Table 1
Performance of in silico systems

	Ames result	+	−	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
CGX database								
DEREK	+	288	64	352				
	−	69	267	336	81.8	79.5	80.7	97.9
	Total	357	331	688				
MCase	+	235	32	267				
	−	6	249	255	88.0	97.6	92.7	74.3
	Total	241	281	522				
AWorks	+	267	89	356				
	−	149	187	336	75.0	55.7	65.6	98.4
	Total	416	276	692				
ECJ database								
DEREK	+	19	7	26				
	−	21	159	180	73.1	88.3	86.4	100.0
	Total	40	166	206				
MCase	+	13	7	20				
	−	13	133	146	65.0	91.1	88.0	80.6
	Total	26	140	166				
AWorks	+	19	7	26				
	−	54	124	178	73.1	69.7	70.1	99.0
	Total	73	131	204				

MCase: MultiCASE; AWorks: ADMEWorks.

number of chemicals evaluated; and N_{all} is total number of chemicals subjected.

3. Results

Among the set of 703 CGX chemicals with published Ames data, 358 were positive and 345 were negative. The results of the in silico evaluation are summarized in Table 1. The highest sensitivity, specificity, and concordance with Ames assay results was provided by MCase, then followed by DEREK. However, the systems that showed the best applicability were AWorks and (almost the same) DEREK, then followed by MCase. For the database of 206 ECJ chemicals, 26 were positive and 180 were negative. The outcomes of the in silico analyses are summarized in Table 1. The pattern of performance was very similar to that with the 703 chemicals in the CGX database.

Fig. 1 shows the cumulative percent of Ames positive chemicals against molecular weight. It can be seen that 87.1% of those positive chemicals had molecular weights less than 1000, and 96.4% had molecular weights less than 3000; in other words, only 3.6% of the chemicals with a molecular weight >3000 gave a positive response in the Ames assay. Seven of 194 Ames positive chemicals had a molecular weight >3000 and four of these seven polymers had epoxy groups.

When we combined the in silico systems, the performance was different from that when assessed individually (Table 2). If we considered the in silico mutagenicity as positive (or negative) when two or more systems gave positive (or negative) evaluations, 87.8

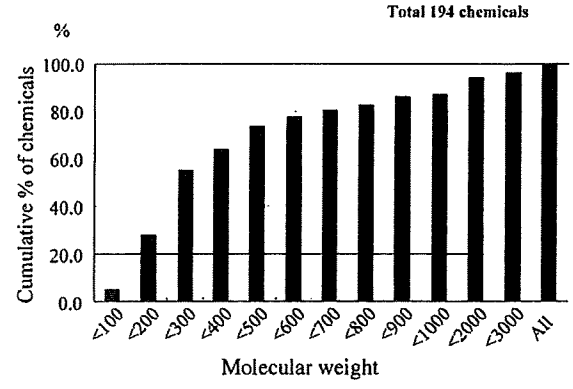

Fig. 1. Cumulative percentage of chemicals based on their molecular weight. 194 Ames positive chemicals were analyzed. 7/194 chemicals were more than 3000 molecular weight and Ames positive and 4/7 contained epoxy groups.

Table 2
Performance of in silico systems after combined

CGX database

In silico / Ames	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	279	40	319				
-	42	249	291	**87.8**	**85.6**	**86.7**	**86.8**
Total	321	289	610				
	+++	---					
+	166	1	167				
-	3	127	130	**99.4**	**97.7**	**98.7**	**42.2**
Total	168	129	297				

ECJ database

In silico / Ames	++ or +++	-- or ---	Total	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)
+	19	7	26				
-	23	147	170	**73.1**	**86.5**	**84.7**	**95.1**
Total	42	154	196				
	+++	---					
+	13	2	15				
-	5	94	99	**86.7**	**94.9**	**93.9**	**55.3**
Total	18	96	114				

Table 3
Performances of DEREK and MCase in several published papers.

Target compounds	In silico system	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)	Reference
394 Drugs	DEREK	52	75	74	94[a]	[11]
	MCase	48	93	90	91[a]	
217 Non-drugs	DEREK	86	50	81	100[a]	[10]
	MCase	91	62	83	100[a]	
520 Drug candidates	DEREK	28	80	72	100	[13]
	MCase	50	86	81	41	
	DEREK + MCase	29	95	88	29	
	DEREK + Mcase + TOPKAT	75	96	95	15	
123 Drug candidates	DEREK	8[b]	31[c]	61	100[d]	[4]
	MCase (A2H)	13[b]	15[c]	72	97[d]	
	Topcat (Ames Mut)	18[b]	15[c]	67	98[d]	
	DEREK + MCase	6[b]	19[c]	75	97[d]	
	DEREK + Mcase + TOPKAT	5[b]	9[c]	86	46[d]	
94 Non-drugs	DEREK	63	81	76	100	[13]
	MCase	40	90	76	75	
	DEREK + MCase	47	100	85	56	
	DEREK + Mcase + TOPKAT	55	100	86	37	
516 Non-drugs	DEREK	6[b]	24[c]	70	100[d]	[4]
	MCase (A2H)	7[b]	12[c]	81	98[d]	
	Topcat (Ames Mut)	25[b]	19[c]	56	97[d]	
	DEREK + MCase	2[b]	16[c]	82	98[d]	
	DEREK + Mcase + TOPKAT	7[b]	10[c]	83	43[d]	

[a] Calculated by us
[b] % False negative.
[c] % False positive.
[d] (1-Indeterminate).

and 73.1% sensitivity, 85.6 and 86.5% specificity, 86.7 and 84.7% concordance, and 86.8 and 95.1% applicability were obtained for the CGX and ECJ databases, respectively. If we considered the in silico mutagenicity as positive (or negative) only when all three systems gave positive (or negative) evaluations, all performance measures (sensitivity, specificity, etc.) increased up to 98.7 and 93.9%. However, applicability decreased to 42.2 and 55.3%, which meant only about half of the chemicals in the CGX and ECJ databases could be evaluated. One chemical, *o*-phenylphenol [90-43-7], was positive in the Ames test but negative by all three in silico systems and three chemicals, carboxymethylnitrosourea [60391-92-6], methidathion [950-37-8], 1-nitroso-3,5-dimethyl-4-benzoylpiperazine [61034-40-0], were negative in the Ames test although all three in silico system gave positive evaluation for mutagenicity in the CGX database. When we used the ECJ database, 2-amino-1-naphthalenesulfonic acid [81-16-3] and 2-vinylpyridine [100-69-6] were positive in the Ames test but negative by all three in silico systems and there was no chemical that was negative in the Ames assay and all positive in in silico system. These exceptional chemicals are listed in Table 3 together with such chemicals taken from literatures.

4. Discussion

It is important to construct a strategy for efficient evaluation of the toxicity of a large number of existing chemicals. Even so-called short-term assays, e.g., Ames assay and in vitro chromosomal aberration assay, can practically assess only 100 chemicals per year according to our experiences in Japan. In this case, it will take 180 years to assess the outstanding 18,000 existing chemicals for genotoxicity, and it will take even longer when repeat dose toxicity tests are also performed, as these are not short-term assays. We therefore need higher-throughput systems to assess chemical safety, or at least to set priorities for those chemicals that should be tested in in vitro and/or in vivo tests. In silico systems have the capability for high throughput but have not yet been well validated for assessment of human hazard, although some regulatory bodies have started to use these methods.

Correlation between the Ames assay result and molecular weight could be explained by the lack of membrane permeability of high molecular weight chemicals, making it more difficult for them to reach target molecules such as DNA and proteins that contribute to the fidelity of cell division. Therefore, only a few chemicals with molecular weight >3000 gave positive responses in the Ames assay. This phenomenon is also true for induction of chromosomal aberrations in vitro (data not shown). The other important issue is the contribution of epoxy group in the polymer. Although of molecular weight >3000, some polymers with an epoxy group gave positive results in both the Ames and chromosomal aberration assays. Epoxy embedding reagents employed in electron microscopy (e.g., epon and araldite) have been reported as mutagenic in the Ames assay [8]. According to these findings, we should include a step to evaluate molecular weight and existence of any epoxy groups in the molecule.

In the present study, we used the CGX database recently published by Kirkland et al. [1] for microbial mutagenicity data on 358 carcinogens and 345 non-carcinogens for validation of three commercially available in silico (Q)SAR systems. When applied individually, MCase gave high sensitivity, specificity, and concordance compared to other two systems. One of the reasons may be because the CGX database contained many results from the U.S. National Toxicology Program (NTP), and the learning dataset of MCase would have used many of the same results. Therefore, some of them were evaluated by direct matching. Moreover, the applicability of MCase was relatively low compared with the other systems in this study (Table 1). MCase judged 119 chemicals as inconclusive and one chemical as marginal, and could not evaluate 67 chemicals. Such selectivity in MCase may contribute to the high concordance. On the other hand, the other systems were not influenced directly by the NTP data. We applied the in silico systems to another dataset, the ECJ database, that does not contain the NTP data and we obtained similar patterns of sensitivity, specificity, etc.

Each in silico system showed different outcomes on some chemicals complimentary by some extent. These different evaluation patterns were mainly due to the different evaluation rules. The DEREK is a rule-based system, AWorks is a discriminant-based system mainly depending on physicochemical descriptors, and MCase is a hybrid system based on a database. Therefore, we concluded that in silico evaluation could be optimized by combining the evaluations from the three systems. Sensitivity, specificity and concordance were increased when we combined the three in silico systems to make a final conclusion of mutagenicity (Table 1). Concordance was much higher after combining but the applicability became poor (42.2%). When two of the in silico systems gave the same evaluations, the applicability (86.8%) was good but the concordance was lower (86.7%) than when all three were combined (98.7).

Recently, several in silico studies for prediction of mutagenicity have been conducted on drugs or non-

Table 4
Exceptional chemicals that showed Ames test gave positive but all three in silico systems (DEREK, MCase, TOPKAT/AWorks) gave negative and Ames test gave negative but all three systems gave positive

Compound	CAS	Ames test	DEREK	MCase	TOPKAT/Aworks	Source[a]
Bupropion	34911-55-2	+	–	–	–	1
Citalopram	59729-33-8	+	–	–	–	1
Naloxone	465-65-6	+	–	–	–	1
Oxcarbazepime	28721-07-5	+	–	–	–	1
Quetiapine	111976-69-7	+	–	–	–	1
Rabeprazole	117976-89-3	+	–	–	–	1
Zolmitriptan	139264-17-8	+	–	–	–	1
2-(2-Methylpropyl) thiazole	18640-74-9	+	–	–	–	2
2-Chloropyridine	109-09-1	+	–	–	–	2
Pyrogallol	87-66-1	+	–	–	–	2
o-Phenylphenol	90-43-7	+	–	–	–	3
2-Amino-1-naphthalenesulfonic acid	81-16-3	+	–	–	–	3
2-Vinylpyridine	100-69-6	+	–	–	–	3
Fosfomycin	23155-02-4	–	+	+	+	1
Toremifene	89778-26-7	–	+	+	+	1
Poly (2-hydroxypropyl methacrylate)	25703-79-1	–	+	+	+	2
Carboxymethylnitrosourea	60391-92-6	–	+	+	+	3
Methidathion	950-37-8	–	+	+	+	3
1-Nitroso-3,5-dimethyl-4-benzoylpiperazine	–	+	+	+	3	

[a] 1: Synder et al. [11] (with TOPKAT), 2: White et al. [13] (with TOPKAT), 3: this study (with AWorks).

drug chemicals with commercially available programs, e.g., DEREK, MCase or TOPKAT, or newly developed computational approaches [4,9–12]. The performances of DEREK and MCase in several of these studies are summarized in Table 4. Generally, similar performance in sensitivity, specificity, concordance, and applicability were shown between DEREK and MCase but with some exceptions, e.g., sensitivity in 520 drug candidates [13], specificity in 516 non-drugs [4], and applicability in 520 pharmaceutical drug candidates and 94 non-drugs [13]. These differences might be due to the chemical class of target compounds in each database. However, there was no remarkable difference in performance whether the chemical was intended for use as a pharmaceutical, agricultural, or industrial agent. Our results on performance of in silico systems showed similarity with the published analyses. With respect to the combination of in silico prediction systems, White et al. [13] reported that combination improved the overall accuracy and specificity, but sensitivity was barely above the 50% level (Table 4). On the other hand, their analysis showed quite low applicability in the combination of three prediction systems, DEREK, MCase and TOPKAT. Our analysis of the combination of DEREK, MCase and AWorks showed good improvements in sensitivity, specificity and concordance, but applicability was low, especially in the 3-system combination.

Exceptional chemicals that gave positive Ames results but were negative in all three in silico systems (DEREK, MCase, TOPKAT/AWorks), and those that were negative in the Ames test but gave positive evaluations in all three systems, are summarized in Table 4. This table, which includes data from Synder et al. [11] and White et al. [13] shows there are 19 exceptional chemicals from both drug and non-drug families. Although it would be unrealistic to expect zero exceptions using this approach, further improvement of the prediction systems is needed. We do not have good reasons to explain the discordance, therefore we will verify the results from both sides, i.e., in silico system and Ames test.

Considering these outcomes, we propose a decision tree (Fig. 2), in order to evaluate chemical induction of gene mutation. We may use the decision tree to prioritize chemicals to be assayed by in vitro and/or in vivo tests. A final goal being that eventually, chemical mutagenicity will be evaluated by in silico systems alone for regulatory use. The decision tree consists of three steps; namely to assess the molecular weight, the existence of epoxy groups, and the in silico evaluation for genotoxicity. Based on the purpose of the in silico evaluation, the tree might be altered by the different final call of the in silico evaluation, i.e., regarding as positive (negative) all three systems show positive (negative). The choice of definition for final call applying to the decision tree should be based on the balance between accuracy of eval-

Arch Toxicol (2005) 79: 627–635
DOI 10.1007/s00204-005-0677-1

REGULATORY TOXICOLOGY

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Ability of the Hershberger assay protocol to detect thyroid function modulators

Received: 27 December 2004 / Accepted: 19 April 2005 / Published online: 10 June 2005

Abstract In vivo screening methods for detection of thyroid function modulators are now under development in many research laboratories. We assessed the applicability of the Hershberger assay protocol to screen for thyroid function modulators. In experiment 1, castrated male BrlHan WIST@Jcl (GALAS) rats were administered a potent thyroid peroxidase inhibitor, 3-amino-1,2,4-triazole (AT), in doses of 0, 40, 200, and 1,000 mg/kg/day with gravimetric endpoint, and in experiment 2, castrated and intact male rats were administered in doses of 0, 40, and 200 mg/kg/day, with quantification of the extent of hypertrophy of the thyroid epithelium, to assess the effects of castration, by gavage to 8-week-old for 10 consecutive days. At necropsy of both experiments, the thyroid glands and hypophysis were collected and fixed with 10% neutral-buffered formalin. To avoid crushing during weighing because of their fragility, the thyroid glands and hypophysis were weighed approximately 24 h after fixation with 10% neutral-buffered formalin. All animals were sacrificed approximately 24 h after the final dose. In experiment 2, the thyroid glands of all animals were stained with hematoxylin and eosin for histological examination and morphometry of follicular epithelial height. In experiment 1, absolute and relative thyroid weights in all of the AT groups were statistically increased in a dose-dependent manner, regardless of the testosterone propionate (TP)-injection. In experiment 2, the results showed a significant increase in thyroid weight in the 200 mg/kg groups of both castrated and intact rats. Hypophyseal weight was unaltered by AT, but comparison of vehicle-treated groups showed that the hypophyseal weight of the castrated rats was greater than that of the intact rats. Enlarged thyroid glands were observed in the AT-treated rats at necropsy. Histological examination of the thyroid glands of all the AT-treated animals showed hypertrophy and hyperplasia of the follicular epithelial cells, and the height of follicular epithelium of the thyroid glands increased in a dose-dependent manner in both the castrated and intact rats. In experiment 1, assessment of the (anti-) androgenic action of AT in seminal vesicle weight revealed a significant increase in the 200 and 1,000 mg/kg + TP groups in a dose-dependent manner. These results suggest that the effect of AT can be detected by the Hershberger assay 10-day administration protocol and may be useful for screening for thyroid function modulators regardless of whether the animals have been castrated.

Keywords Hershberger assay · Thyroid function modulators · 3-amino-1,2,4-triazole · Castrated rat · Intact rat

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Introduction

Currently, there is a great deal of concern that certain environmental chemicals may have the ability to impair normal sexual differentiation and development in humans and wildlife (McLachlan 1993; McLachlan and Korach 1995). Endocrine Disruptor Screening and Testing Committee (EDSTAC) is developing a tiered chemical screening and testing program to evaluate endocrine disrupting effects (EDSTAC 1998). In this program, high priority chemicals will be evaluated for hormonal activity in the early screening stage, and chemicals positive in the screening stage will be tested for hazard in a definitive test (EDSTAC 1998; Gray et al. 2002).

A Hershberger assay in surgically castrated male rats was proposed by the Organization for Economic

Cooperation and Development (OECD) and EDSTAC of the US Environmental Protection Agency as an in vivo screening method to detect the (anti-) androgenic activity of the chemicals acting mainly via androgen receptor (AR)-mediated mechanisms (Hershberger et al. 1953; Dorfman 1969; OECD 1997, 2001, 2003; EDSTAC 1998). Focusing on the disruption of androgenic system, the proposed endpoints of this assay are the weight of the ventral prostate, seminal vesicles, bulbocavernosus/levator ani (BC/LA) muscles, glans penis, Cowper's glands, and liver. The gravimetric endpoint has the advantage of enabling the detection of (anti-) androgenic action of a chemical at low cost in a short time. The Hershberger assay will be used in the early stage of a chemical screening and testing program after the in vitro screening assays, such as the AR binding assay and the AR reporter gene assay (Gray et al. 2002).

In vitro and in vivo screening methods for the detection of thyroid function modulators are now under development in many research laboratories, and a variety of in vivo screening methods have been proposed, e.g., enhanced TG407 and male or female pubertal assay. But they have the disadvantage such as long experimental periods or low cost-performance, and there have been no breakthroughs thus far. Yamada et al. (2004) first reported that the enhanced Hershberger assay evaluation of thyroid histopathology and weights, and determination of serum hormone levels, appears to be reliable for screening thyroid modulators with a single dose of propylthiouracil (PTU), 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE) and phenobarbital (PB). The original objective of the Hershberger assay was to screen for (anti-) androgenic activity of the chemicals in male rats subjected to castration, which is essential for the detection of androgenic activities. If the Hershberger assay is capable of detecting the (anti-) androgenic activity and thyroid hormone modulating activity of the chemicals at the same time, it will be possible to screen for hormonal activity of the chemicals rapidly and with better cost-performance.

Because of the well-known presence of androgen receptors in the thyroid gland of mammalians (Pellietier 2000; Banu et al. 2002), the thyroid gland is speculated to be one of the target organs of androgenic compounds. Moreover, testosterone has a stimulatory effect of on the expression of the TSH mRNA (Ross 1990), and testosterone administration results in a significant decrease in serum T3 and T4 levels in 15-day intact male assays (O'Connor et al. 2000). Thus, if we intend to screen chemicals for (anti-) androgenic activity based on their thyroid hormone modulating activity, effect of castration of male rats on the thyroid should be evaluated. It appears rather unlikely that thyroid-specific endpoints can be used to specifically detect (anti-) androgenicity. However, it is necessary to evaluate whether thyroid-active compounds affect the classical parameters of the Hershberger assay and thus confound the detection of (anti-) androgenicity.

We performed two experiments. Experiment 1 was designed to assess whether thyroid hormone modulating activity of 3-amino-1,2,4-triazole (AT), which is known to inhibit the synthesis of T3 and T4 by peroxidase inhibiting in the thyroid gland (Ealey et al. 1984; Krauss and Eling 1987; Reader et al. 1987; Masuda and Goto 1994; Santini et al. 2003), could be detected by the thyroid gravimetric method, and whether the dose-dependent AT-induced effect on thyroid gland would be confirmed by the Hershberger assay in castrated male rats. Experiment 2 was designed to assess the influence of castration on the AT-induced effect on thyroid gland, and thus, both castrated and intact male rats were subjected to the same protocol to elucidate the effect of castration on the thyroid.

Materials and methods

Chemicals

AT (CAS No. 61-82-5, 99% pure) was obtained from Tokyo Kasei Kougyou (Tokyo, Japan), and olive oil was obtained from Fujimi Pharmaceutical Company (Osaka, Japan). The physical stability of AT was assessed with a Fourier transform infrared spectrophotometer (FTS-135, Nippon Bio-Rad Laboratories K.K., Tokyo, Japan), and the stability, homogeneity, and concentration of each AT suspension prepared for administration were confirmed by HPLC.

Animals

Seven-week-old BrlHan WIST@Jcl (GALAS) rats castrated at 6 weeks of age, and 7-week-old intact BrlHan WIST@Jcl (GALAS) rats were purchased from Clear Japan Inc. (Fuji, Japan). The animals were housed, three per cage, in stainless steel, wiremesh cages throughout the study. The rats were weighed, weight-ranked, and randomly assigned to one of the treatment or control groups. Body weight and clinical signs were recorded daily throughout the study. Rats were given ad libitum access to tap water and a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) and ad libitum access to water from an automatic dispenser. The animal room was maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 5%, and it was artificially illuminated with fluorescent light on a 12-h light/dark cycle (0600–1800 hours). All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by the Japanese Association for Laboratory Animal Science.

Study design

Experiment 1: Hershberger assay

The experimental design of our study followed the OECD validation of the rodent Hershberger bioassay: phase-2 protocol (OECD 2002). Six castrated rats/group

were given AT orally at doses of 40, 200, or 1,000 mg/kg daily via a stomach tube for 10 consecutive days beginning at 9 weeks of age with or without s.c. injection of 0.2 mg/kg testosterone propionate (TP). The volume of the olive oil solutions of AT was 5 ml/kg for oral administration and 5 ml/kg for s.c. injection. A vehicle control group given olive oil alone was also established. The dosage was adjusted daily for body weight change. Approximately 24 h after the final dose, the animals were killed by bleeding from the abdominal vein under deep ether anesthesia. At necropsy, the ventral prostate, seminal vesicles, BC/LA muscles, glans penis, Cowper's gland, and liver were removed and weighed. The thyroid glands and hypophysis were collected and fixed with 10% neutral-buffered formalin, and to avoid crushing during weighing because of their fragility, they were weighed approximately 24 h after fixation.

Experiment 2: quantification of the extent of hypertrophy of the thyroid epithelium

The same experimental procedures, as described below, were performed on intact rats and castrated rats.

The rats were given AT in doses of 40 or 200 mg/kg daily. Administration and necropsy were executed according to the same procedure as experiment 1. At necropsy, the thyroid glands and hypophysis were removed and fixed with 10% neutral-buffered formalin. To avoid crushing during weighing because of its fragility, the thyroid glands and hypophysis were weighed approximately 24 h after fixation, stained with hematoxylin and eosin, and examined under a light microscope. Morphometry of the thyroid epithelium was performed to quantify the extent of hypertrophy. The height of the epithelial cells of all rats was measured under a microscope with Scion Image image analysis software (Scion Corporation, MA, USA). Fifty points/rat (25 left, 25 right) of the thyroid epithelium were measured.

Statistical analyses

Body weight, organ weights, and height of thyroid follicular epithelium of the rats in the experimental groups and corresponding vehicle control groups were compared, i.e., data obtained from the castrated group were compared with data from the castrated control group. Data from the castrated control group and intact control group were also compared. Each endpoint was analyzed by Bartlett's test for homogeneity of variance. If the variances were homogeneous at the 5% level of significance, one-way analysis of variance was performed, and if it revealed a significant difference, the difference between the control group and each of the experimental groups was analyzed by Dunnett's test. If the variances were not homogeneous, the Kruskal–Wallis test was used, and if it showed a significant difference, the difference between the control group and each of the experimental groups was analyzed by the nonparametric Dunnett's test.

Results

Experiment 1: Hershberger assay

General observation revealed no abnormal findings in the AT-treated castrated rats. Irrespective of TP-treatment, the body weight of the AT-treated castrated rats remained normal throughout the study (Table 1).

Organ weight changes are shown in Tables 1 and 2. Absolute and relative thyroid weights in all the AT groups were statistically increased in a dose-dependent manner, regardless of the TP-injection, and increases were statistically significant.

In the classical parameters of the Hershberger assay, absolute seminal vesicle weights were significantly higher in the 200 mg/kg + TP group, and absolute and relative seminal vesicle weights were significantly higher in the 1,000 mg/kg + TP group when compared to each corresponding vehicle control group. In the 1,000 mg/kg + TP group, relative liver weights were significantly higher. Besides, absolute seminal vesicle weights were significantly lower in the 1,000 mg/kg group, absolute and relative weights were significantly higher in the 40 mg/kg group, absolute glans penis weights and relative BC/LA weights were significantly lower, and absolute and relative liver weights were significantly higher in the 40 mg/kg + TP group, and absolute and relative BC/LA weights were significantly higher in the 200 mg/kg + TP group when compared to each corresponding vehicle control group.

Gross examination revealed enlargement of the thyroid glands in 4/6 rats in the 40 mg/kg group, in all rats in the 200 and 1,000 mg/kg group, in 5/6 rats in the 40 mg/kg + TP group, and in all rats in the 200 and 1,000 mg/kg + TP group (Table 3).

Experiment 2: quantification of the extent of hypertrophy of the thyroid epithelium

Castrated rats

General observation revealed no abnormal findings in the AT-treated castrated rats. The body weight of the AT-treated castrated rats remained normal throughout the study (Fig. 1).

Organ weight changes are shown in Fig. 2. Absolute and relative thyroid weights were significantly higher in the 200 mg/kg group. No statistically significant differences were detected in hypophysis weight.

Gross examination revealed enlargement of the thyroid glands in 4/6 rats in the 40 mg/kg group and in all rats in the 200 mg/kg group (Table 3).

Histopathological findings in the thyroid glands of the AT-treated castrated rats are shown in Fig. 3 (left panel); the thyroid glands of all AT-treated rats

Table 1 Absolute organ weight in AT-treated castrated male rats

Dose (mg/kg/day)	Thyroid (mg)	Ventral prostate (mg)	Seminal vesicle (mg)	Bulbocavernosus/ levator ani muscles (mg)	Glans penis (mg)	Cowper's galnd (mg)	Liver (g)	Final body weight (g)
VC	16.4 ± 5.9	15.1 ± 1.7	29.3 ± 4.2	146.0 ± 17.1	39.0 ± 3.0	3.9 ± 1.5	11.0 ± 0.6	281.2 ± 13.3
40	27.8 ± 8.8*	14.1 ± 6.1	27.1 ± 3.0	148.4 ± 19.2	41.3 ± 4.2	5.3 ± 0.9	12.0 ± 0.8	12.0 ± .08*
200	50.2 ± 11.0**	17.1 ± 1.9	25.3 ± 2.8	144.2 ± 23.1	38.2 ± 5.3	4.5 ± 0.8	11.6 ± 0.9	11.6 ± 0.9
1,000	56.5 ± 19.1**	15.9 ± 2.5	24.0 ± 4.1*	139.8 ± 24.6	39.6 ± 4.4	4.9 ± 1.0	11.2 ± 1.1	11.2 ± 1.1
VC + TP	14.8 ± 2.3	108.2 ± 9.4	232.2 ± 30.2	368.7 ± 23.8	78.5 ± 6.4	23.7 ± 3.8	11.0 ± 0.9	11.0 ± 0.9
40 + TP	27.1 ± 6.2##	118.9 ± 14.2	275.5 ± 66.1	344.7 ± 28.0	70.5 ± 6.4	24.3 ± 3.2	12.6 ± 1.2#	12.6 ± 1.2#
200 + TP	45.8 ± 16.8##	109.0 ± 13.8	285.4 ± 45.0#	317.5 ± 14.8##	71.4 ± 5.1	23.3 ± 3.8	11.7 ± 1.1	11.7 ± 1.1
1,000 + TP	45.4 ± 9.3##	113.3 ± 25.6	307.5 ± 39.5##	348.9 ± 32.2	73.3 ± 3.4	26.3 ± 2.3	12.2 ± 1.5	12.2 ± 1.5

AT, 3-amino-1,2,4-triazole; VC, vehicle control; TP, testosterone propionate.
*,** Significantly different from VC at $p < 005$ and $p < 0.01$, respectively.
#,## Significantly differently from VC + TP at $p < 0.05$ and $p < 0.01$, respectively.

Table 2 Relative organ weight in AT-treated castrated male rats

Dose (mg/kg/day)	Thyroid (mg/100 g b.w.)	Ventral prostate (mg/100 g b.w.)	Seminal vesicle (mg/100 g b.w.)	Bulbocavernosus/ levator ani muscles (mg/100 g b.w.)	Glans penis (mg/100 g b.w.)	Coeper's gland (mg/100 g b.w.)	Liver (mg/100 g b.w.)
VC	5.8 ± 1.7	5.4 ± 0.8	10.5 ± 1.8	52.0 ± 6.7	13.9 ± 1.3	1.4 ± 0.5	3.9 ± 0.2
40	9.9 ± 3.3*	4.9 ± 2.0	9.5 ± 0.9	51.9 ± 4.6	14.5 ± 1.2	1.8 ± 1.2	4.2 ± 0.2*
200	17.7 ± 3.2**	6.1 ± 0.8	9.0 ± 1.4	50.9 ± 6.0	13.6 ± 2.3	1.6 ± 0.3	4.0 ± 0.2
1,000	20.1 ± 5.5**	5.7 ± 0.8	8.6 ± 1.2	50.1 ± 6.0	14.4 ± 2.3	1.8 ± 0.4	1.8 ± 0.2
VC + TP	5.2 ± 0.9	37.7 ± 4.9	81.0 ± 14.6	127.9 ± 9.2	27.3 ± 3.3	8.2 ± 1.4	3.8 ± 0.2
40 + TP	9.1 ± 1.9##	39.9 ± 5.7	92.2 ± 21.5	115.5 ± 9.3#	23.7 ± 2.4	8.2 ± 1.4	4.2 ± 0.3#
200 + TP	15.9 ± 5.2##	38.0 ± 4.9	99.6 ± 16.3	110.8 ± 6.6##	24.9 ± 1.8	8.1 ± 1.1	4.1 ± 0.2
1,000 + TP	15.8 ± 3.6##	39.3 ± 9.4	106.5 ± 13.9#	120.7 ± 10.6	25.4 ± 1.7	9.1 ± 0.9	4.2 ± 0.3#

AT, 3-amino-1,2,4-triazole; VC, vehicle control; TP, testosterone propionate.
*,** Significantly different from VC at $p < 0.05$ and $p < 0.01$, respectively.
#,## Significantly different from VC + TP at $p < 0.05$ and $p < 0.01$, respectively.

Table 3 Gross pathological findings in AT-treated rats (No. of animals = 6)

	Experiment 1								Experiment 2					
	AT (mg/kg/day)				AT + TP (mg/kg/day)				Castrated (mg/kg/day)			Intact (mg/kg/day)		
	VC	40	200	1,000	VC	40	200	1,000	VC	40	200	VC	40	200
No abnormalities detected	6	2	0	0	6	1	0	0	6	2	0	6	0	0
Thyroid enlargement	0	4	6	6	0	5	6	6	0	4	6	0	6	6

AT, 3-amino-1,2,4-triazole; VC, Vehicle control; Tp, testosterone propionate.

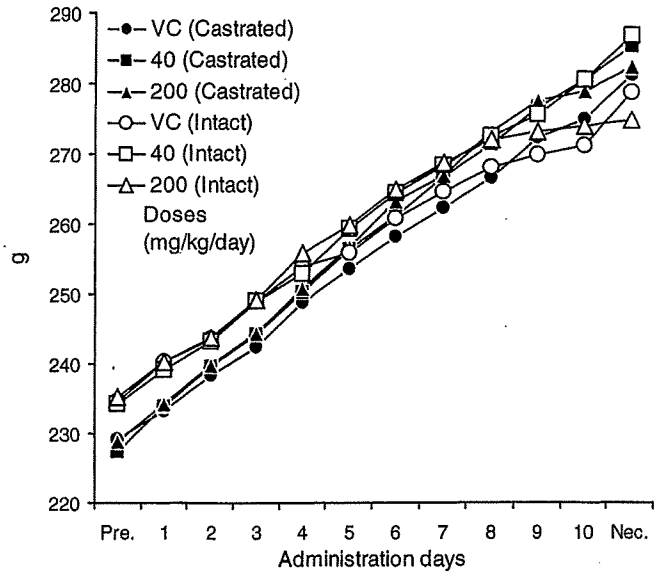

Fig. 1 Body weight changes in AT-treated castrated and intact male rats

exhibited hypertrophy and hyperplasia of the thyroid follicular epithelial cells.

The follicular epithelial cell heights are shown in Fig. 4. The height of the follicular epithelial cell in all AT-treated groups was increased in a dose-dependent manner by AT (40 mg/kg group, 200.8% of the control; 200 mg/kg group, 225.7% of the control), and the increase were statistically significant.

Intact rats

General observation revealed no abnormal findings in the AT-treated intact rats. The body weight of the AT-treated rats remained normal throughout the study (Fig. 1).

Organ weight changes are shown in Fig. 2. Absolute and relative thyroid weights were significantly higher in the 200 mg/kg group, and absolute thyroid weight in the 40 mg/kg group. No statistically significant differences in hypophyseal weights were detected. Comparison between the VC groups, however, revealed significantly decreased absolute and relative hypophyseal weights in the intact VC group.

Gross examination revealed enlargement of the thyroid glands in all rats in all the AT-treated groups (Table 3). Histopathological findings in the thyroids of the AT-treated intact rats are shown in Fig. 3 (right panel). The thyroid glands of all AT-treated animals exhibited hypertrophy and hyperplasia of the thyroid follicular epithelial cells.

The height of the follicular epithelial cell is shown in Fig. 4. Follicular epithelial cell height in all AT-treated groups was significantly increased in a dose-dependent manner by AT (40 mg/kg group, 149.6% of the control; 200 mg/kg group, 195.0% of the control).

Discussion

We examined the applicability of the Hershberger assay 10-day administration protocol to screening of chemicals for a thyroid hormone modulator. The thyroid peroxidase inhibitor, AT, a widely used herbicide found to produce thyroid and liver tumors in rodents and classified as possibly carcinogenic to humans, was investigated to acquire further information about its mechanism of action (Mattioli et al. 1994). In this study, AT was used as the test substance to determine whether the Hershberger assay can detect thyroid hormone modulation caused by a mechanism different from that of PTU, *p, p'*-DDE, and PB, such as by the mechanisms of modulation of iodination of thyroglobulin and of the coupling reaction and metabolism enhancers. Both castrated and intact male rats were subjected to the same protocol to elucidate the effect of castration on the thyroid.

The organ weight changes showed that thyroid weight increased in all AT-treated groups in a dose-dependent manner, and thyroid gland enlargement in the AT-treated rats. Histologically, the thyroid glands of all AT-treated rats exhibited hypertrophy and hyperplasia of the follicular epithelial cells, and their height increased in a dose-dependent manner in both the castrated and intact rats. AT is known to inhibit the synthesis of T3 and T4 by peroxidase inhibiting in the thyroid gland (Ealey et al. 1984; Krauss and Eling 1987; Reader et al. 1987; Masuda and Goto 1994; Santini et al. 2003). It has been reported that the administration of AT to rats is followed by enlargement of the thyroid

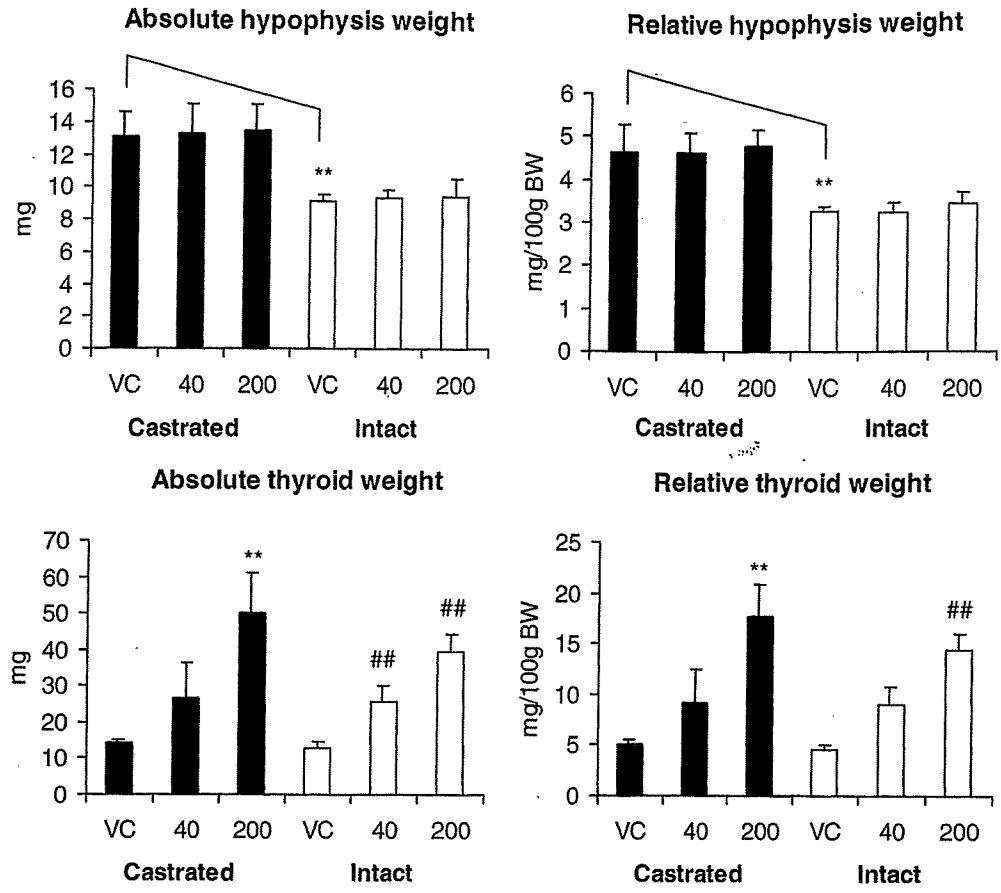

Fig. 2 Hypophyseal and thyroid weights of AT-treated castrated and intact male rats. **Significantly different from the castrated vehicle control at $p < 0.01$.## Significantly different from the intact vehicle control at $p < 0.01$

gland, a decrease in colloid content, and proliferation of the follicular epithelium (Masuda and Goto 1994). The results of the present study showed that the Hershberger assay 10-day administration protocol clearly detected thyroid hormone modulating effect of AT on the thyroid glands. Moreover, the reproducibility of the results was demonstrated by experiments 1 and 2. Yamada and colleagues (2004) first reported that the Hershberger assay enhanced by evaluation of thyroid histopathology and weights and serum hormone levels appears to be a reliable method of screening for thyroid modulators. PTU is not only an inhibitor of type I deiodinase inhibitor, but also an inhibitor of iodination of thyroglobulin and of the coupling reaction, whereas peroxidase inhibition seems not to occur under in vivo inhibition (Shiroozu et al. 1983; Moura et al. 1990; Taurog et al. 1995). *p,p'*-DDE and PB are the thyroid hormone metabolism enhancers (McClain et al. 1989; Barter and Klaassen 1994; Capen 1997). Thus, the thyroid inhibiting effects of several thyroid modulators have already been detected by the Hershberger assay. It therefore seems possible to use the Hershberger assay to assess the potency of anti-thyroid chemicals and perhaps to bioassay them.

The workshop on Screening Methods for Chemicals that Alter Thyroid Hormone Action, Function and Homeostasis (DeVito et al. 1997, 1999) concluded that at least a 2–6-week dosing period is necessary to be able to observe consistent thyroid responses. Other assays, such as the male and female pubertal assays (Goldman et al. 2000; Stoker et al. 2000) and enhanced TG 407 (OECD 1999), require a 20–30-day administration period and are not convenient screening methods for evaluating large numbers of chemicals for the thyroid hormone modulating activity. If the Hershberger assay is used to screen for thyroid function modulating activity and (anti-) androgenic compounds at the same time, more chemicals can be screened for its (anti-) androgenic activity and/or thyroid hormone modulating activity.

It was reported that castration induced a dramatic increase in the size and proportion of LH-immunoreactive cells present within the adenohypophysis of control rats (Kirby et al. 1997) and the castration induces an immediate increase in the serum levels and pituitary content of the gonadotropins, luteinizing hormone, and follicle-stimulating hormone as well as a concomitant rise in the steady state levels of the messenger RNAs directing their synthesis (Emanuele et al. 1996; Valenti et al. 1997). It is speculated that an increased hypophysis weight in castrated rats may be a result of the removal of testicular negative feedback. Histologically, however, the thyroid glands of all AT-treated rats exhibited follicular epithelial cell hypertrophy and hyperplasia, and the height of the follicular epithelium of the thyroid glands increased in a dose-dependent manner in both the

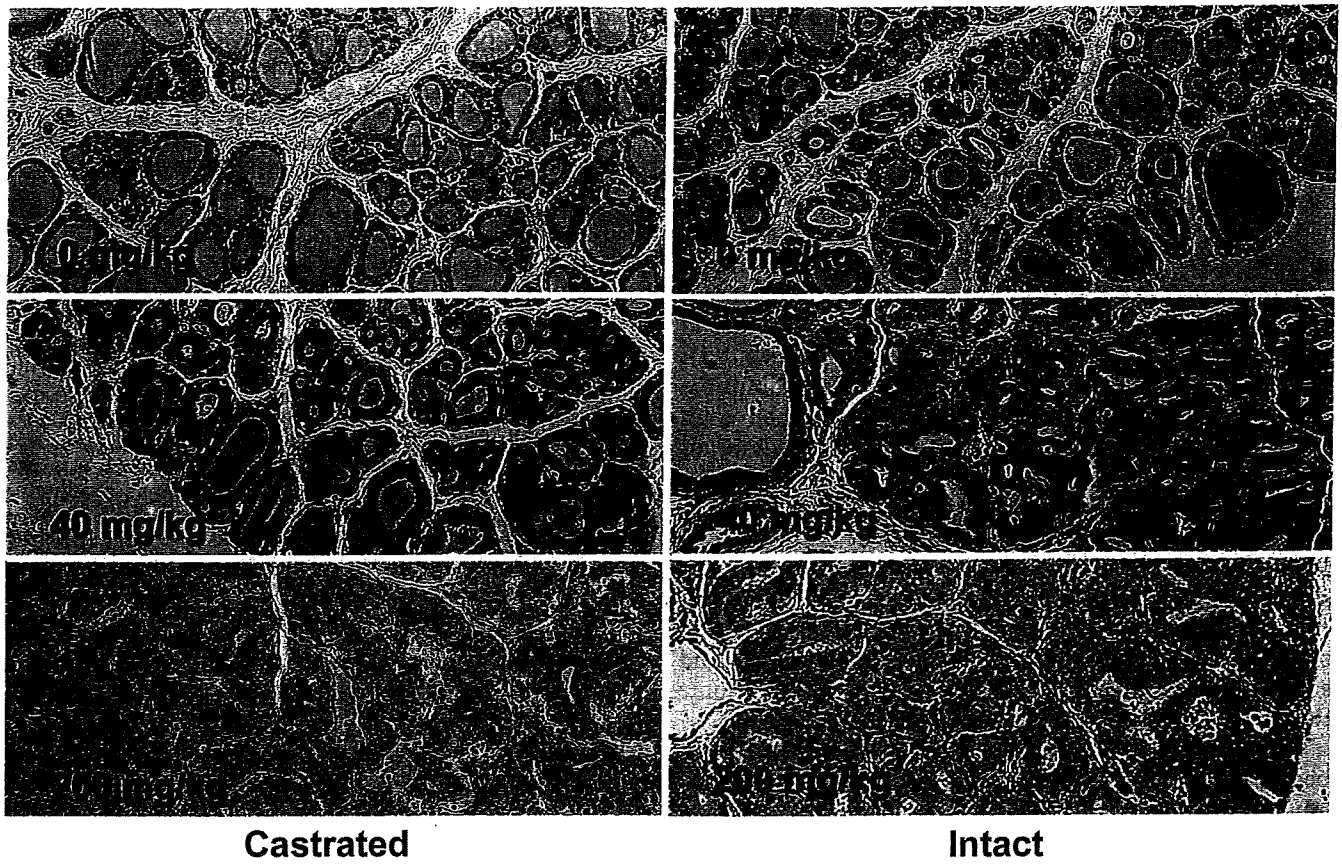

Fig. 3 Micrographs of the thyroid gland of an AT-treated castrated rat and an AT-treated intact male rat. Magnification (125 x)

castrated and intact rats. Moreover, results of morphometry of the thyroid epithelium showed almost same sensitivity in both the castrated rats and intact rats. These results suggest that the effect of AT can be detected by the Hershberger assay 10-day administration protocol regardless of castration. The other assays to detect the thyroid modulating effect, such as male or female pubertal assay (Goldman et al. 2000; Stoker et al. 2000), and enhanced TG 407 (OECD 1999) are used in intact rats. Our results emphasize the possibility of applying the Hershberger assay protocol to the detection of thyroid modulators. At the same time, the 10-day oral administration protocol in intact male rats may provide a new approach to screening for thyroid hormone modulators. In terms of prevention of surgical intervention of animals, it is valuable to develop screening methods that do not involve surgical invasion, such as castration, ovariectomy, etc. Thus, further study is needed, including evaluation of other compounds with mechanisms of action different from those of AT, such as thyroid hormone metabolism enhancers and thyroid hormone antagonist etc.

In addition, assessment of the (anti-) androgenic action of AT in seminal vesicle weight revealed a significant increase in the 200 and 1,000 mg/kg + TP groups in a dose-dependent manner. The mechanisms and biological or toxicological significance of the increase of seminal vesicle weight in AT + TP groups were unclear and there were no AT-related changes in other androgen-sensitive organs, but this seminal vesicle weight change was not considered to be disregarded

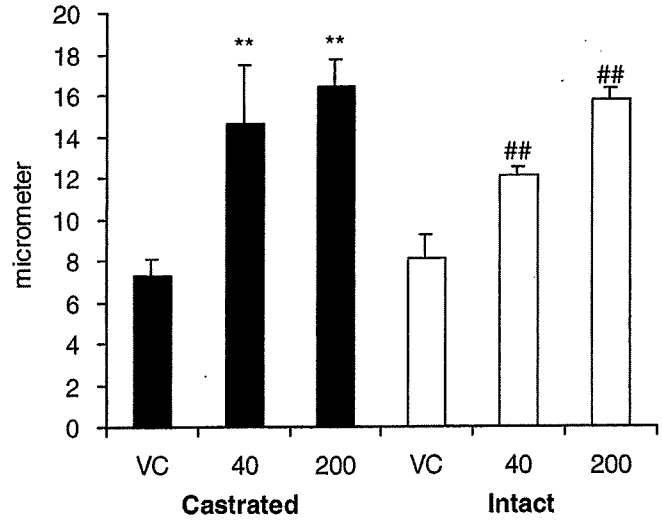

Fig. 4 Height of thyroid follicular epithelial cells of an AT-treated castrated rat and an AT-treated intact male rat. **Significantly different from the castrated vehicle control at $p<0.01$. ## Significantly different from the intact vehicle control at $p < 0.01$

because dose dependency was confirmed, although the study to determine the reproducibility of the data will be needed to confirm these findings in the present study. Other organ weight changes, excluding the seminal vesicle weight changes in the AT + TP groups, were judged to be of no toxicological significance, since there were no dose relationships. Based on this finding, it might be appropriate to conclude that the androgen action of TP is reinforced or synergized. The original Hershberger assay is a screening method that evaluates the hormonal activity of chemicals based on changes in accessory sex organs alone (Hershberger et al. 1953; Dorfman 1969; OECD 1997; Gray et al. 2002), and it is impossible to speculate on the mechanism of changes in the accessory sex organs. We performed the AR binding assay and AR reporter gene assay of AT, and AT showed lack of binding affinity to AR and did not exhibit any agonist or antagonist activity in the reporter gene assay in these in vitro assays (data not shown). These results point to the existence of an endocrine-disrupting mechanism other than the AR-mediated mechanism in the Hershberger assay and suggest the importance of the in vitro assay to evaluate the androgen action by the Hershberger assay.

In conclusion, we wish to emphasize the that Hershberger assay has the ability to be used to assess the potency, and possibly to bioassay not only of the (anti-) androgenic activity of chemicals but of thyroid hormone modulators, although further studies will be needed to confirm the reproducibility of the findings in the present study, and evaluated another endpoints, such as hormonal evaluation, and other chemicals with thyroid hormone modulating effects to evaluate the value of the Hershberger assay protocol as a method to screen thyroid function modulators. Moreover, 10-day oral administration protocol in intact male rats may enable a new approach to screening for thyroid hormone modulators, although other compounds with different mechanisms of action than those of AT, such as thyroid hormone metabolism enhancers and thyroid hormone antagonists should be tested.

Acknowledgements This study was supported by a Grant-in-Aid from the Ministry of Economy, Trade and Industry, Japan.

References

Banu SK, Govindarajulu P, Aruldhas MM (2002) Testosterone and estradiol up-regulate androgen and estrogen receptors in immature and adult rat thyroid glands in vivo. Steroids 67:1007–1014

Barter RA, Klaassen CD (1994) Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. Toxicol Appl Pharmacol 128:9–17

Capen CC (1997) Mechanistic data and risk assessment of selected toxic endpoints of the thyroid gland. Toxicol Pathol 25:39–48

DeVito M, Crofton K, McMaster S (1997) Workshop report: screening methods for chemicals that alter thyroid hormone action, function and homeostasis. Durham, NC, June 23–25, 1997. United States Environmental Protection Agency. EPA/600/R-98/057

DeVito M, Biegel L, Brouwer A, Brown S, Brucker-Davis F, Cheek AO, Christensen R, Colborn T, Cooke P, Crissman J, Crofton K, Doerge D, Gray E, Hauser P, Hurley P, Kohn M, Lazar J, McMaster S, McClain M, McConnell E, Meier C, Miller R, Tietge J, Tyl R (1999) Screening methods for thyroid hormone disruptors. Environ Health Perspect 107:407–415

Dorfman RI (1969) Androgens and anabolic agents. In: Dorfman RI (ed) Methods in hormone research, vol IIA. Academic, San Diego, pp. 151–220

Ealey PA, Henderson B, Loveridge N (1984) A quantitative study of peroxidase activity in unfixed tissue sections of the guinea-pig thyroid gland. Histochem J 16(2):111–122

EDSTAC (1998) Endocrine Disruptor Screening and Testing Committee (EDSTAC). Final report

Emanuele NV, Jurgens J, La Paglia N, Williams DW, Kelley MR (1996) The effect of castration on steady state levels of luteinizing hormone-releasing hormone (LHRH) mRNA and pro-LHRH processing: time course study utilizing semi-quantitative reverse transcription/polymerase chain reaction. J Endocrinol 148(3):509–515

Goldman JM, Laws SC, Balchak SK, Cooper RL, Kavlock RJ (2000) Endocrine-disrupting chemicals: prepubertal exposures and effects on sexual maturation and thyroid activity in the female rat. A focus on the EDSTAC recommendations. Crit Rev Toxicol 30:135–196

Gray LE, Ostby J, Wilson V, Lambright C, Bobseine K, Hartig P, Hotchkiss A, Wolf C, Furr J, Price M, Parks L, Cooper RL, Stoker TE, Laws SC, Degitz SJ, Jensen KM, Kahl MD, Korte JJ, Makynen EA, Tietge JE, Ankley GT (2002) Xenoendocrine disrupters-tiered screening and testing filling key data gaps. Toxicology 181(182):371–382

Hershberger LG, Shipley EG, Eyer RK (1953) Mytrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. Proc Soc Exp Biol Med 83:175–180

Kirby JD, Arambepola N, Porkka-Heiskanen T, Kirby YK, Rhoads ML, Nitta H, Jetton AE, Iwamoto G, Jackson GL, Turek FW, Cooke PS (1997) Neonatal hypothyroidism permanently alters follicle-stimulating hormone and luteinizing hormone production in the male rat. Endocrinology 138:2713–2721

Krauss RS, Eling TE (1987) Macromolecular binding of the thyroid carcinogen 3-amino-1,2,4-triazole (amitrole) catalyzed by prostaglandin H synthase, lactoperoxidase and thyroid peroxidase. Carcinogenesis 8(5):659–664

Masuda H, Goto N (1994) Comparative study of goitrogenic actions of 3-substituted 1, 2, 4-triazoles in rats. J Vet Med Sci 56(2):341–346

Mattioli F, Robbiano L, Fazzuoli L, Baracchini P (1994) Studies on the mechanism of the carcinogenic activity of amitrole. Fundam Appl Toxicol 23(1):101–106

McClain RM, Levin AA, Posch R, Downing JC (1989) The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. Toxicol Appl Pharmacol 99:216–228

McLachlan JA (1993) Functional toxicology: a new approach to detect functionally active xenobiotics. Environ Health Prespect 101:386–387

McLachlan JA, Korach KS (1995) Estrogens in the environment: global health implications. Environ Health Perspect 103:3–4

Moura EG, Pazos-Moura CC, Dorris ML, Taurog A (1990) Lack of effect of propylthiouracil and methylmercaptoimidazole on thyroglobulin biosynthesis. Proc Soc Exp Biol Med 194(1):48–53

O'Connor JC, Davis LG, Frame SR, Cook JC (2000) Evaluation of a Tier I screening battery for detecting endocrine-active compounds (EDCs) using the positive controls testosterone, coumestrol, progesterone, and RU486. Toxicol Sci 54:338–354

OECD (1997) Draft detailed review paper: appraisal of test methods for sex-hormone disrupting chemicals. Environmental Health and Safety Publications, Paris

OECD (1999) EMSG OECD draft proposal for testing of adequacy of an enhanced OECD protocol, repeated dose (28 days) toxicity (oral) study, based on OECD 407, April 19–20

OECD (2001) Third meeting of the validation management group for the screening and testing of endocrine disrupters (mammalian effects), March 26–27

OECD (2002) Final report of the work towards the validation of the rat Hershberger assay: phase-1, androgenic response to testosterone propionate, and anti-androgenic effects of flutamide. ENV/JM/TG/EDTA(2002)1/REV2. Paris

OECD (2003) Fourth meeting of the validation management group for the screening and testing of endocrine disupters (mammalian effects), April 14–15

Pelletier G (2000) Localization of androgen and estrogen receptors in rat and primate tissues. Histol Histopathol 15:1261–1270

Reader SC, Carroll B, Robertson WR, Lambert A (1987) Assessment of the biopotency of anti-thyroid drugs using porcine thyroid cells. Biochem Pharmacol 36(11):1825–1828

Ross DS (1990) Testosterone increases TSH-beta mRNA, and modulates alpha-subunit mRNA differentially in mouse throtrophic tumor and castrate rat pituitary. Horm Metab Res 22:163–169

Santini F, Vitti P, Ceccarini G, Mammoli C, Rosellini V, Pelosini C, Marsili A, Tonacchera M, Agretti P, Santoni T, Chiovato L, Pinchera A (2003) In vitro assay of thyroid disruptors affecting TSH-stimulated adenylate cyclase activity. J Endocrinol Invest 26(10):950–955

Shiroozu A, Taurog A, Engler H, Dorris ML (1983) Mechanism of action of thioureylene antithyroid drugs in the rat: possible inactivation of thyroid peroxidase by propylthiouracil. Endocrinology 113(1):362–370

Stoker TE, Parks LG, Gray LE, Cooper PL (2000) Endocrine-disrupting chemicals: prepubertal exposures and effects on sexual maturation and thyroid function in the male rat. A focus on the EDSTAC recommendations. Crit Rev Toxicol 30:197–252

Taurog A, Dorris ML, Hu WX, Guziec FS Jr (1995) The selenium analog of 6-propylthiouracil. Measurement of its inhibitory effect on type I iodothyronine deiodinase and of its antithyroid activity. Biochem Pharmacol 49(5):701–709

Valenti S, Sarkissian A, Giusti M, Giordano G, Dahl KD (1997) Immunoreactive and bioactive LH release from pituitaries of intact or castrated male rats: effect of in vitro GnRH and KCl administration. J Endocrinol Invest 20(7):381–386

Yamada T, Kunimatsu T, Miyata K, Yabushita S, Sukata T, Kawamura S, Seki T, Okuno Y, Mikami N (2004) Enhanced rat Hershberger assay appears reliable for detection of not only (anti-) androgenic chemicals but also thyroid hormone modulators. Toxicol Sci 79:64–74

Toxicology Letters
Article in Press, Corrected Proof - Note to users

doi:10.1016/j.toxlet.2005.10.001 Cite or Link Using DOI

OECD validation of the Hershberger assay in Japan: Phase 3. Blind study using coded chemicals

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Received 3 August 2005; revised 6 October 2005; accepted 6 October 2005. Available online 10 November 2005.

Abstract

The Organization for Economic Co-operation and Development (OECD) has initiated the development of new guidelines for the screening and testing of potential endocrine disrupters. The Hershberger assay is one of the assays selected for validation based on the need for in vivo screening to detect androgen agonists or antagonists by measuring the response of five sex accessory organs and tissues of castrated juvenile male rats: the ventral prostate, the seminal vesicles with coagulating glands, the levator ani and bulbocavernosus muscle complex (LABC), Cowper's glands, and the glans penis. The Phase 1 feasibility demonstration stage of the Hershberger validation program has been successfully completed with a single androgen agonist and a single antagonist as reference substances. The Phase 2 validation study was performed, employing a range of additional androgen agonists and antagonists. Recently, the Phase 3 validation study was conducted and performed in several International laboratories. Three Japanese laboratories have contributed to the blind study using coded materials of Phase 3 validation. Four coded test substances in the agonistic version and seven substances in the antagonistic version were orally administered by gavage for 10 consecutive days, respectively. In the antagonist version of the assay, 0.2 mg/kg/day of testosterone propionate (TP) was coadministered by subcutaneous injection. All five accessory sex reproductive organs and tissues consistently responded with statistically significant changes in weight within a narrow window in both versions. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects.

Keywords: Blind study; Endocrine; Hershberger assay; OECD validation

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Arch Toxicol (2005) 79: 627–635
DOI 10.1007/s00204-005-0677-1

REGULATORY TOXICOLOGY

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Ability of the Hershberger assay protocol to detect thyroid function modulators

Received: 27 December 2004 / Accepted: 19 April 2005 / Published online: 10 June 2005

Abstract In vivo screening methods for detection of thyroid function modulators are now under development in many research laboratories. We assessed the applicability of the Hershberger assay protocol to screen for thyroid function modulators. In experiment 1, castrated male BrlHan WIST@Jcl (GALAS) rats were administered a potent thyroid peroxidase inhibitor, 3-amino-1,2,4-triazole (AT), in doses of 0, 40, 200, and 1,000 mg/kg/day with gravimetric endpoint, and in experiment 2, castrated and intact male rats were administered in doses of 0, 40, and 200 mg/kg/day, with quantification of the extent of hypertrophy of the thyroid epithelium, to assess the effects of castration, by gavage to 8-week-old for 10 consecutive days. At necropsy of both experiments, the thyroid glands and hypophysis were collected and fixed with 10% neutral-buffered formalin. To avoid crushing during weighing because of their fragility, the thyroid glands and hypophysis were weighed approximately 24 h after fixation with 10% neutral-buffered formalin. All animals were sacrificed approximately 24 h after the final dose. In experiment 2, the thyroid glands of all animals were stained with hematoxylin and eosin for histological examination and morphometry of follicular epithelial height. In experiment 1, absolute and relative thyroid weights in all of the AT groups were statistically increased in a dose-dependent manner, regardless of the testosterone propionate (TP)-injection. In experiment 2, the results showed a significant increase in thyroid weight in the 200 mg/kg groups of both castrated and intact rats. Hypophyseal weight was unaltered by AT, but comparison of vehicle-treated groups showed that the hypophyseal weight of the castrated rats was greater than that of the intact rats. Enlarged thyroid glands were observed in the AT-treated rats at necropsy. Histological examination of the thyroid glands of all the AT-treated animals showed hypertrophy and hyperplasia of the follicular epithelial cells, and the height of follicular epithelium of the thyroid glands increased in a dose-dependent manner in both the castrated and intact rats. In experiment 1, assessment of the (anti-) androgenic action of AT in seminal vesicle weight revealed a significant increase in the 200 and 1,000 mg/kg + TP groups in a dose-dependent manner. These results suggest that the effect of AT can be detected by the Hershberger assay 10-day administration protocol and may be useful for screening for thyroid function modulators regardless of whether the animals have been castrated.

Keywords Hershberger assay · Thyroid function modulators · 3-amino-1,2,4-triazole · Castrated rat · Intact rat

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Introduction

Currently, there is a great deal of concern that certain environmental chemicals may have the ability to impair normal sexual differentiation and development in humans and wildlife (McLachlan 1993; McLachlan and Korach 1995). Endocrine Disruptor Screening and Testing Committee (EDSTAC) is developing a tiered chemical screening and testing program to evaluate endocrine disrupting effects (EDSTAC 1998). In this program, high priority chemicals will be evaluated for hormonal activity in the early screening stage, and chemicals positive in the screening stage will be tested for hazard in a definitive test (EDSTAC 1998; Gray et al. 2002).

A Hershberger assay in surgically castrated male rats was proposed by the Organization for Economic

Cooperation and Development (OECD) and EDSTAC of the US Environmental Protection Agency as an in vivo screening method to detect the (anti-) androgenic activity of the chemicals acting mainly via androgen receptor (AR)-mediated mechanisms (Hershberger et al. 1953; Dorfman 1969; OECD 1997, 2001, 2003; EDSTAC 1998). Focusing on the disruption of androgenic system, the proposed endpoints of this assay are the weight of the ventral prostate, seminal vesicles, bulbocavernosus/levator ani (BC/LA) muscles, glans penis, Cowper's glands, and liver. The gravimetric endpoint has the advantage of enabling the detection of (anti-) androgenic action of a chemical at low cost in a short time. The Hershberger assay will be used in the early stage of a chemical screening and testing program after the in vitro screening assays, such as the AR binding assay and the AR reporter gene assay (Gray et al. 2002).

In vitro and in vivo screening methods for the detection of thyroid function modulators are now under development in many research laboratories, and a variety of in vivo screening methods have been proposed, e.g., enhanced TG407 and male or female pubertal assay. But they have the disadvantage such as long experimental periods or low cost-performance, and there have been no breakthroughs thus far. Yamada et al. (2004) first reported that the enhanced Hershberger assay evaluation of thyroid histopathology and weights, and determination of serum hormone levels, appears to be reliable for screening thyroid modulators with a single dose of propylthiouracil (PTU), 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE) and phenobarbital (PB). The original objective of the Hershberger assay was to screen for (anti-) androgenic activity of the chemicals in male rats subjected to castration, which is essential for the detection of androgenic activities. If the Hershberger assay is capable of detecting the (anti-) androgenic activity and thyroid hormone modulating activity of the chemicals at the same time, it will be possible to screen for hormonal activity of the chemicals rapidly and with better cost-performance.

Because of the well-known presence of androgen receptors in the thyroid gland of mammalians (Pellietier 2000; Banu et al. 2002), the thyroid gland is speculated to be one of the target organs of androgenic compounds. Moreover, testosterone has a stimulatory effect of on the expression of the TSH mRNA (Ross 1990), and testosterone administration results in a significant decrease in serum T3 and T4 levels in 15-day intact male assays (O'Connor et al. 2000). Thus, if we intend to screen chemicals for (anti-) androgenic activity based on their thyroid hormone modulating activity, effect of castration of male rats on the thyroid should be evaluated. It appears rather unlikely that thyroid-specific endpoints can be used to specifically detect (anti-) androgenicity. However, it is necessary to evaluate whether thyroid-active compounds affect the classical parameters of the Hershberger assay and thus confound the detection of (anti-) androgenicity.

We performed two experiments. Experiment 1 was designed to assess whether thyroid hormone modulating activity of 3-amino-1,2,4-triazole (AT), which is known to inhibit the synthesis of T3 and T4 by peroxidase inhibiting in the thyroid gland (Ealey et al. 1984; Krauss and Eling 1987; Reader et al. 1987; Masuda and Goto 1994; Santini et al. 2003), could be detected by the thyroid gravimetric method, and whether the dose-dependent AT-induced effect on thyroid gland would be confirmed by the Hershberger assay in castrated male rats. Experiment 2 was designed to assess the influence of castration on the AT-induced effect on thyroid gland, and thus, both castrated and intact male rats were subjected to the same protocol to elucidate the effect of castration on the thyroid.

Materials and methods

Chemicals

AT (CAS No. 61-82-5, 99% pure) was obtained from Tokyo Kasei Kougyou (Tokyo, Japan), and olive oil was obtained from Fujimi Pharmaceutical Company (Osaka, Japan). The physical stability of AT was assessed with a Fourier transform infrared spectrophotometer (FTS-135, Nippon Bio-Rad Laboratories K.K., Tokyo, Japan), and the stability, homogeneity, and concentration of each AT suspension prepared for administration were confirmed by HPLC.

Animals

Seven-week-old BrlHan WIST@Jcl (GALAS) rats castrated at 6 weeks of age, and 7-week-old intact BrlHan WIST@Jcl (GALAS) rats were purchased from Clear Japan Inc. (Fuji, Japan). The animals were housed, three per cage, in stainless steel, wiremesh cages throughout the study. The rats were weighed, weight-ranked, and randomly assigned to one of the treatment or control groups. Body weight and clinical signs were recorded daily throughout the study. Rats were given ad libitum access to tap water and a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) and ad libitum access to water from an automatic dispenser. The animal room was maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 5%, and it was artificially illuminated with fluorescent light on a 12-h light/dark cycle (0600–1800 hours). All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by the Japanese Association for Laboratory Animal Science.

Study design

Experiment 1: Hershberger assay

The experimental design of our study followed the OECD validation of the rodent Hershberger bioassay: phase-2 protocol (OECD 2002). Six castrated rats/group

were given AT orally at doses of 40, 200, or 1,000 mg/kg daily via a stomach tube for 10 consecutive days beginning at 9 weeks of age with or without s.c. injection of 0.2 mg/kg testosterone propionate (TP). The volume of the olive oil solutions of AT was 5 ml/kg for oral administration and 5 ml/kg for s.c. injection. A vehicle control group given olive oil alone was also established. The dosage was adjusted daily for body weight change. Approximately 24 h after the final dose, the animals were killed by bleeding from the abdominal vein under deep ether anesthesia. At necropsy, the ventral prostate, seminal vesicles, BC/LA muscles, glans penis, Cowper's gland, and liver were removed and weighed. The thyroid glands and hypophysis were collected and fixed with 10% neutral-buffered formalin, and to avoid crushing during weighing because of their fragility, they were weighed approximately 24 h after fixation.

Experiment 2: quantification of the extent of hypertrophy of the thyroid epithelium

The same experimental procedures, as described below, were performed on intact rats and castrated rats.

The rats were given AT in doses of 40 or 200 mg/kg daily. Administration and necropsy were executed according to the same procedure as experiment 1. At necropsy, the thyroid glands and hypophysis were removed and fixed with 10% neutral-buffered formalin. To avoid crushing during weighing because of its fragility, the thyroid glands and hypophysis were weighed approximately 24 h after fixation, stained with hematoxylin and eosin, and examined under a light microscope. Morphometry of the thyroid epithelium was performed to quantify the extent of hypertrophy. The height of the epithelial cells of all rats was measured under a microscope with Scion Image image analysis software (Scion Corporation, MA, USA). Fifty points/rat (25 left, 25 right) of the thyroid epithelium were measured.

Statistical analyses

Body weight, organ weights, and height of thyroid follicular epithelium of the rats in the experimental groups and corresponding vehicle control groups were compared, i.e., data obtained from the castrated group were compared with data from the castrated control group. Data from the castrated control group and intact control group were also compared. Each endpoint was analyzed by Bartlett's test for homogeneity of variance. If the variances were homogeneous at the 5% level of significance, one-way analysis of variance was performed, and if it revealed a significant difference, the difference between the control group and each of the experimental groups was analyzed by Dunnett's test. If the variances were not homogeneous, the Kruskal–Wallis test was used, and if it showed a significant difference, the difference between the control group and each of the experimental groups was analyzed by the nonparametric Dunnett's test.

Results

Experiment 1: Hershberger assay

General observation revealed no abnormal findings in the AT-treated castrated rats. Irrespective of TP-treatment, the body weight of the AT-treated castrated rats remained normal throughout the study (Table 1).

Organ weight changes are shown in Tables 1 and 2. Absolute and relative thyroid weights in all the AT groups were statistically increased in a dose-dependent manner, regardless of the TP-injection, and increases were statistically significant.

In the classical parameters of the Hershberger assay, absolute seminal vesicle weights were significantly higher in the 200 mg/kg + TP group, and absolute and relative seminal vesicle weights were significantly higher in the 1,000 mg/kg + TP group when compared to each corresponding vehicle control group. In the 1,000 mg/kg + TP group, relative liver weights were significantly higher. Besides, absolute seminal vesicle weights were significantly lower in the 1,000 mg/kg group, absolute and relative weights were significantly higher in the 40 mg/kg group, absolute glans penis weights and relative BC/LA weights were significantly lower, and absolute and relative liver weights were significantly higher in the 40 mg/kg + TP group, and absolute and relative BC/LA weights were significantly higher in the 200 mg/kg + TP group when compared to each corresponding vehicle control group.

Gross examination revealed enlargement of the thyroid glands in 4/6 rats in the 40 mg/kg group, in all rats in the 200 and 1,000 mg/kg group, in 5/6 rats in the 40 mg/kg + TP group, and in all rats in the 200 and 1,000 mg/kg + TP group (Table 3).

Experiment 2: quantification of the extent of hypertrophy of the thyroid epithelium

Castrated rats

General observation revealed no abnormal findings in the AT-treated castrated rats. The body weight of the AT-treated castrated rats remained normal throughout the study (Fig. 1).

Organ weight changes are shown in Fig. 2. Absolute and relative thyroid weights were significantly higher in the 200 mg/kg group. No statistically significant differences were detected in hypophysis weight.

Gross examination revealed enlargement of the thyroid glands in 4/6 rats in the 40 mg/kg group and in all rats in the 200 mg/kg group (Table 3).

Histopathological findings in the thyroid glands of the AT-treated castrated rats are shown in Fig. 3 (left panel); the thyroid glands of all AT-treated rats

Table 1 Absolute organ weight in AT-treated castrated male rats

Dose (mg/kg/day)	Thyroid (mg)	Ventral prostate (mg)	Seminal vesicle (mg)	Bulbocavernosus/ levator ani muscles (mg)	Glans penis (mg)	Cowper's galnd (mg)	Liver (g)	Final body weight (g)
VC	16.4 ± 5.9	15.1 ± 1.7	29.3 ± 4.2	146.0 ± 17.1	39.0 ± 3.0	3.9 ± 1.5	11.0 ± 0.6	281.2 ± 13.3
40	27.8 ± 8.8*	14.1 ± 6.1	27.1 ± 3.0	148.4 ± 19.2	41.3 ± 4.2	5.3 ± 0.9	12.0 ± 0.8	12.0 ± .08*
200	50.2 ± 11.0**	17.1 ± 1.9	25.3 ± 2.8	144.2 ± 23.1	38.2 ± 5.3	4.5 ± 0.8	11.6 ± 0.9	11.6 ± 0.9
1,000	56.5 ± 19.1**	15.9 ± 2.5	24.0 ± 4.1*	139.8 ± 24.6	39.6 ± 4.4	4.9 ± 1.0	11.2 ± 1.1	11.2 ± 1.1
VC +TP	14.8 ± 2.3	108.2 ± 9.4	232.2 ± 30.2	368.7 ± 23.8	78.5 ± 6.4	23.7 ± 3.8	11.0 ± 0.9	11.0 ± 0.9
40 +TP	27.1 ± 6.2##	118.9 ± 14.2	275.5 ± 66.1	344.7 ± 28.0	70.5 ± 6.4	24.3 ± 3.2	12.6 ± 1.2#	12.6 ± 1.2#
200 +TP	45.8 ± 16.8##	109.0 ± 13.8	285.4 ± 45.0#	317.5 ± 14.8##	71.4 ± 5.1	23.3 ± 3.8	11.7 ± 1.1	11.7 ± 1.1
1,000 +TP	45.4 ± 9.3##	113.3 ± 25.6	307.5 ± 39.5##	348.9 ± 32.2	73.3 ± 3.4	26.3 ± 2.3	12.2 ± 1.5	12.2 ± 1.5

AT, 3-amino-1,2,4-triazole; VC, vehicle control; TP, testosterone propionate.
*,** Significantly different from VC at $p < 005$ and $p < 0.01$, respectively.
#,## Significantly differently from VC+TP at $p < 0.05$ and $p < 0.01$, respectively.

Table 2 Relative organ weight in AT-treated castrated male rats

Dose (mg/kg/day)	Thyroid (mg/100 g b.w.)	Ventral prostate (mg/100 g b.w.)	Seminal vesicle (mg/100 g b.w.)	Bulbocavernosus/ levator ani muscles (mg/100 g b.w.)	Glans penis (mg/100 g b.w.)	Coeper's gland (mg/100 g b.w.)	Liver (mg/100 g b.w.)
VC	5.8 ± 1.7	5.4 ± 0.8	10.5 ± 1.8	52.0 ± 6.7	13.9 ± 1.3	1.4 ± 0.5	3.9 ± 0.2
40	9.9 ± 3.3*	4.9 ± 2.0	9.5 ± 0.9	51.9 ± 4.6	14.5 ± 1.2	1.8 ± 1.2	4.2 ± 0.2*
200	17.7 ± 3.2**	6.1 ± 0.8	9.0 ± 1.4	50.9 ± 6.0	13.6 ± 2.3	1.6 ± 0.3	4.0 ± 0.2
1,000	20.1 ± 5.5**	5.7 ± 0.8	8.6 ± 1.2	50.1 ± 6.0	14.4 ± 2.3	1.8 ± 0.4	1.8 ± 0.2
VC +TP	5.2 ± 0.9	37.7 ± 4.9	81.0 ± 14.6	127.9 ± 9.2	27.3 ± 3.3	8.2 ± 1.4	3.8 ± 0.2
40 +TP	9.1 ± 1.9##	39.9 ± 5.7	92.2 ± 21.5	115.5 ± 9.3#	23.7 ± 2.4	8.2 ± 1.4	4.2 ± 0.3#
200 +TP	15.9 ± 5.2##	38.0 ± 4.9	99.6 ± 16.3	110.8 ± 6.6##	24.9 ± 1.8	8.1 ± 1.1	4.1 ± 0.2
1,000 +TP	15.8 ± 3.6##	39.3 ± 9.4	106.5 ± 13.9#	120.7 ± 10.6	25.4 ± 1.7	9.1 ± 0.9	4.2 ± 0.3#

AT, 3-amino-1,2,4-triazole; VC, vehicle control; TP, testosterone propionate.
*,** Significantly different from VC at $p < 0.05$ and $p < 0.01$, respectively.
#,## Significantly different from VC+TP at $p < 0.05$ and $p < 0.01$, respectively.

Table 3 Gross pathological findings in AT-treated rats (No. of animals = 6)

	Experiment 1								Experiment 2					
	AT (mg/kg/day)				AT + TP (mg/kg/day)				Castrated (mg/kg/day)			Intact (mg/kg/day)		
	VC	40	200	1,000	VC	40	200	1,000	VC	40	200	VC	40	200
No abnormalities detected	6	2	0	0	6	1	0	0	6	2	0	6	0	0
Thyroid enlargement	0	4	6	6	0	5	6	6	0	4	6	0	6	6

AT, 3-amino-1,2,4-triazole; VC, Vehicle control; Tp, testosterone propionate.

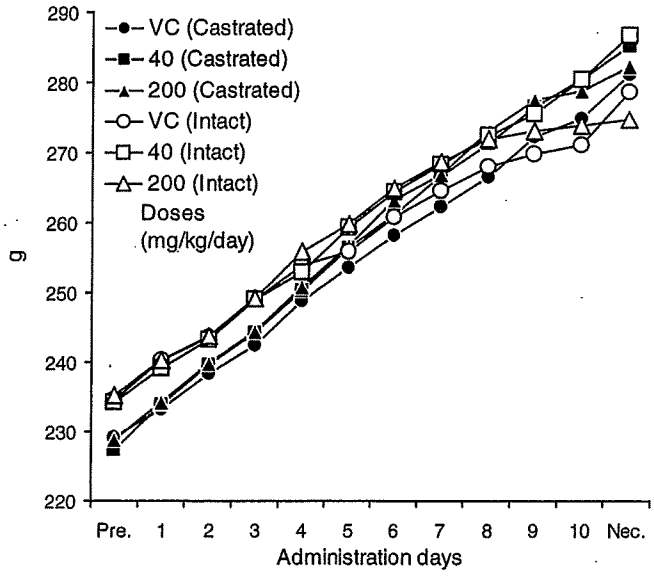

Fig. 1 Body weight changes in AT-treated castrated and intact male rats

exhibited hypertrophy and hyperplasia of the thyroid follicular epithelial cells.

The follicular epithelial cell heights are shown in Fig. 4. The height of the follicular epithelial cell in all AT-treated groups was increased in a dose-dependent manner by AT (40 mg/kg group, 200.8% of the control; 200 mg/kg group, 225.7% of the control), and the increase were statistically significant.

Intact rats

General observation revealed no abnormal findings in the AT-treated intact rats. The body weight of the AT-treated rats remained normal throughout the study (Fig. 1).

Organ weight changes are shown in Fig. 2. Absolute and relative thyroid weights were significantly higher in the 200 mg/kg group, and absolute thyroid weight in the 40 mg/kg group. No statistically significant differences in hypophyseal weights were detected. Comparison between the VC groups, however, revealed significantly decreased absolute and relative hypophyseal weights in the intact VC group.

Gross examination revealed enlargement of the thyroid glands in all rats in all the AT-treated groups (Table 3). Histopathological findings in the thyroids of the AT-treated intact rats are shown in Fig. 3 (right panel). The thyroid glands of all AT-treated animals exhibited hypertrophy and hyperplasia of the thyroid follicular epithelial cells.

The height of the follicular epithelial cell is shown in Fig. 4. Follicular epithelial cell height in all AT-treated groups was significantly increased in a dose-dependent manner by AT (40 mg/kg group, 149.6% of the control; 200 mg/kg group, 195.0% of the control).

Discussion

We examined the applicability of the Hershberger assay 10-day administration protocol to screening of chemicals for a thyroid hormone modulator. The thyroid peroxidase inhibitor, AT, a widely used herbicide found to produce thyroid and liver tumors in rodents and classified as possibly carcinogenic to humans, was investigated to acquire further information about its mechanism of action (Mattioli et al. 1994). In this study, AT was used as the test substance to determine whether the Hershberger assay can detect thyroid hormone modulation caused by a mechanism different from that of PTU, *p*, *p'*-DDE, and PB, such as by the mechanisms of modulation of iodination of thyroglobulin and of the coupling reaction and metabolism enhancers. Both castrated and intact male rats were subjected to the same protocol to elucidate the effect of castration on the thyroid.

The organ weight changes showed that thyroid weight increased in all AT-treated groups in a dose-dependent manner, and thyroid gland enlargement in the AT-treated rats. Histologically, the thyroid glands of all AT-treated rats exhibited hypertrophy and hyperplasia of the follicular epithelial cells, and their height increased in a dose-dependent manner in both the castrated and intact rats. AT is known to inhibit the synthesis of T3 and T4 by peroxidase inhibiting in the thyroid gland (Ealey et al. 1984; Krauss and Eling 1987; Reader et al. 1987; Masuda and Goto 1994; Santini et al. 2003). It has been reported that the administration of AT to rats is followed by enlargement of the thyroid