Project Periodic Report
Publishable Summary

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Project acronym: ESNATS

Project title: EMBRYONIC STEM CELL-BASED NOVEL ALTERNATIVE TESTING STRATEGIES

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Periodic report 1st ☐ 2nd ☐ 3rd ☐ 4th ☒ 5th ☐

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Embryonic Stem cell-based Novel Alternative Testing Strategies

Background and objectives

The aim of the ESNATS project is to develop a novel toxicity test platform based on embryonic stem cells (ESCs), in particular human ESC (hESCs), to streamline the drug development R&D process and evaluation of drug toxicity in clinical studies, reduce related costs, and thus, to not only increase the safety of patients but also to reduce the number of animals normally used to these aims due to earlier detection of adverse effects.

ESNATS addresses current shortcomings in toxicity testing:

- A major part of safety testing takes place late in the research and development cycle, implying protracted experimentation involving high numbers of animals and generating significant costs.
- Some *in vitro* assays rely on cells lines of malignant origin or primary cells that are hard to standardise and that are limited in terms of quantity, homogeneity and genetic diversity.
- Existing assay systems based on primary animal and human cell lines do not reliably represent the human physiological situation of cells in native tissue.

To reach the project goals, a battery of toxicity tests is being developed using ESC lines subjected to standardised culture and differentiation protocols. Tests will cover ESCs in several stages of development as well as differentiated derivatives, including neuronal lineages, complemented with systems for hepatic metabolism. Genomics approaches will be used to determine predictive toxicoproteomics and –toxicogenomics signatures. The individual tests will be integrated into an "all-in-one" testing strategy. To ensure practical usage in the pharmaceutical industry, concepts for automated ESC culture will be developed and the test systems will be scaled up. In a later stage of the project, the predictivity, quality and reproducibility of the test strategy will be evaluated in a “proof of concept” study.

ESNATS objectives will be achieved in a five-year multidisciplinary collaboration of leading European researchers in alternative testing, toxicology, ESC research, genomics, modelling, and automation. The consortium also includes representatives from regulatory bodies, the pharmaceutical industry and ethical advisors to provide guidance to ensure rapid applicability of the developed test systems.

Progress and main results

To streamline the overall strategy towards the eventual accomplishment of a meaningful result, during the 3rd project year, the consortium had decided to concentrate its efforts on prenatal toxicity with focus on the nervous system, and how to most efficiently feed suitable tests concertedly into the approach, observing the coverage of all critical windows of neuronal cell differentiation. It had also been decided to set up a specific “biomarker study” which would complement the all-in-one test battery by focusing on gene expression analysis to establish an algorithm that allows identifying compounds that act by a certain toxic mechanism or induce a specific phenotype in a pathway-based approach.

Biomarker Study

The four partners participating in the biomarker study tested 2 positive and 1 negative control compounds on their test systems (the so-called “2+1 study”). A biostatistical analysis was carried out to identify the IC_{10} concentrations in all the compounds. It also allowed identifying the gene expression signatures and affected toxicity signal transduction pathways of prenatal toxicants on the respective test systems. It was established that:

- Some of the reference toxic compounds affected specific pathways, e.g. ETP to affect T-cell and B-cell receptor signalling pathways;

- All reference compounds affected a few common pathways like focal adhesion pathways, p53 signalling and ECM-receptor pathways. Also some of the cancerous signal transduction pathways, such as bladder cancer and colorectal cancer pathway were affected.

Assessment of ESNATS test systems for inclusion in the all-in-one test battery

In the 3rd period, an evaluation of the ESNATS test systems had allowed establishing that some additional optimisation of most test systems needed to be done before they could be candidates for the final test battery. Therefore, during the 4th period, a second evaluation was carried out on test systems that had not already been selected for participating either in the biomarker study or in the test battery in the first evaluation. The evaluation was made by the ESNATS evaluation group composed of representatives of the project. The following evaluation criteria were applied:

- Availability of SOP
- Reliability and robustness of the test system
- Acceptance criteria
- Negative and positive controls
- Non-specific controls (depending on system)
- Biological relevance of the test system

Experimental design for toxicity tests participating in the battery approach is based on the following:

- Definition of the test method including its biological basis (test system) and a rationale for the relevance of the results produced such as the endpoints to be measured and a rationale or decision criteria for how the results are to be interpreted.
- Definition of the toxicity range of test compounds in the test system.
- Definition of basic characteristics of the test system and test method: dynamic range of the endpoint, detection limit, stability of the readout.
- Data on response characteristics of the endpoint.
- Data quality and statistical evaluation.
- Capacity of testing at least 20-30 compounds

The recommendation from the Evaluation Group was that the test systems UKK1, UKN1, UKN2, JRC and UNIGE1 should take part in the final test battery. All other test systems should support the core efforts of the project by producing data complementary to the primary focus of work. This recommendation was approved by the ESNATS Executive Board.

Further development and implementation of the ESNATS overall test strategy

In order to challenge and validate the results obtained from the 2+1 study, a biomarker extension study is being carried out in the last project period. This study is expected to help identify a pattern in the gene expression signatures identified in the previous study, and thus, allow identification of compounds that act by a specific toxic mechanism, or induce a specific phenotype.

Additionally, to demonstrate a synergy between the ESNATS test systems, the all-in-one test battery strategy has been further fine-tuned, and it was agreed to focus on compounds of pharmaceutical interest. The further development and implementation of the overall test strategy has been described in two concept papers on a) the biomarker study and b) the test battery approach where the detailed planning of each step of the test strategy is described.

Figure 1 below shows an overview of the overall test strategy: the main steps in the biomarker study, and the test battery and how they are linked together.

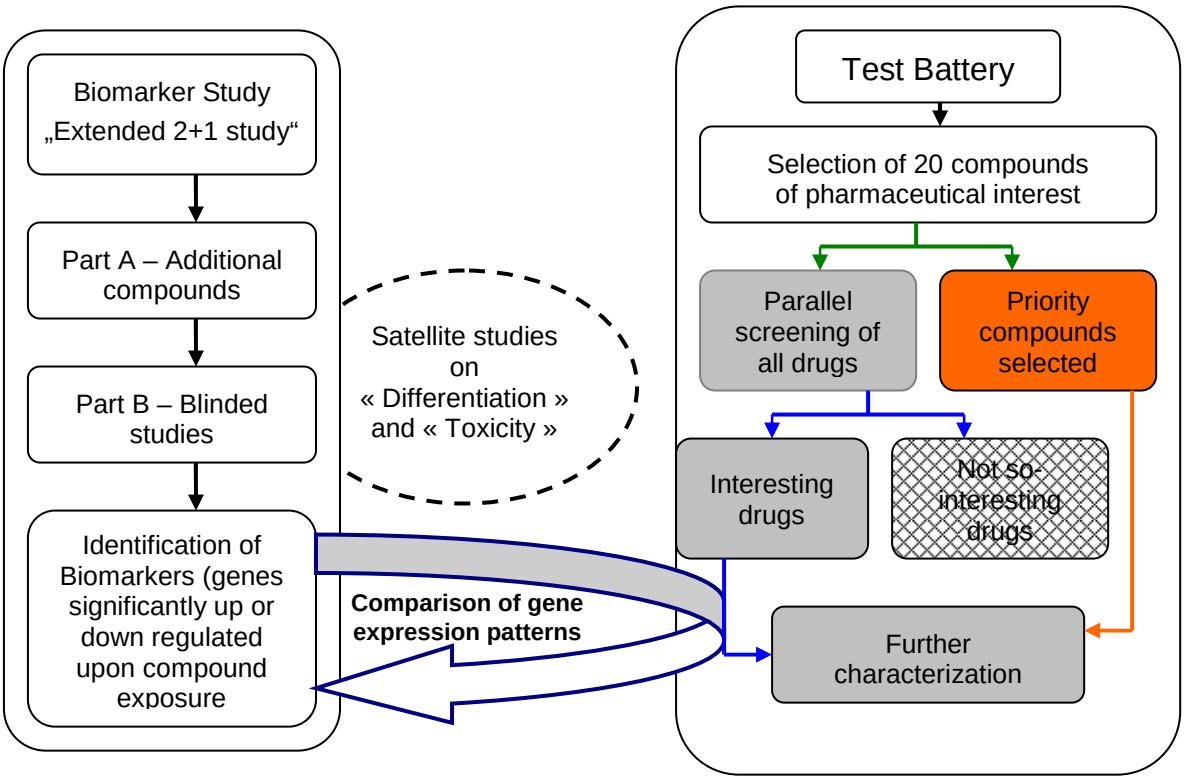

Figure 1: Overview of ESNATS test strategy

The test battery will assess different aspects of prenatal toxicity such as functional impairments and changes in the differentiation capacity after exposure to well selected reference compounds of pharmaceutical interest. The test battery and the gene array chip will then be challenged with compounds under blinded conditions and the predictivity of the tests will be assessed. Biostatistics will be applied to evaluate specificity, sensitivity and predictive capacity of the tests.

PBPK modelling with a simulated pre-incubation of test compounds with hepatocytes will allow the extrapolation of *in vitro* data to the *in vivo* situation, and to be more comprehensive, including a study of the metabolising effects.

It would be feasible to carry out most steps of the test battery above within the project duration. However, in order to further characterise interesting drugs and to test additional compounds to have more confirmed data and relevant results, the ESNATS consortium has decided to request for an extension of the project of six months.

ESNATS partners

The ESNATS consortium is composed of the following organisations:

- Universität zu Köln – Universitätsklinikum (UKK)
- Commission of the European Communities – Directorate General Joint Research Centre JRC (JRC)
- University of Newcastle upon Tyne (UNEW) (Until July 2010)
- Université de Genève (UNIGE)
- Forschungsgesellschaft für Arbeitsphysiologie und Arbeitschutz e.V. (IFADO)
- European Consensus Platform on 3R Alternatives to Animal Experimentation (ecopa)
- The Automation Partnership (Cambridge) Limited (TAP)
- OÜ Quretec (QURE)
- ProteoSys AG (PSY)
- Université de Liège (Ulg) (Until October 2009)

- Consorzio per l'incremento Zootecnico SRL (LTR) (Until March 2009)
- Cellartis AB (Cellartis)
- Cell Cure Neurosciences Ltd. (CELL CURE)
- Universität Konstanz (UKN)
- Health Protection Agency (HPA)
- Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek – TNO (TNO)
- The University of Edinburgh (UEDIN)
- Vrije Universiteit Brussel (VUB)
- Technische Universität München, Klinikum Rechts der Isar (TUM) (Until August 2011)
- ARTTIC (ARTTIC)
- The School of Pharmacy, University of London (ULSOP) (Until September 2011)
- N.V. Organon (Org) (Until September 2011)
- Läkemedelsverket / Medical Products Agency (MPA)
- H. Lundbeck A/S (Lundbeck)
- In Vitro Testing Industrial Platform (IVTIP)
- Bundesinstitut für Risikobewertung (BfR)
- Edinethics Ltd (Edinethics)
- Gottfried Wilhelm Leibniz Universität Hannover (LUH)
- F. Hoffmann-La Roche, Ltd. (Roche)
- Avantea srl (Avantea)
- Brunel University (UBRUN) (From August 2011)
- Eberhard Karls Universität Tübingen (EKUT) (From September 2011)

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