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IMPORTANCE AND THE UNIQUE ASPECTS OF MODALITIES FOR CONDUCTING NON-CLINICAL AND PRE-CLINICAL STUDIES OF COMPARTMENTAL AND NON-COMPARTMENTAL ANALYSIS

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Abstract

Pre-clinical and nonclinical studies are a fundamental step to assess the safety and quality of ingredients new to infant formulas. In drug development, pre-clinical and non-clinical studies are the stages of research that must be performed before an ingredient can be considered for clinical studies in humans in order to determine the potential toxicity of the ingredient, its metabolites, and its matrix. The FDA Redbook II and Redbook 2000 provide comprehensive guidelines for pre-clinical studies. Mainly, two levels of pre-clinical assessment are recommended. Level 1 assessment suggests standard measures for each organ system (e.g., gastrointestinal, blood, kidney, immune, endocrine, brain) and are required for any new ingredient. Level 2 assessments include in-depth measures of organ systems that would be used to explicate equivocal level 1 findings or specific theoretical concerns not typically addressed by level 1 test. A distinct set of procedures using appropriate non-clinical and pre-clinical studies at relevant developmental stages should be included in studies to assess safety following established guidelines. Rats and mice are commonly used in pre-clinical studies, but there are some limitations to achieve a developmental activity due to the difficulty to feed the active ingredients of a new formula to a preweanling rodent. The non-human primate and the piglet are more conformable for these types of studies.

Keywords: *non-clinical; pre-clinical; compartment; risk assessment; toxicogenetic study*

Introduction

The non- and pre-clinical developments, in essence of drug discovery and development are the stage of research that begins before clinical trials (testing in humans) can begin, and during which important feasibility, iterative testing and drug safety data are collected (Emanuel, 2015). Pre-clinical studies are a fundamental step to assess the safety and quality of ingredients new to infant formulas. They must be performed before an ingredient can be considered for clinical studies in humans in order to determine the potential toxicity of the ingredient, its metabolites, and its matrix (Deckelbaum et al., 2004). Guidelines for these studies to assess the safety of infant formulas must be based on considerations of the diversity of potential new ingredients and the ingredients' source and matrix (Haslberger, 2003).

The main goals of pre-clinical studies are to determine the safe dose for first-in-man study and assess a product's safety profile (Atanasov et al., 2015). Products may include new medical devices, drugs, gene therapy solutions and diagnostic tools. The discovery and development are related to the frequent or repetitive pre-clinical trials (Munro et al., 1999). The FDA Redbook II and Redbook 2000 (OFAS, 2001, 2003) provide comprehensive guidelines for conducting pre-clinical studies to test the safety of food and color additives (Hinton, 2000). Current regulatory guidelines for pre-clinical studies are described, and a two-level assessment process is proposed. The recommended two-level process is a flexible approach that can accommodate a variety of potential ingredients (Merrill and Francer, 2000).

Typically, both *in vitro* and *in vivo* tests are performed frequently to check the toxicity of drugs. It is due to check the organ protectivity, as well as if there are any long-term carcinogenic effects or toxic effects on the mammalian reproductive system (Olson et al., 2000).

This paper describes the importance and the unique aspects and modalities of conducting non-clinical and pre-clinical studies to assess the safety of infant formulas in both compartmental and non-compartmental analysis.

Recommended levels of compartmental and non-compartmental assessment

A hierarchy of two levels of pre-clinical assessment, using techniques from cellular-molecular studies through whole-animal studies, should be implemented to assess the safety of ingredients new to infant formulas for developing organ systems (Abrams et al., 2007). Level 1 assessments are suggested standard measures for each organ system (e.g., gastrointestinal, blood, kidney, immune, endocrine, and brain) and are required for any new ingredient. Level 2 assessments, in-depth measures of organ systems or functions that would be performed to explain abnormalities found in level 1 assessments and specific theoretical concerns not typically addressed by level 1 test. These are suggested measures to assess any new ingredient that primarily interacts with an organ system, has a metabolite that interacts with an organ system, or stimulates or changes the synthesis of factors (e.g., hormones, cytokines, immunoglobulin, and endotoxin) that interact with an organ system.

Non-clinical studies

Non-clinical testing is conducted on a stage of medicines development that uses animals and/or cells or tissues. It does not involve testing in humans. The main goal of non-clinical tests is to determine the safety of a medicine. Non-clinical testing will investigate any harmful effects of the medicine on the body due to the medicine's pharmacology.

Structure, stability, and solubility

The complete chemical structure and functional groups and the purity and stability of the intended and non-targeted ingredients present in the matrix must be determined using well-established physical methods (Zschocke et al., 1998). The high

performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and thin layer chromatography (TLC) are commonly used to assist the structure, molecular mass, and purity of most classes of compounds because derivatization is not necessary.

Genetic tests

To cause molecular changes in the deoxyribonucleic acid (DNA) or to cause structural changes in the chromosomes of cells needs, evaluating all ingredients, their metabolites, or their secondary effectors for their ability (Bachevalier, 2001). These changes may include forward and reverse mutations, point mutations, deletion mutations, chromosomal aberrations, micronuclei deletions, polymorphisms, DNA strand breaks, or unscheduled DNA synthesis (Derisi and Iyer, 1999).

Cellular studies

It is often most efficient to perform *in vitro* studies of metabolism before whole-animal (oral dosing) studies to provide information about future *in vivo* studies and estimate dosages to be used in pre-clinical animal studies (Potter, 1951). *In vitro* work and pharmacokinetic modeling can be used to predict the potential toxicity and *in vivo* kinetics of the ingredients and the matrix (Macgregor et al., 2001).

Pre-clinical studies

Pre-clinical studies refer to the testing of a drug, procedure or other medical treatment in animals before trials may be carried out in humans. During pre-clinical drug development, the drug's toxic and pharmacological effects need to be evaluated through *in vitro* and *in vivo* laboratory animal testing.

Toxicological studies

Toxicology is a discipline, overlapping with biology, chemistry, pharmacology, and medicine, that involves the study of the adverse effects of chemical substances on living organisms and the practice of diagnosing and treating exposures to toxins and toxicants (Lee et al., 2017). Several toxicity studies must be performed in animals to ensure the safety of ingredients new to infant formulas. These toxicological studies are described below.

Acute, sub-chronic, and chronic toxicity studies

For all levels of toxicity studies, the route of administration of the supplement should approximate that of normal human exposure as closely as possible (e.g., through the diet in the case of infant formulas). If there is no information that can be used to determine the appropriate dose levels for short-term or sub-chronic toxicity levels, toxicity studies should begin with tests of acute toxicity, followed by sub-chronic, and finally chronic assessments (Weathereholtz, 1997).

Developmental toxicity studies

In the evaluation of ingredients new to infant formulas, the developmental toxicity study is used to evaluate the effects of the ingredient on developing fetuses that result from exposure of either parent prior to conception or to mothers during gestation (Knudsen et al., 2009). The main manifestations of an effect on the developing organism are death, structural abnormality, altered or retarded growth, and functional deficiency.

Gastrointestinal tract function studies

The development of the infant's gastrointestinal tract is essentially complete at birth and, therefore, assessments of its proper development will involve ensuring that its functions (e.g., digestion, absorption, secretion) have not been impaired by the addition of an ingredient new to infant formulas (Palmer et al., 2007). **Table 3** provides several examples of the types of tests that could be used in level 1 and level 2 assessments of the gastrointestinal tract.

Hepatic function tests

The liver is involved in synthesis, metabolism, and excretion. Therefore, along with the above mentioned histology evaluation, tests that account for each of these functions must be performed as part of the level 1 assessment of liver health. Level 2 tests should be used to explicate equivocal level 1

findings or specific theoretical concerns not typically addressed by level 1 test (Silen et al., 1957; Cho et al., 1976; O'Reilly et al., 1996). **Table 4** provides some examples of the types of tests that could be used in level 1 and level 2 assessments of liver health.

Hematological function tests

Ingredients new to infant formulas or their metabolites may have profound effects on the bone marrow. **Table 5** provides some examples of the types of tests that could be used in level 1 and level 2 assessments of hematological function (Silen et al., 1957; Cho et al., 1976; Palmer et al., 2007).

Immunological function tests

The immunological system is highly complex and has been shown to be sensitive to nutritional manipulation (Miles and Calder, 1998). The various effects of nutrients in the immunological system can be divided into those mediated by antigen-specific immunoglobulin (Ig) E (allergic reactions), other antibodies, T-cells, cytokines, and chemokines, and those mediated by non-immunological mechanisms. **Table 6** provides unknown allergenic properties that could be used in level 1 and level 2 assessments (Miles and Calder, 1998).

Endocrine function tests

Growth abnormalities of the test animal are an important early indication of a possible effect of a new ingredient in the endocrine system. Because endocrine effects may not be immediately apparent in growth changes, nor in other metabolic functions, some screening tests are indicated (Boyar et al., 1973; Spiegel et al., 1999). **Table 7** provides some examples of the types of tests that could be used in level 1 and level 2 assessments of endocrine function.

Other pharmacological studies

Animal models are used as a tool in initial toxicology studies before human clinical trials are conducted. The most commonly used animal models for general toxicological studies are the rat and mouse because the biological characteristics of them are similar to human. The advantages and challenges of using each animal model have been summarized in **Table 8**.

Rats and mice are commonly used in pre-clinical studies, but there are some limitations to achieve a developmental activity due to the difficulty to feed the active ingredients of a new formula to a preweanling rodent. The non-human primate and the piglet are more conformable for these types of studies. **Table 9** provides some toxicogenetic studies using different animal models.

Final considerations

Non-clinical and pre-clinical studies are an exigent first step to assess the safety and quality of ingredients new to infant formulas. Non-clinical and pre-clinical studies must be based on the regulatory guidelines and the FDA Redbook guidelines. Regulatory guidelines and FDA Redbook guidelines provide comprehensive guidelines for conducting non-clinical and pre-clinical studies based on the considerations of diversity of the potential new ingredients and the ingredients' source and matrix.

It is concluded that on the basis of biological insight, the importance and the unique aspects and modalities of conducting non-clinical and pre-clinical studies to assess the safety of infant formulas in both compartmental and non-compartmental analysis, meaningful non-clinical and pre-clinical systems can be developed and identified, which constitute a scientific basis for the development and clinical implementation of novel systems therapeutic interventions.

Competing interests

The authors declare that they have no competing interests.

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Table 1. Examples of potential in vitro tests to assess genetic toxicity

Test Name	Function of Test	References
Ames test	Microsomal reverse mutation	Aeschbacher et al., 1983
Mouse lymphoma thymidine kinase gene mutation assay	Genetic forward mutations	McGregor et al., 1987, 1988a, 1988b; Myhr and Caspary, 1988; Myhr et al., 1990
Mammalian erythrocyte micronucleus test	Micronuclei deletions, chromosomal aberrations	Schlegel and MacGregor, 1982; Schlegel et al., 1986
Polymerase chain reaction	Changes in gene expression and deoxyribonucleic (DNA) sequence, polymorphisms, point mutations	Innis et al., 2012
DNA microarray	Identifies genes that are up or down regulated	DeRisi and Iyer, 1999; Perou et al., 1999; Williams, 1999; Lee et al., 2000; Wang, 2000; Guengerich, 2001; Moreno-Aliaga et al., 2001; Cohen et al., 2002; Daniel, 2002
Proteonomics	Identifies proteins that are altered after exposure to the ingredient	Anderson and Anderson, 1998; Hochstrasser, 1998; Jungblut et al., 1999; Govorun and Archakov, 2002; Bogyo and Hurley, 2003; MacBeath, 2002

Table 2. Examples of acute, sub-chronic, chronic, and developmental toxicity studies

Study	Example
Acute toxicity	Single dose known to be toxic to the species, followed by observation of the animals for at least 2 weeks and establishment of the lethal dose for 50% of the animals (LD_{50}) for the ingredient, known bioactive metabolites, and biomass (source)
Subchronic toxicity	Generally conducted for 90 days in rats using doses established with the acute toxicity studies
Chronic toxicity	Can follow the subchronic and are usually carried out beyond the 90-day period and perhaps to adulthood
Developmental toxicity	Multigenerational study endpoints: generation toxicity F_0 (parental generation) and F_1 (second generation) Reproductive toxicity study endpoints: fertility, live born, weaning, viability indices, and male reproductive indices (e.g., testicular spermatid numbers)

Data source: OFAS (2001, 2003).

Table 3. Gastrointestinal assessment: examples of tests in level 1 and level 2

Level	Assessment
Level 1	• Absorption • Cell culture • Organ weight/histology
Level 2	• Isotopic absorption tests • Microarray/proteonomics • Receptor expression • Specific histology stains • Permeability tests

Table 4. Hepatic assessment: examples of tests in level 1 and level 2

Level	Assessment
Level 1	• Liver weight/histology, cell culture/mutagenicity • Assessment of synthetic function: serum ALAT, ASAT, ornithine carbamyltransferase, albumin:globulin, coagulation profile, prothrombin time, partial thromboplastin time, radioactive amino acids, electrophoresis techniques for serum proteins • Assessment of excretion function: gamma-glutymltransferase, LDH, bilirubin, alkaline phosphatase • Assessment of metabolic function: total protein, albumin, fasting glucose, urea nitrogen, triglycerides (LDL, VLDL), cholesterol (HDL and LDL)
Level 2	• Metabolism assessments • Microarray/proteonomics • Protein electrophoresis • Special clotting factor levels • Special imaging studies • Special stains on histology

Here, ALAT = alanine amino transferase, ASAT = aspartate amino transferase, LDH = lactate dehydrogenase, LDL = low-density lipoprotein, VLDL = very low-density lipoprotein, HDL = high-density lipoprotein.

Table 5. Hematology assessment: examples of tests in level 1 and level 2

Level	Assessment
Level 1	• Bone marrow histology • Assessment of hematopoietic system: whole blood hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, total red cell count, red cell morphology • Assessment of thrombopoietic system: platelet count, platelet morphology • Assessment of white cells: total white cell count and the differential • Assessment of the clotting system: prothrombin time, partial thromboplastin time, bleeding time
Level 2	• Colony forming units • Microarray/proteonomics • Special bone marrow stains

Table 6. Immunology assessment: examples of tests in level 1 and level 2

Level	Assessment
Level 1	• T-/B-cell quantitation and function (immunological analysis of B- and T-lymphocytes and T-lymphocytes subsets [Th+Ts or CD4 and CD8]) • Thymus, spleen, bone marrow, lymph nodes, tissue histology • Electrophoresis (e.g., for changes in levels of γ-globulin fractions [IgG, IgM, IgA, IgE]) • Total serum complement and components of complement (e.g., C3 from CH_{50} determinations) • Levels of prostoglandin E_2, balance of LTB_4 and LTB_5 • Immunochemical assays of γ-interferons and serum autoantibodies (antinuclear, antimitochondrial, antiparietal antibodies of B-lymphocytes) • In vitro assays of activity of natural killer cells • Mitogenic stimulation assay of B- and T-lymphocytes • Macrophage activity assays • Stem cell assays • In vitro assays to assess allergenicity (source of the protein, amino acid sequence homology analysis, physicochemical properties)
Level 2	• Microarray/proteonomics • Special histology stains • Stimulation tests of immune function using the T-dependent or independent antigens or human vaccines • Cell-mediated immune reactivity and host-resistance assays

Here, Th = T helper cells, Ts = T suppressor cells, CD4 = cell differentiation antigen 4, CD8 = cell differentiation antigen 8, IgG = immunoglobulin G, IgM = immunoglobulin M, IgA = immunoglobulin A, IgE = immunoglobulin E, LTB_4 = leukotriene B_4, LTB_5 = leukotriene B_5.

Table 7. Endocrine assessment: examples of tests in level 1 and level 2

Level	Assessment
Level 1	• Hormone levels (e.g., T4, TSH, LH, FSH, GH, CCK, NPY, cortisol, leptin), blood sugar, insulin • Organ weight/histology (e.g., adrenals, ovaries, testes, pancreas, thyroid)
Level 2	• Microarray/proteonomics • Provocative endocrine tests (e.g., cosyntropin stimulation) • Special histology stains

Here, T4 = thyroxine, TSH = thyrotropin, LH = luteinizing hormone, FSH = follicle-stimulating hormone, GH = growth hormone, CCK = cholecystokinin, NPY=neuropeptide Y.

Table 8. Summary of animal models used in preclinical studies

Animals	Advantages	Challenges
Chicken	Immunology	No relevance to human nutrition, genetics
Dogs	Metabolism, immunology, organs	Behavior, genetics
Hamsters	Lipid metabolism	Immunology, genetics
Mice	Genetics, molecular analysis, mechanisms, organs	Learning paradigms, developmental
Nonhuman primates	Functional/behavioral relationship to humans, similar diet as humans, immunological studies, dermatological studies, renal function, kidney biopsy, invasive assessment	Basic biochemistry, histopathology, genetics; expense and ethical concerns for neural tests
Pigs	Comparable size to neonatal humans, lipid biochemistry, organs	Learning paradigms, poor model for genetics
Rabbits	Biochemistry, immunology	No relevance to human metabolism
Rats	Cellular, molecular analysis, behavior, organs, physiological studies; brain development is similar to human infant	Higher-level learning, genetics, developmental

Table 9. Toxicogenetic studies in animal models

Test	Animal	References
Repeated-dose toxicity	Rats and mice are a common choice of animal model. Non-rodent animals such as the beagle dogs, pigs, marmosets or macaques may also be used to test certain classes of chemical, such as agrochemicals and pharmaceuticals.	Smith et al., 1967; Roe, 1993; Morris et al., 2002; Manna et al 2004; National Research Council, 2004
Single-dose toxicity	Rodents: rats, mice and hamster are generally used to identify doses that causing no adverse effect and doses causing serious toxicity. Non-rodents: dogs, non-human primates, minipigs are also selected to test.	Smith et al., 1967; Manna et al 2004; Marshall et al., 2010; Liu et al 2011
Carcinogenicity	Rats, mice, or hamsters are commonly used for long-term carcinogenicity studies. Additional assessments of carcinogenic potential typically use transgenic mice in short-term study designs.	Haseman et al., 1985; Ennever et al., 1987; Whitaker et al., 1995; Kirkland et al., 2007; Brambilla et al., 2009
Genotoxicity	Mice are usually used only when one or more in vitro tests has given a positive result, and with the purpose of short-term studies investigating interactions with genetic material (DNA and chromosomes).	Ennever et al., 1987; Kirkland et al., 2007; Pfuhler et al., 2010
Reproductive and developmental toxicity	Rats and mice are a common choice of animal model for the assessment of effects on fertility, embryo-foetal development, and pre- and post-natal toxicity. Rabbits are commonly selected as a second	Gaylor, 1989; Daston et al., 1997; Seiler et al., 2004; Spielmann, 2009; Knapen et al., 2015
Local tolerance	Rats, mice, dogs, monkeys and rabbits most commonly used species. Localtolerance studies are applicable to all types of drug products, including biotechnology-derived pharmaceuticals, chemicals and herbal products.	Eckstein et al., 1969; Kaestner, 1992; Van Der Laan et al., 2008