/2}$ *(hour)*	$AUC_{(0-48)}$ *(ng eqh/mL)*	$AUC_{(0-\infty)}$ *(ng eqh/mL)*
Control	2	15.2	19.3	287	347
Nelfinavir	2	12.2	17.3	217	253

[a]Rats ($n = 12$/group) were treated with the vehicle control (1% CMC) or nelfinavir (1000 mg/kg per day) for 4 weeks. Two animals in the nelfinavir group died as a result of intubation errors. At the end of the exposure period, the remaining animals in both groups were injected intravenously with [^{125}I]-thyroxine. Blood (1 mL) was collected from the first half of the animals in each group at 2, 8, and 24 hours following the administration of the radiolabel. Blood was collected from the remaining animals in each group at 4, 12, and 48 hour following the administration of the radiolabel. Pharmacokinetic parameters were calculated from the determined [^{125}I]-thyroxine concentration (ng equivalents/mL).

compared to humans (5–9 days) and the serum TSH levels are approximately 25-fold greater than in man.[21] This is supported by the differences in the histomorphologic architecture of the thyroid in these species. Primates typically have large follicles with abundant colloid, while the rodent thyroid has large follicles only on the periphery of the gland and comparatively small follicles on the interior that contain small amounts of colloid. These data indicate that the rodent thyroid is significantly more active than the human thyroid and functions at a higher level with respect to thyroid hormone turnover. This is supported by a 2% spontaneous incidence of thyroid follicular cell neoplasia (0.8% carcinoma and 1% adenoma) in the rat compared to approximately 0.004% in humans.[22–24] Additionally, while epidemiologic evidence linking endemic goiter in humans living in iodine-deficient areas to human thyroid cancer has not been able to demonstrate a clear association, rats living in the same areas exhibit a high incidence of thyroid gland neoplasia. Taken together, these data demonstrate that there are clear, significant species differences between humans and rats with respect to the development of thyroid neoplasia secondary to thyroid hormone imbalance.[20] These data also support the hypothesis that small changes in TSH in the rodent may have a significant impact on the development of thyroid neoplasia in this species.

Serum TSH levels were similar between treated and control groups in the carcinogenicity study. In contrast, serum TSH levels were elevated in the definitive 4-week thyroid function study. This apparent discrepancy may be due to methodological factors involved in measuring TSH in the rat, which are variable, or possibly a time-dependent evolution of a new steady state for TSH, a phenomenon that can occur in rats and has been observed for phenobarbital, a drug known to produce thyroid tumors in rats.[25] The statistically significant increase in serum TSH levels without a statistically significant decrease in T_4 or T_3 levels suggests that by 4 weeks of treatment with nelfinavir, a compensated state has been achieved to maintain the normal physiologic levels of T_4 and T_3. Similar effects have been observed with phenobarbital.[6]

The moderate increase in the plasma clearance of T_4 likely resulted from the induction of metabolic mechanisms for thyroxine clearance (e.g., UDPGT). Microsomal enzyme induction by a variety of compounds has been identified as a mechanism that predisposes rats, but not humans, to thyroid neoplasms.[5,6,26,27] The impact of the induction is the enhanced disposition of thyroid hormones resulting in hormone imbalance and a compensatory stimulation in TSH synthesis and secretion. Augmentation of glucuronidation of thyroid hormones in rat liver by UDPGT is directly linked to this mechanism.[19,27–33] A previous study at doses similar to those used in the carcinogenicity study revealed that nelfinavir caused an increase in liver weight and related elevations in hepatic CYP450 activities (unpublished observations). Since the data suggested that nelfinavir might cause thyroid tumors in rats secondary to hormone imbalance via microsomal enzyme induction, the effect of nelfinavir on enzyme induction, including UDPGT, was determined in both rat liver slices and isolated hepatocytes. Similar to phenobarbital, nelfinavir produced an increase in UDPGT, indicating that this may be responsible for the altered thyroid function in rats. Another protease inhibitor, indinavir, also produced an increase in UDPGT. This is consistent with the observation of thyroid hyperplasia and thyroid adenomas in the chronic and carcinogenicity studies performed in rats with this drug.[34] The lower level of induction (4-fold) compared with nelfinavir (13-fold) may be related to the selection of the 20 μM indinavir dose as representative of the high end of human exposure at 800 mg tid. Exposures in the rat carcinogenicity study may have been 2- to 3-fold higher. Additionally, indinavir has been shown as a competitive inhibitor of UDPGT in some *in vitro* systems.[35]

In the present study, toxicokinetic evaluation confirmed the presence of nelfinavir in rats of all dose groups on Day 1 at 8 hours after the first dose. Prior to the morning dose on the following day (Day

1, 24 hours) plasma levels were below the limit of detection in all but the high dose group. On Day 1, plasma nelfinavir concentrations (C_{8hr}) in male and female rats were approximately 0.5- to 0.9-fold (100 mg/kg per day), 1.3- to 2.1-fold (300 mg/kg per day), or 3.3- to 6.5-fold (1000 mg/kg per day) the C_{max} observed in healthy and HIV-infected individuals dosed with either 750 mg tid or 1250 mg bid nelfinavir each day for 28 days.[36] Plasma nelfinavir concentrations in rats were lower in the eighteenth month of dosing compared to Day 1, an observation consistent with previous repeated dosing of nelfinavir in rats,[3] (unpublished observations) and is probably due to induction of metabolic enzyme systems. Despite the decline in systemic drug exposure over time in nelfinavir-treated rats, C_{8hr} values following nelfinavir treatment at 1000 mg/kg per day were 2- to 3-fold the C_{max} values in humans. C_{8hr} values following nelfinavir treatment at 100 or 300 mg/kg per day were approximately 1- to 1.5-fold the C_{max} values in humans. At all doses on Day 1 and at Month 18, C_{8hr} values were comparable to or significantly exceeding the reported human trough levels.[36] While these nelfinavir exposures may seem relatively low, they are generally consistent with exposures obtained in long-term repeat dose studies evaluating other marketed protease inhibitors (data from FDA Summary Basis of Approvals, EMEA Scientific Discussion, and EMEA Summary of Product Characteristics for available protease inhibitors).

In summary, administration of nelfinavir by oral gavage to rats for 2 years resulted in an increased incidence of thyroid follicular cell hypertrophy and hyperplasia and follicular cell neoplasms at 300 mg/kg per day (males) and 1000 mg/kg per day (males and females). The NOAEL for general toxicity was considered to be 1000 mg/kg per day. Nelfinavir is considered a moderate microsomal enzyme inducer as evidenced by increased liver weights, increased incidence of hepatocellular hypertrophy, the pattern of gene expression in *in vitro*-exposed rat liver slices and hepatocytes, and increased plasma clearance of thyroxine in nelfinavir-treated rats. Nelfinavir treatment clearly results in thyroid hormone imbalance in rats as evidenced by a statistically significant increase in serum TSH and the presence of thyroid follicular cell hypertrophy in most of the nelfinavir-treated rats and follicular cell hyperplasia in several treated rats. Based on these data, the thyroid follicular cell neoplasms observed in the carcinogenicity study are considered to be secondary to thyroid hormone imbalance, and that microsomal enzyme induction in nelfinavir-treated rats is likely to contribute to the effect on thyroid gland function. These results are consistent with a well-recognized rat-specific susceptibility for the induction of thyroid follicular cell neoplasms. Due to the fact that the evidence supports the conclusion that nelfinavir treatment results in thyroid follicular cell tumors in rats secondary to thyroid hormone imbalance and the marked species differences in thyroid function and susceptibility to tumor induction between rats and humans, it is unlikely that nelfinavir presents a human thyroid cancer risk.

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