Demystifying juvenile toxicity study designs for regulatory success

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Neonatal toxicity studies pose some unique challenges that do not exist in adult toxicity studies

Children are not just small adults – need to be considered as a separate entity due to degree of organ system maturity and the rapid changes they undergo.
Understanding organ system maturation is critical. “Standard” designs do not really exist:

**Possibilities are almost endless:**
- Routine toxicity parameters?
- Satellite group needed for bleeds?
- Behavioral assessments?
- Reproductive assessment?
- Immunology assessment?
- Recovery?

Key is to tailor design according to test material class, known effects in adult pre-clinical studies, age of target population, duration of patient treatment.
This presentation will mainly focus on the rodent model and will cover:

+ A brief overview of organ system development comparisons
+ General design/planning considerations
  + E.g. species selection, dose route
+ Practical challenges
  + What can and cannot be done
Until ~14 years ago, targeted pediatric drug development significantly lacking – extensive “off-label” use.

Problem – this assumes that pediatric patients will exhibit similar disease progression and respond similarly to the intended therapeutic intervention as adult patients.

Estimated 50% - 90% of drugs never specifically evaluated for pediatric use.

How can we be sure that adult/pediatric toxicity profiles are the same?

Inherent differences between mature and immature systems means that there is a risk of:

- Unique toxicity profile in children
- Poor efficacy
- Exaggerated pharmacology
- Unexpected adverse effects, even death.

Guidance/Guidelines

FDA issued formal guidance in 2006:
+ “Guidance for Industry – Non-clinical Safety Evaluation of Pediatric Drug Products”.

EMA guideline issued in 2008:
+ “Guideline on the need for non-clinical testing in juvenile animals of pharmaceuticals for paediatric indications”

Oct 2012: Japanese MHLW guideline issued:
+ “Guideline on the Non-clinical Safety Study in Juvenile Animals for Pediatric Drugs”.

2015:
+ Discussions began to create an ICH guideline – introduction is imminent.

Now, drug development programs for a pediatric population must take into consideration possible effects on developmental processes specific to the relevant age groups.
Why do we need them?

To obtain information on potentially different safety profiles from those seen in adults – bridging the gap between reprotoxicity and repeat dose toxicity studies.
When are they needed?

Situations that would justify toxicity studies in juvenile animals include, but are not limited to:

+ When the indication is specifically targeted for children
+ Findings in non-clinical studies that indicate target organ or systemic toxicity relevant for developing systems.
+ Possible effects on growth and development in the intended age group.
+ If a pharmacological effect of the test compound could/would affect developing organs.
+ Unique chemical class or unique combination product.
The original proposal was that the conduct of juvenile toxicity studies should be considered on a case-by-case basis.

The emphasis has changed though. Rather than questioning whether studies need to be conducted, there is now an assumption that these are required unless you can justify why they are not!

Study design/content must be discussed with, and approved by, the Regulatory Agencies (FDA/EMA).

In the EU, as part of regulation a Pediatric Investigation Plan must be submitted and agreed by the Pediatric Committee (PDCO).

These are also required by FDA although in a slightly different format.
Challenges

There are many!

- Children are not “miniature adults”
- Predicting responses in children based on adult data can be difficult and unreliable
- Known cases of different sensitivity between children and adults
Examples of different sensitivity

Chloramphenicol (broad spectrum antibiotic)
Associated with “Grey baby syndrome” in premature babies because of limited clearance → exposure increased due to a longer $T_\frac{1}{2}$ (26 h) compared to adults (4h).

Valproic acid (anti-convulsant - epilepsy)
Young children treated with VPA appear disproportionally vulnerable to fatal hepatotoxicity.

Aspirin
↓ susceptibility due to abnormal fat accumulation in liver and other organs, and severe increase in intracranial pressure (Reye’s syndrome)

(Quoted in US FDA CDER Guidance 2006)
Post-natal growth and development can affect drug disposition and action:

- Metabolism (maturation rate of Phase I/II enzyme activities)
- Body composition (water and lipid partitions)
- Receptor expression and function
- Growth rate
- Organ functional capacity

These are all susceptible to modification or disruption by drugs.
### Where does a child begin and end?

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Pathophysiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm or Newborn</td>
<td>Unique pathophysiology</td>
</tr>
<tr>
<td></td>
<td>Difficult to extrapolate from adults/children</td>
</tr>
<tr>
<td>Infant &lt; 1 month</td>
<td>Blood-brain barrier (BBB) immature</td>
</tr>
<tr>
<td></td>
<td>Altered pharmacodynamics</td>
</tr>
<tr>
<td></td>
<td>Less predictable absorption</td>
</tr>
<tr>
<td>Infants/Toddlers (1 m - 1 year)</td>
<td>Developing CNS, immune system, growth</td>
</tr>
<tr>
<td></td>
<td>Altered pharmacodynamics</td>
</tr>
<tr>
<td>Children/Adolescents (&gt;2 yrs)</td>
<td>Mainly growth phase</td>
</tr>
<tr>
<td></td>
<td>Developing repro system</td>
</tr>
<tr>
<td></td>
<td>↑ compliance issues</td>
</tr>
</tbody>
</table>
Comparative age categories

Based on CNS and reproductive system development
Study design considerations

There is no such thing as a “standard” study design - each study is uniquely tailored according to the test material, target population, organ system of interest & duration of use.

Age of animals at start of dosing
+ To match lowest age of target patient group

Duration of dosing period
+ To cover developing organ system(s) of interest
+ To cover all target pediatric groups (age range)
+ To reflect planned clinical use (e.g. single dose)
Number of animals

Determined by age at start of treatment and investigations required:

+ Pre-weaning – some dose routes ↑ risk of dosing trauma.

+ Blood sampling:
  TK or hematology/blood chemistry?
  + Pre-weaning currently most likely to be terminal. Pool samples?
  + Post-weaning may be only 1 sample/occasion due to limited circulating blood volume.
    If haem/blood chem needed, may still need to be terminal prior to Day 35 of age.

+ Reproductive/behavioral/recovery assessments:
  + Group size must be sufficient to allow meaningful assessment and strong statistical power (for repro, remember ICH requirements).

+ Multiple cohorts? (e.g. where repeat daily dose not possible, but still need to cover every developmental stage)
Main study endpoints – routine

+ Growth (bodyweight and limb length)
+ Development (external indices of sexual maturation)
+ Clinical/post-dosing signs
+ Food intake (post-weaning only)
+ Hematology/blood chemistry/urinalysis (usually post-weaning)
+ Organ weights
+ Macropath/micropath investigations
+ Reversibility
+ Toxicokinetics (critical endpoint)
Neurotoxicity and immunotoxicity assessments:
Triggered if the class of compound or previous studies in humans or animals give cause for concern
+ Behavioral tests usually in recovery, but can be evaluated during treatment (generally for CNS active drugs)

Reproductive function:
If positive signal on fertility study (ICH 4.1.1) or among F1 selected animals on pre and post natal study (ICH 4.1.2), histopathological changes in male/female reproductive organs in adult studies or delays in attainment of puberty
+ Needs group size of 16+ (ICH 4.1.1 – 4.1.3 Repro guidelines)
+ Usually only assessed after a period of recovery
To provide cover for 1 month old babies and older, dosing of rat pups could start on Day 7 of age.

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<td>6M+6F</td>
</tr>
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<td>18M+18F in treated groups; 6M+6F in Controls</td>
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## Will be required if bioanalytical method is not compatible with microsampling: up to two 35 µl non terminal blood samples can be obtained from the tail artery of juvenile rats on Day 7 of age, when samples are taken at least 2 hours apart.
US FDA CDER Guidance 2006:

“For Developmental neurotoxicity assessments, well-established methods should be used to monitor key central nervous system functions, including, assessments of reflex ontogeny, sensorimotor function, locomotor activity, reactivity, learning and memory.”

A neurobehavioral testing battery could be:
+ Ontogeny of Air righting reflex from Day 14 of age
+ Auditory startle response and pupil closure reflex on Day 20 of age
+ Post weaning automated motor activity monitoring
+ FOB – In The Hand and Arena observations
+ Learning and Memory – Morris water maze place task
+ Automated auditory startle response pre pulse inhibition
Study design – neurotoxicity endpoints

**Group size:**
We suggest that this should be 20 males and 20 females to match the group size on the Pre and Post natal study (ICH 4.1.2) for medicinal products and the group size for most of the neurobehavioral tests on the OECD 426 Developmental Neurotoxicity study for chemicals.

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Our Services – Automated Motor activity monitoring
Motor activity monitoring

– High beam breaks (rearing activity) – positive control data

![High beam breaks graph](image-url)
## Arena observations – positive control data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Grade</th>
<th>Group/sex:</th>
<th>1M</th>
<th>2M</th>
<th>3M</th>
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</thead>
<tbody>
<tr>
<td>Group/sex:</td>
<td></td>
<td>Control</td>
<td>Acrylamide</td>
<td>Acrylamide</td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td></td>
<td></td>
<td>Acrylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose (mg/kg/occasion)</td>
<td></td>
<td>0</td>
<td>12.5</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>Number of animals:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parameter</td>
<td>Grade</td>
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<td>Palpebral closure (0-3)</td>
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<td>7</td>
<td>8</td>
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<td>0</td>
<td>3</td>
<td>1</td>
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<td>2</td>
<td>0</td>
<td>0</td>
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<td>Posture</td>
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<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Gait (U, 0-3)</td>
<td></td>
<td>U</td>
<td>1</td>
<td>3</td>
<td>4</td>
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<tr>
<td></td>
<td></td>
<td>0</td>
<td>9</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Arousal (1-5)</td>
<td></td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>10</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Activity count</td>
<td>Mean</td>
<td>Wi</td>
<td>12.6</td>
<td>6.7*</td>
<td>7.6*</td>
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<tr>
<td></td>
<td>SD</td>
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<td>6.7</td>
<td>4.1</td>
<td>5.6</td>
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<tr>
<td>Rearing count</td>
<td>Mean</td>
<td>Wi</td>
<td>8.2</td>
<td>3.4**</td>
<td>3.4**</td>
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<tr>
<td></td>
<td>SD</td>
<td></td>
<td>5.3</td>
<td>1.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Tremor (0-3)</td>
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<td>0</td>
<td>9</td>
<td>5</td>
<td>6</td>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>4</td>
</tr>
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</table>
Test of learning and memory
Automated auditory startle response testing
Study design – Reproductive function evaluated

Usually evaluated at Day 14 of gestation as per the fertility study ICH 4.1.1

Evaluation can be more extensive with potential effects upon parturition and post natal survival and development of the offspring assessed for example if these stages of the life cycle have not been evaluated on earlier studies:

+ Needs group size of 16+ (ICH 4.1.1 – 4.1.3 Repro guidelines)
+ Usually only assessed after a period of recovery
+ Quite common to pair treated males with untreated females and treated females with untreated males

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<td>20F</td>
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Evaluations during treatment and recovery could include:

+ Immunophenotyping of peripheral blood leukocytes
+ T-cell dependent antibody response (TDAR) to Keyhole limpet hemocyanin (KLH) = IgM response

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</table>
Mean female serum concentrations (ng/mL) of T-cell dependent IgM antibodies following IV administration of KLH to Sprague Dawley rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose (µg/animal/occasion)</th>
<th>Pre-treatment</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 29</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0</td>
<td>Mean</td>
<td>34100</td>
<td>45000</td>
<td>65500</td>
<td>70700</td>
<td>56700</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S.D.</td>
<td>18100</td>
<td>19700</td>
<td>38900</td>
<td>28400</td>
<td>27900</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>%CV</td>
<td>53.0</td>
<td>43.9</td>
<td>59.5</td>
<td>40.1</td>
<td>49.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>KLH</td>
<td>300</td>
<td>Mean</td>
<td>39400</td>
<td>50300</td>
<td>144000</td>
<td>169000</td>
<td>70000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S.D.</td>
<td>24300</td>
<td>15600</td>
<td>73600</td>
<td>320000</td>
<td>118000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>%CV</td>
<td>61.6</td>
<td>31.1</td>
<td>51.1</td>
<td>189.6</td>
<td>168.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>KLH/Cyclophosphamide</td>
<td>30/10</td>
<td>Mean</td>
<td>36700</td>
<td>34200</td>
<td>18000</td>
<td>12500a</td>
<td>7760a</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>S.D.</td>
<td>17000</td>
<td>8250</td>
<td>2640</td>
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<td>N.A.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>%CV</td>
<td>46.4</td>
<td>24.1</td>
<td>14.6</td>
<td>N.A.</td>
<td>N.A.</td>
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<td></td>
<td></td>
<td></td>
<td>N</td>
<td>7</td>
<td>7</td>
<td>7</td>
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</tr>
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</table>
Incorporates all aforementioned evaluations: neurotoxicity, reproductive function and immune system. The design would be as follows:

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Age at start of treatment will affect how animals are sourced:

Pre Day 10: time-mated females:
+ Day post coitum limit on dispatch from supplier?
+ Spares in case of non-pregnancy? Sex ratio issues?
+ Day 10 onwards: dams with litters:
  + Age limit on dispatch from supplier?
  + Supplier litter randomization?
  + Post-weaning start – natural litters ok (allocate 1+1/group).

Age stagger on arrival dependent on investigations required during study.
Selection of species

- Must be appropriate for evaluating tox endpoints relevant for intended pediatric population
- Rats are traditionally the species of first choice
- Dogs are usually the second species
- Testing in one appropriate species using both sexes will normally be sufficient (but not always)
  - CNS drug: FDA Neurology Division require rat for behavioral assessment (e.g. Morris maze), but dog for CNS maturation (pathology)
Why the rat?

- Major species in general toxicity data
- Studies run in a short time-frame
- Can synchronize breeding/animal supply
- Pup dosing possible from an early age
- Good statistical power by using a large number of animals (the number of animals determined by consideration of accuracy of evaluation and the principles of the 3Rs (reduce, refine, replace))
- Established functional/behavioral tests
- Reproductive function easily assessable
- Can easily randomize litters pre-weaning:
  - Spreads genetic pool, eliminating genetic bias
  - Can use all litters born (no wastage due to abnormal sex ratio/litter size)
Why not the rat?

+ May not respond to compounds in similar way to man
+ Small size
  + may compromise some routes of dose administration
  + makes toxicokinetic and/or clinical pathology sampling difficult:
    + uses many animals because pre-weaning bleeds are generally terminal
    + achievable blood volumes low, so may need to pool samples
Dog

- Usually performed at the request of the regulator to answer a specific question, e.g. recent request from FDA to perform pre-weaning study in dogs to assess CNS pathology, to further investigate findings in the rat juvenile studies.
- Not always necessary to perform all 'standard' toxicology endpoints.
- Long lead-in time
  - Suppliers do not wish to release their breeding females.
- Cannot synchronize breeding of parent females
  - Supply will be over several weeks.
- Long duration studies due to longer maturation.
- Low statistical power.
- Behavioral/reproductive assessments difficult.
Minipig

+ Usually for dermal studies, however, this may change as more minipig toxicity studies are performed instead of the dog
+ Feasibility study demonstrated that they can be cross-fostered and hand-reared
+ Hand-rearing may be important in dermal studies, as grooming by the sow would lead to oral dosing that could possibly increase the exposure to the piglets through the milk
Avoiding genetic bias

Rat and minipig: Randomization at early age
+ Mix-up pups to derive new litter
+ Maternal rejection uncommon for young offspring
+ Single dose group for litter (prevent cross-contamination)

Dog: Randomization not appropriate
+ Difficult to synchronize the time of birth in dogs and cross-fostering needs to occur when the pups are very young to avoid rejection
Dose route/volume rat

Stage of physical development determines feasibility of dose route:

+ SC/IP/Oral (buccal cavity): Day 1 of age
+ Oral gavage: From ~Day 14 of age
  + Dose volume ideally 5 mL/kg for oral route
  + 10 mL/kg possible with care (if maximum practical formulation concentration is low)
+ Intravenous: single dose from Day 14 of age; repeat dose from Day 21 of age
+ Intramuscular: Day 21 of age
+ Inhalation: 1-hr snout only exposure feasible from Day 2 of age

Use intended clinical route, where practical. Sometimes use substitute route until clinical route is feasible, e.g. subcutaneous administration instead of repeat IV dosing pre-weaning.
Oral dosing of beagle puppies

Experience of dosing from Day 22 of age, but dosing could start earlier
Inhalation administration

Poses some unique challenges when compared to other routes:

Size of animals
- Young rat pups much smaller than mice (handling issues)
- No fur to ~Day 11 of age (maintenance of body temp during exposure)

Age of start of treatment?
- Is there time for and/or is it practical to acclimatise to the restraint tubes?

Duration of removal from dam?
- Dose determined by length of exposure due to maximum practical concentration
- Needs to be minimised to help maintain body temperature and to prevent maternal rejection
Inhalation administration

Method of exposure:

+ Snout only a challenge due to size (pivot ability → turn around in tubes; tubes small enough?)
+ Whole body → risk of maternal exposure through grooming → possible “double-hit” exposure through milk
+ We have now successfully completed four main GLP-compliant juvenile studies (and associated prelims) using snout-only inhalation (3 x pre-weaned; 2 x post-weaned) with exposure for 1 hour
Formulations

Restrictions in rat studies:

+ Earlier treatment starts, the more restrictions apply.
+ Solutions only for the youngest rats, whether by gavage or subcutaneous administration.
+ Smooth, non-viscous suspension suitable for gavage from mid-lactation.
+ Restricted by the small bore of the dosing catheter.

Other species would not have these restrictions, in principle.
Practicality of procedures

Ophthalmoscopy:
+ Eyes closed at birth, so not possible prior to Day 15, so pre-treatment assessment often not feasible; can confound data interpretation

Urine collection:
+ Not practicable prior to weaning
+ Early post-weaning: individual volumes low - pooled sample/limited list of parameters

Behaviour assessments:
+ Essential to tailor to age at testing
+ During treatment - conducted prior to dosing

Ensuring correct identification of pups pre-weaning:
+ Accurate toe marking (or alternative) required
Blood sample collection in the rat:

+ Samples for young rats currently need to be pooled to achieve volume unless microsampling can be used
+ Samples are generally terminal
+ Currently working on additional method - different sampling points and microsampling

Day 1 toxicokinetic sampling:

+ Blood sample site: Decapitation
+ No anesthetic
+ Up to 0.5 mL
+ Terminal procedure

Week 4 toxicokinetic sampling:

+ Blood sample site: Lateral tail vein
+ No anaesthetic
+ Up to 0.5 mL
+ Multiple sampling occasions
+ Warming chamber required
Blood sample collection (cont’d)

- Microsampling
- Blood sample of ≤75 µL
- Capillary action
- 10-25 µL of plasma
- Will replace conventional TK where 200-300 µL of plasma was required
Other study design considerations

Dose selection
+ Exaggerated toxicity not desirable, aim is to detect any possible increase in sensitivity of young vs adults.

Preliminary study essential
+ To assess tolerability/dose-range

Inclusion of TK essential

Endpoints
+ Numerous and flexible
  + Each study has tailor-made design

Numbers per sex per group not standard
+ Depends on endpoints

Practical issues… !!
Practical issues

- Study organization
- Dosing procedure
- Formulation
- Blood sampling
- Laboratory capabilities
- Husbandry
Summary

+ Neonatal toxicity studies by any dose route are logistically challenging
+ There is no such thing as a “standard” study design
  + Each study is uniquely tailored according to the test material, target population, organ system of interest & duration of use.
+ Must recognize that organ systems differ between babies and adults. So, variation in anatomical and functional maturation is an important consideration in extrapolation of animal data to man.
+ Examples of design considerations and practical issues are not an exhaustive list, but they are some of the more important factors.
+ Endpoints must be appropriate for age.
+ Use all available adult data to design study.
+ Careful and timely planning is vital!!
+ Expect the unexpected and be prepared for any and every eventuality!!
References

+ Cappon et al. Juvenile animal toxicity study designs to support paediatric drug development. Prepared for publication in Birth Defects Research, Developmental and Reproductive Toxicology.


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