Follicular cell activation and proliferation of the thyroid gland in laboratory animals: How to name and interpret these data today?

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Introduction

Altered thyroid glands – so what?
We surely have something in the liver!

Alterations in the morphology of the thyroid gland in rats are still frequently considered as a species specific change with no toxicological relevance for humans.

One frequently cited article in this respect might be the one from RM McClain from 1995 and its sentence “There are important species differences in thyroid gland physiology between rodents and humans that may account for a marked species difference in the inherent susceptibility for neoplasia to hormone imbalance.”

RM McClain, 1995: Mechanistic considerations for the relevance of animal data on thyroid neoplasia to human risk assessment. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, Vol. 333, Pages 131–142
Agenda

- Introduction – Anatomy and Physiology
- Current Nomenclature (goRENI / INHAND)
- Examples
- Difficulties in Assessment
- Conclusion
Introduction
Thyroid and Thyroid Hormones

The Thyroid Gland
• Phylogenically the oldest gland (all vertebrates, many invertebrates)
• Largest hormone producing (endocrine) gland in mammals

In contrast to mammals, in (zebra-) fish the follicles are not concentrated together but are diffusely distributed along the ventral aorta.

Menke AL et al. 2011: Normal Anatomy and Histology of the Adult Zebrafish. Toxicologic Pathology, 39: 759-775,
Introduction
Thyroid and Thyroid Hormones

Fixation:
• Bouin‘s: +++
• Davidson‘s: ++(+)
• Formaldehyde: +

Introduction
Thyroid and Thyroid Hormones

• Composed of Follicles and Interstitial (C-) Cells (Baber-Nonidez-cells, Parenchymatous or Parafollicular Cells)

• Compared to plasma, increased (20-50-fold) iodine concentration
• Mainly (90%) organically bound iodine, 10% iodide
• Follicle as thyroid hormone producing functional unit
goRENI/INHAND: Non-neoplastic Terms

- Alteration, colloid (rat)
- Amyloid (mainly mouse)
- Dilatation, follicle, diffuse (goiter)
- Dysplasia, thyroid (rat)
- Follicle, cystic
- Hypertrophy, follicular cell
- Infiltrate, inflammatory cell
- Inflammation
- Mineralization
- Pigment
- Cyst, ultimobranchial
- Duct, thyroglossal, persistent
- Tissue, ectopic, thymus

INHAND GESC plays advisory role for the FDA Standard for the Exchange of Nonclinical Data (SEND) initiative

INHAND – International HArmonization of Nomenclature and Diagnostic Criteria
GESC – Global Editorial Steering Committee
goRENI/INHAND: Non-neoplastic Terms

- Alteration, colloid:
  Degeneration of colloid in thyroid follicles due to rapid turnover of colloid
- Mineralization
  Aging change, may be increased in stimulated thyroid follicles.
- Pigment (tetracyclines)
goRENI/INHAND: Non-neoplastic Terms

- Inflammation

Minipig, due to blood sampling
goRENI/INHAND: Terms

- Hypertrophy, follicular cell  (DD: Dysplasia, thyroid)
- Hyperplasia, follicular cell  
  Modifier: Focal; Cystic; **Diffuse**.
- Adenoma, follicular cell  
  Modifier: Follicular; Solid.
- Carcinoma, follicular cell  
  Modifier: Follicular; Pleomorphic; Solid.

- Dilatation, follicle, diffuse (goiter)
- Follicle, cystic
- Hyperplasia, follicular cell  
  Modifier: **Focal; Cystic**; Diffuse.
- Adenoma, follicular cell  
  Modifier: Cystic; Papillary;

- Duct, thyroglossal, persistent
Follicular cell hypertrophy is a common change seen in toxicity studies in rats induced with a variety of chemicals, such as hepatic microsomal enzymes inducers, which is often associated with hepatocyte hypertrophy. Follicular cell hypertrophy is expected to return to normal if the stimulus is withdrawn.
Hyperplasia, follicular cell

Prolonged follicular cell hypertrophy can progress to **diffuse follicular cell hyperplasia** and they often occur together.
goRENI/INHAND: Non-neoplastic Terms

- A. Dilatation, follicle, diffuse (goiter)
- B. Follicle, cystic
- C. Hyperplasia, follicular cell adjacent to cystic follicle
goRENI/INHAND: Non-neoplastic Terms

- Mouse, Duct, thyroglossal, persistent
• Mouse, Duct, thyroglossal, persistent
goRENI/INHAND: Non-neoplastic Terms

- Mouse, Duct, thyroglossal, persistent with inflammation
Dog, Duct, thyroglossal, persistent with inflammation – note more squamous cell differentiation
• Minipig, Hyperplasia, follicular cell, cystic
goRENI/INHAND: Proliferative Terms

- Hyperplasia, follicular cell, focal cystic, papillary
Adenoma, follicular cell, focally cystic, partly solid
goRENI/INHAND: Proliferative Terms

- Adenoma, follicular cell
• Adenocarcinoma, follicular cell
Thyroid Hormones

- Important role in mammals regarding metabolism, calorigenesis, and growth
- Most important role regarding mental and physical development
  Deficiency: delayed development, reduced growth, intellectual retardation
- Strong binding to plasma proteins (T4: 99,95%; T3: 99,5%)
- Complex homeostasis
- Unique chemistry

L-Thyroxine (T4)  3,3′,5-L-Triiodothyronine (T3)
Introduction
Thyroid and Thyroid Hormones

• Deiodinase 1
  (Liver, kidney, thyroid)

• 5’-Deiodinase 2
  (Brain, pituitary, placenta, thyroid, skeletal muscle, brown fat)

• Deiodinase 3
  (Pregnant uterus, fetus, placenta, brain)
Regulation of Thyroid Hormone Homeostasis
Transporters, Enzymes, Receptors, Binding Proteins

Sites of Interaction

① Hormone Synthesis
② Hormone Release
③ Metabolic Disposition
④ Peripheral 5'-Deiodination
⑤ Plasma Binding Sites
⑥ Hormone Uptake
⑦ Central 5'-Deiodination
⑧ Receptor Interactions
### Selected Modes of Interaction
Maximun Expected In Vivo / Ex Vitro Findings

<table>
<thead>
<tr>
<th>Target affected Mechanism</th>
<th>Hormonal Changes</th>
<th>Findings for Thyroid</th>
<th>Findings for Other Organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na / I Symporter inhibition</td>
<td>T3↓, T4↓, TSH↑</td>
<td>Weight↑ Hypertrophy</td>
<td>(TSH producing cell hypertrophy)</td>
</tr>
<tr>
<td>TPO Inhibition</td>
<td>T3↓, T4↓, TSH↑</td>
<td>Weight↑ Hypertrophy</td>
<td>(TSH producing cell hypertrophy)</td>
</tr>
<tr>
<td>5’-Deiodinase inhibition</td>
<td>T3↓, T4↑, TSH↑</td>
<td>Weight↑ Hypertrophy</td>
<td>(TSH producing cell hypertrophy)</td>
</tr>
<tr>
<td>Increased hepatic disposition</td>
<td>T3↓, T4↓, TSH↑</td>
<td>Weight↑ Hypertrophy</td>
<td>Liver weight↑ Liver hypertrophy (TSH prod. cell hypertrophy)</td>
</tr>
<tr>
<td>T3 Receptor agonism</td>
<td>T3↓, T4↓, TSH↓</td>
<td>Weight (↓) Atrophy</td>
<td>Heart weight↑ Myocard hypertrophy (TSH producing cell atrophy)</td>
</tr>
</tbody>
</table>
Thyroid gland – Species differences

<table>
<thead>
<tr>
<th>Species</th>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid gland activity</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Half time T3 [h]</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>Half time T4 [h]</td>
<td>5 - 9</td>
<td>0.5 - 1</td>
</tr>
<tr>
<td>Serum-TSH-level</td>
<td>1x</td>
<td>6 – 60x</td>
</tr>
<tr>
<td>Substitution level T4 [µg kg⁻¹ d⁻¹]</td>
<td>2.2</td>
<td>20</td>
</tr>
<tr>
<td>Specific binding protein</td>
<td>TBG</td>
<td>-</td>
</tr>
<tr>
<td>TPO - Inhibition</td>
<td>Monkey: strong</td>
<td>Very weak</td>
</tr>
<tr>
<td>Tumor promotion via TSH</td>
<td>Very unlikely</td>
<td>Yes</td>
</tr>
</tbody>
</table>

TBG = thyroid binding globulin; TPO = thyreoperoxidase
Evaluation of Thyroid Gland Effects - I
From Rat Findings to Human Relevance

Best use of in vivo / ex vivo rat data as far as available

• Indices of thyroid function like T3, T4, TSH levels and others (rT3, cholesterol !)
• Gross pathology, weight and histopathology of the thyroid
• Gross pathology, weight and histopathology of other organs (liver, heart)
• Hepatic drug metabolizing enzyme activities

Generation of mechanistic hypothesis for observed rat thyroid effect

• Comparison of results with established mechanistic in vivo/ ex vivo fingerprints
  • Cave: Several mechanisms represented by one fingerprint
  • Cave: Problematic fit in case of weak activity, lack of data
• Evaluate mechanistic information if available
• Design proper mechanistic studies
  • Most likely mechanism as starting point
  • Use of chemical alerts
Structural alerts
Overview by mode of action

Iodine uptake
• SCN⁻, other complex ions, (cyanide)

TPO inhibition
• Aromatic and aryl amines
• Resorcinols and flavonoids
• Certain thiourea derivatives

Iodination
• Thiourea and thionamide derivatives
• Certain flavonoids

Coupling
• Sulfonamides
• Certain thiourea derivatives
• Flavonoids

5’-Deiodinase inhibition
• Certain thiourea derivatives (type 1)
• Iodinated contrast agents
• Flavonoids
• Iodinated biphenylethers

Induction of hepatic glucuronidation
• Activators of AHR, PXR, CAR

Hepatic extraction of T4
• Histamin receptor antagonists

Displacement from binding proteins
• p-Hydroxy halogenated biphenyls

Receptor interaction
• Thyromimetics (lipid lowering drugs)
The Available Tool Box
Mechanistic In Vivo / In Vitro and Cellular Assays

Assays validated through OECD or others
• None

Research Type and Well-Tried Assays
• In vivo / ex vitro assays
  • $^{131}$Iodide uptake
  • Ex vivo TPO, 5′-deiodinase and hepatic glucuronyltransferase assays
  • Genexpression for relevant mRNAs
• In vitro assays
  • TPO and 5′-deiodinase inhibition assays
  • Cellular (lines/thyrocytes) assays for iodide uptake, TPO, hormone synthesis and hormone metabolism and transport
  • Binding protein displacement assays
  • Thyroid hormone binding and transactivation assays
Evaluation of Thyroid Gland Effects - II
From Rat Findings to Human Relevance

Evaluation of established mechanism in the rat

- Potential species differences
  - Sensitivity of the target
  - Differences in regulation

Evaluation of dose / kinetics in rats and humans

- Comparison of intended human dose to rat thyroid effective dose

Evaluation of human relevance

- Based on dose/kinetics, mechanistic considerations and potential species differences
Conclusions

Thyroid hormones (THs) play an important role in mammalian organisms regarding metabolism, calorogenesis, growth and development.

Synthesis and regulation of thyroid hormone (TH) levels is complex and involves regulatory hormones, synthesizing and metabolizing enzymes, binding proteins, transporters and T3 receptors.

TH Homeostasis can readily be disturbed by a broad variety of compounds acting via different mechanisms, especially in the rat.

The available assays – though not validated yet – allow a mechanistic evaluation of observed interactions.

The relevance of rat findings for the human situation can be assessed based on mechanistic and dose / kinetic considerations.

Species differences between rat and humans do exist in certain cases, but must be worked out on a case by case basis.
Example 1

2 Week Rat Study – Study Outline

• Compound primarily developed as an agrochemical but then switched and now intended for use as an animal health product against certain ectoparasites.

• 2 week rat study (oral, gavage, daily)
  • Male and female Wistar Crl:WI (Han) rats (n = 5/dose/sex, + 3 m/f per dose for TK)
  • Dose groups: 0, 20, 100, 400 mg/kg bw
  • Vehicle: Ethanol/Kolliphor HS15\textsuperscript{®}/tap water (10/40/50)
  • Endpoints: clinical parameters (mortality, general observation, food and water intake, body weight, ophthalmology), clinical pathology and full post mortem examination, histopathology on selected organs/tissues, evaluation of selected liver enzymes and mRNA levels
Example 1

2 Week Rat Study - Results

• Survival was not affected
• Food and water intake within normal range
• Body weight: no differences
• Hematology: Hemoglobin ↓ (M+F) – Thrombocyte count ↑
• Toxicokinetics: No sex difference on day 1 but ↑ in F on day 10, no dose dependent increase in AUC, after repeated dosing slight decrease in AUC

• Liver:
  • Weight (abs. / rel.) ↑ 60 % in M; > 90 % in F starting at the low dose
  • Gross Observation: Liver enlarged, discoloration
  • Gamma-GT ↑ in M+F, Cholesterol ↑ in F,
  • Epoxide Hydrolase, Glutathione-S-transferase, UDP-Glucuronyltransferase ↑ in M+F
  • Induction of Cyp3a1, Cyb2b1 and Ugt1a1 mRNAs suggest PXR and CAR activation
  • Histopathology: Hepatocellular hypertrophy, cytoplasmic change, increased glycogen (F)

• → Clear effect on the liver
Example 1

2 Week Rat Study – Histopathology: Liver

Control, female  High dose, female  Low dose, female
Example 1

2 Week Rat Study – Histopathology: Thyroid

Control, male

Control, female
Example 1

2 Week Rat Study – Histopathology: Thyroid

Control, male

Control, female
Example 1
2 Week Rat Study – Histopathology: Thyroid

High dose, male

Low dose, female
Example 1

2 Week Rat Study – Histopathology: Thyroid

High dose, male  High dose, female
Example 1

Summary

• Increased liver weight, induction of liver enzymes, activation of liver mRNA, hepatocellular hypertrophy and cytoplasmic change were observed.

• Results point to a massive and rapid induction of liver enzyme activities (especially phase II) resulting in an increased clearance of thyroidal hormones.

• This is leading to a hypothyroid status (↑ TSH; ↑ TSH-stimulating cells; follicular cell hypertrophy/hyperplasia).

• This mechanism is considered to be rat specific and accepted to be not relevant to humans.

• However: EFSA is currently planning to re-consider !
Example 2

Overview

• Safety assessment to support FiM trial in adult healthy volunteers and clinical trials with repeated administration according to ICH M3
  • 4 week repeat-dose toxicity studies in rats and dogs with once daily oral (gavage) administration
  • Both species responsive to the pharmaceutical activity of the compound
  • Dose selection based on the results of dose-range finding studies with treatment duration of 2 weeks: Rat: 0, 30, 90 and 300 mg/kg
  • No adverse effects were observed in the 2 week studies → Thus, recovery period was not included in the 4 week study
  • Results: Mode of action related-effects in both species with exaggerated pharmacological effects in the rats at the high dose of 300 mg/kg and thus, exceeding the MTD
  • **BUT: Unexpected findings in the thyroid and pituitary gland in the 4 week rat study**
Example 2

4 Week Rat Study - Results

• **Thyroid gland:**
  - ↑ Thyroid Stimulating Hormone (TSH) in M+F at ≥ 30 mg/kg (up to 7x)
  - ↑ Triiodothyronin (T3) in F at 90 mg/kg (1.2x)
  - ↓ Thyroxine (T4) in M at 300 mg/kg (1.8x)
  - Colloidal alteration (slight to moderate), Follicular cell hypertrophy (slight to marked) in M+F at ≥30 mg/kg, but more pronounced in males

• **Pituitary gland:**
  - Activation of TSH producing cells (↑ pale cells, minimal to slight) in M ≥30 mg/kg

• **No** macroscopical changes, **no** effects on organ weights
• **Liver without any abnormal finding**
Example 2

4 Week Rat Study - Histopathology

Thyroid gland, control male

Thyroid gland, male treated @ 90 mg/kg
- Follicular cell hypertrophy
- Colloidal alteration
Example 2

4 Week Rat Study - Histopathology

Thyroid gland, control male

Thyroid gland, male treated @ 90 mg/kg
  • Follicular cell hypertrophy
  • Colloidal alteration
Example 2

4 Week Rat Study - Histopathology

Pituitary gland, control male

Pituitary gland, male treated @ 300 mg/kg
Increase in Pale cells
Example 2

Questions to be answered:

• Is the finding adverse / not adverse?

• Is the finding reversible?

• Is the finding relevant for humans?

Overall:

What is the consequence with respect to the Risk-Benefit-Assessment?
1. **Retrospective evaluation of 2 week study**
   - Histopathological evaluation of thyroid gland and pituitary gland of exploratory (2 week) rat study (daily, oral, gavage)
     - Dose levels (n = 5): 0, 10, 30, 100 mg/kg bw
     - Results:
       - Pituitary gland: ↑ Pale cells in two M @ 100 mg/kg
       - Thyroid gland: Follicular cell hypertrophy with increased severity in M @ ≥ 30 mg/kg and in F @ 100 mg/kg

   → **Findings already observed after 2 weeks of treatment**

2. **Evaluation of reversibility**
   - 4 week rat study (daily, oral, gavage) + 4 week recovery
     - Dose levels (n = 8): 0, 90 mg/kg bw
     - Results:
       - Thyroid Hormones: no relevant differences between control and treated rats after rec.
       - Histopathology: **Clear tendency towards reversibility of thyroid and pituitary gland effects**
3. Mechanistic *in vitro* studies

- *In vitro* evaluation of iodination inhibition in a chemical model
  - No effect observed

- *In vitro* evaluation of inhibition on hog thyroid peroxidase
  - Method: Incubation of hog thyroid peroxidase with cpd @ different concentration (25 – 50 – 100 – 250 µM), H$_2$O$_2$ and guaiacol or iodide as substrates; positive control (amitrol)
  - Result: Concentration dependent inhibition of iodide oxidation

→ Hints for inhibition of thyroid hormone synthesis with peroxidase as target

However, not at human relevant expected plasma concentration levels
4. Mechanistic *in vivo* study

- 2 week rat study (oral, gavage, daily)
  - Dose levels (n = 20 males/dose): 0, 30, 90, 300 mg/kg bw
  - Endpoints: clinical parameters (mortality, general observation, body weight), TSH, T3, T4 (2 h after administration of test compound on day 1, 3, 8 and 15), liver enzymes and full post mortem examination (macroscopy, histopathology: liver, pituitary gland, thyroid gland)
  - Necropsy: 5 males of each dose group on day 1, 3, 8 and 15

- **Results:**
  - **Time- and dose-dependent onset of thyroid effects** (early ↓T4 decrease, ↑TSH, follicular cell hypertrophy)
  - No effect on liver enzymes and morphology
The observed effects in the thyroid and the pituitary gland are considered to be adverse, but showed to be reversible within a 4 week recovery period.

Results of mechanistic studies point to an inhibition of thyroid hormone synthesis by the test compound leading to a hypothyroid status in a dose- and time-dependent manner (↑ TSH; ↑ TSH-stimulating cells; follicular hypertrophy, colloidal alteration).

Mechanism per se also relevant for humans.

However, rat known to be very sensitive and with respect to functionality not comparable to human situation.

Dog more human-like and comparable – no findings observed in dog up to high dose of 25 mg/kg resulting in MoEs of 59 for $C_{\text{max, u}}$ and 20 for $\text{AUC}_{\text{u}}$ (based on expected human therapeutic exposure).

Findings are considered as not prohibitive for intended administration period.
Example 3
Dog, 4-week Study

Commonly the dog is not susceptible for thyroidal activation and clinical or histopathological changes are rarely seen:

Control, male and female
Example 3

Dog treated, 4 weeks
Conclusion

• The rat thyroid gland is very sensitive to any xenobiotic (and to fixatives!)

• There are no morphologic differences in the hypertrophic follicular cell epithelium of the thyroid gland, irrespective of the mode of action of the test compound

• **Induction of liver enzyme activities** (especially phase II) result in an increased clearance of thyroidal hormones and a secondary hypothyroid status. Measurement of these enzymes at early time-points is considered as useful. This as rat-specific accepted mechanism is currently under discussion (EFSA)

• Direct **inhibition of thyroid hormone synthesis** is leading to a hypothyroid status (↑ TSH; ↑ TSH-stimulating cells; follicular cell hypertrophy/hyperplasia) as well. The measurement of these parameters should be included into the early investigations.

• Direct **binding of the compound to specific receptors** may occur in dogs and other species.
Közsönöm/Thank you!

Ute Bach, Heidrun Ellinger, Alexius Freyberger, Elke Hartmann, Bettina Lawrenz, Laura Popp, Christine Ruehl-Fehlert, and Ludwig Schladt at Bayer AG