ECVAM-Coordinated Prevalidation Study of Three Cell Transformation Assays for Chemical Carcinogenicity Testing

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Summary

ECVAM coordinated a formal prevalidation study of the Syrian hamster embryo (SHE) Cell Transformation Assays (CTAs) performed at pH 6.7 and pH 7.0 and the BALB/c 3T3 CTA. The study was designed to complement the findings of the OECD detailed review paper (DRP) on CTAs, and it was aimed at refining and standardizing existing protocols and assessing their reliability.

The study results were peer-reviewed by the ECVAM Scientific Advisory Committee, which concluded that the study succeeded in generating standardized, transferable, and reproducible SHE protocols. Further evaluation and use of the BALB/c 3T3 protocol was recommended to expand reproducibility data.

The study results, together with the OECD DRP extensive database, support the utility of the SHE assays for carcinogenicity potential assessment. Based on available data and a formal ECVAM Recommendation on CTA status, the OECD will discuss and decide the necessary follow-up work, possibly with view to test guideline development.

Keywords: Cell Transformation Assay, prevalidation, Syrian hamster embryo primary cells, BALB/c 3T3 cell line, carcinogenicity

1. Introduction

As part of the human safety assessment of new chemical compounds, carcinogenicity testing currently is based on the standard 2-year rodent assay (OECD, 2008). However, this assay is time-consuming, costly, and requires a large number of animals. The *in vitro* Cell Transformation Assay (CTA) has been proposed as a valuable alternative to the bioassay. The CTA recapitulates critical stages of *in vivo* carcinogenesis and assesses, as final readout, whether chemicals trigger the generation of transformed cells that can be tumorigenic *in vivo* in suitable hosts (LeBoeuf et al., 1999). It detects both genotoxic and non-genotoxic carcinogens (OECD, 2007). The different variants of the CTA have been in use for more than 40 years and, for instance, the Syrian hamster embryo (SHE) CTAs currently are being used in academia, in the chemical, agro-chemical, cosmetic, pharmaceutical, and tobacco industries, and in Contract Research Organizations (CROs) to screen chemicals for their potential carcinogenicity. In 2007, the Organisation for Economic Co-operation and Development (OECD) concluded a review of existing data on the three main variants of the CTA (Syrian hamster embryo (SHE), BALB/c 3T3, C3H10T1/2), containing an analysis of the predictive capacity of these assays (OECD, 2007). In this detailed review paper (DRP), the OECD expert group concluded that the performances of the SHE and the BALB/c 3T3 CTAs were adequate and that these tests should be developed into official OECD test guidelines.

Following the recommendations of two expert meetings held at the European Centre for the Validation of Alternative Methods (ECVAM) in 1998 (Combes et al., 1999) and 2004, ECVAM coordinated a formal prevalidation study of the SHE and BALB/c 3T3 CTAs (Fig. 1) from 2005 to 2010. Since an extensive body of existing evidence on predictivity of CTAs was available in the OECD DRP (OECD, 2007), the study did not aim at the full validation of the assay but rather at complementing the OECD DRP findings by addressing issues of protocol standardization, within-laboratory reproducibility, test method transferability, and between-laboratory reproducibility. Three protocol variants were evaluated in a multi-laboratory trial with eight chemicals in total (six chemicals per assay) (Vanparys et al., 2010; Corvi et al., in press): the CTA performed in SHE primary cells at pH 6.7 (SHE pH 6.7 CTA), the CTA performed in SHE primary cells at pH 7.0 (SHE pH 7.0 CTA), and the CTA using the BALB/c 3T3 cell line. Details of the study will be published in a Special Issue on CTAs in *Mutation Research*, currently in preparation.

After completion of the study, the three study reports were peer reviewed by the ECVAM Scientific Advisory Committee (ESAC). In agreement with the study's Validation Management Team (VMT), the ESAC concluded in February 2011 that the SHE protocol variants had been improved (e.g., generation of photo catalogues supporting consistent visual scoring) and successfully standardized, as demonstrated by the generation of reproducible and accurate results in the participating labora-

tories, whereas further assessment of the optimized BALB/c 3T3 protocol was recommended. Use of the BALB/c 3T3 assay, however was encouraged to generate more data on reproducibility. The ESAC made further, more detailed suggestions regarding the next necessary steps in view of possible routine use of the CTA.

Based on the ESAC Opinion, the Study Reports, and existing data such as the OECD DRP (OECD, 2007), and ECVAM Workshop Reports (Combes et al., 1999), ECVAM is now drafting its recommendation on the SHE pH 6.7, SHE pH 7.0, and BALB/c 3T3 CTAs. This recommendation will summarize the current validity status of the three assays for *in vitro* carcinogenicity testing and suggest future steps for wider use and regulatory application of the assays. In light of the prevalidation study results, the existing information, and the ECVAM peer review outcome, the OECD will decide on the way forward regarding CTA test guideline development (Fig. 1).

2. Overview of the prevalidation study

An independent VMT was established by ECVAM to manage the study (Corvi et al., in press). The modular approach to validation (Hartung et al., 2004) was followed to evaluate whether the tests would fulfill ECVAM criteria on test validity (Balls et al., 1995). The study focused on the assessment of the first four modules: test definition, within-laboratory reproducibility, transferability, and between-laboratory reproducibility. Since the evaluation of the fifth module, i.e., predictive capacity, had already been addressed extensively in the OECD DRP (OECD, 2007), only a limited number of chemicals (six per assay / eight in total) was tested in the study to generate additional data on reliability. The same six chemicals were used for the SHE pH 6.7 and SHE pH 7.0 CTAs (Pant et al., in press; Maire et al., in press, a): four rodent carcinogens (benzo[a]pyrene, 2,4-diaminotoluene, o-toluidine HCl, and 3-methylcholanthrene) and two

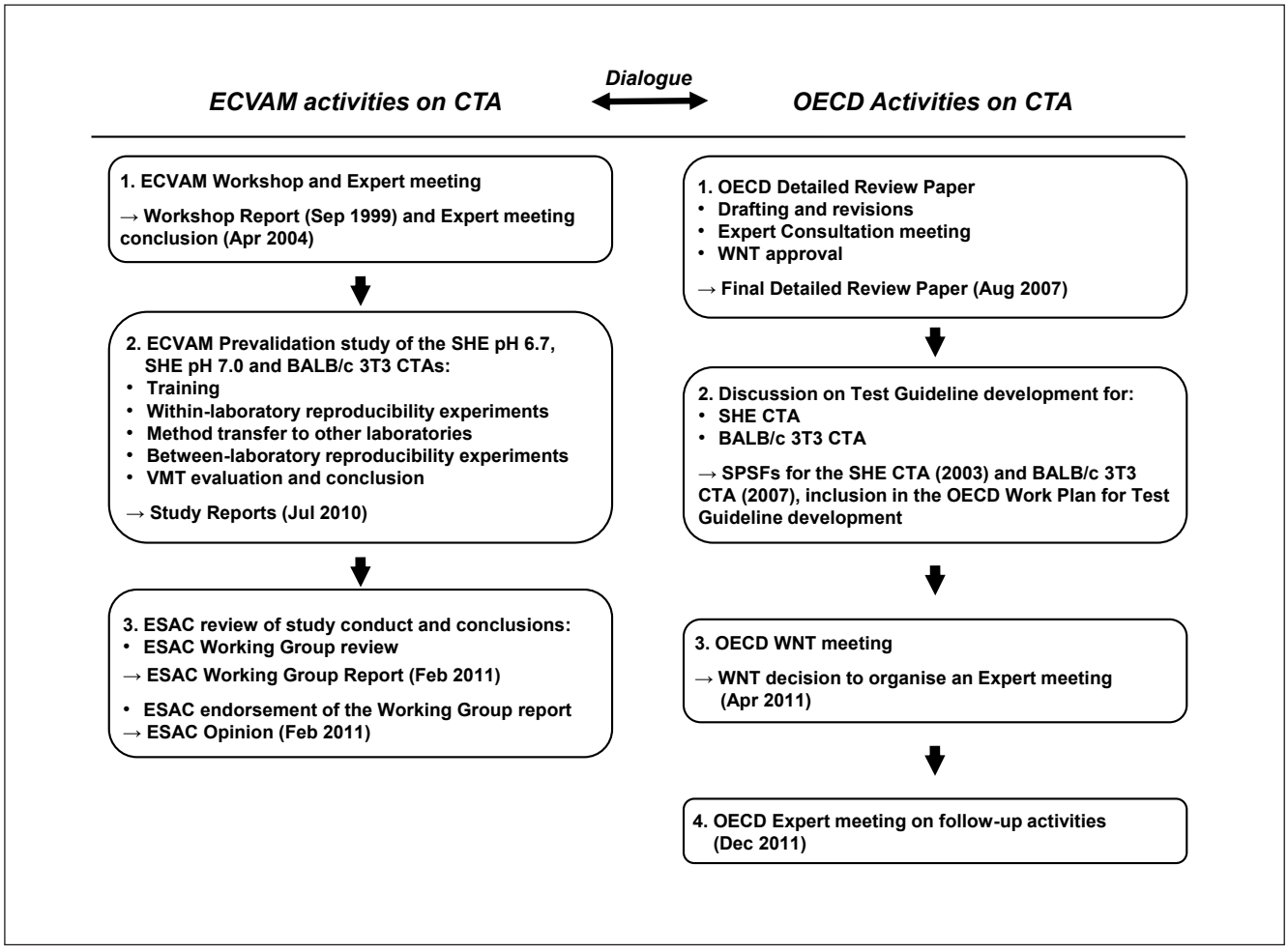

Fig. 1: ECVAM and OECD activities regarding the Cell Transformation Assay

The different projects were led in parallel by both bodies, taking into account the results, recommendations and conclusions from each other as they became available.

CTA = Cell Transformation Assay; ECVAM = European Centre for the Validation of Alternative Methods; ESAC = ECVAM Scientific Advisory Committee; OECD = Organisation for Economic Co-operation and Development; SPSF = Standard Project Submission Form; VMT = Validation Management Team; WNT: Working Group of National Coordinators of the Test Guidelines Programme.

non-carcinogens (anthracene and phthalic anhydride). Where possible, the same chemicals also were selected for the BALB/c 3T3 assay (Tanaka et al., in press): four rodent carcinogens (benzo[a]pyrene, 2-acetylaminofluorene, o-toluidine HCl and 3-methylcholanthrene), and two non-carcinogens (anthracene and phenanthrene).

The study design took into account the objectives, the time, and the resources available. Each variant of the assay was evaluated in three (for the SHE pH 6.7 and the BALB/c 3T3 CTAs) to four (for the SHE pH 7.0 CTA) different laboratories representing industry, academia, CROs, and governmental establishments from the USA, Japan, and Europe (Corvi et al., in press).

Prior to the testing phase, a training session was organized to ensure that all study participants were adequately prepared and that the respective test procedures were sufficiently detailed and clear to allow testing in naïve laboratories. Protocols, acceptance, and assessment criteria were refined and agreed-upon by the participating laboratories. Photo catalogues for each variant of the CTA also were produced as visual aids to support consistency in colony/focus identification and scoring. Subsequently, test definition, within-laboratory reproducibility, and transferability of the assay protocols were characterized based on the results of the respective positive control compounds, tested non-coded and coded. In a second phase, the between-laboratory reproducibility was assessed by the testing of five additional coded compounds.

The study experiments were completed in September 2007 for the SHE pH 6.7 and the BALB/c 3T3 CTAs and in April 2009 for the SHE pH 7.0 CTA. All three study reports were finalized in July 2010.

As the main outcomes of the study, standardized protocols have been established for the SHE assays, an improved protocol has been developed for the BALB/c 3T3 assay, and all variants were assessed for their transferability and reproducibility within and between laboratories. Although data were limited, within-laboratory reproducibility was shown to be satisfactory in all laboratories. The assays were shown to be easily transferable to laboratories that had limited experience with such assays but had basic experience in cell culture techniques. Objectivity and consistency in colony and focus visual scoring was shown to be achievable with appropriate training and the use of the photo catalogues produced during the study. Between-laboratory reproducibility was shown to be satisfactory for the three assays, but only after refinement of the acceptance and assessment criteria for the BALB/c 3T3 CTA, which would need further testing for confirmation.

The concordance between the different assay results and chemical classifications regarding carcinogenicity was looked at as additional information, since it was outside the scope of this prevalidation study. Predictive capacity was judged satisfactory, despite unexpected results produced with the non-carcinogens phthalic anhydride, detected as positive in SHE cells at pH 6.7 (Pant et al., in press), and phenanthrene, detected as positive in BALB/c 3T3 cells (Tanaka et al., in press).

The VMT concluded that, overall, the study results in combination with the extensive database summarized in the OECD DRP (OECD, 2007) support the utility of *in vitro* CTAs for the assessment of carcinogenicity potential (Vanparys et al., 2010; Corvi et al., in press).

Details on the test procedures, acceptance and assessment criteria (Maire et al., in press, b; Sasaki et al., in press, a), the photo catalogues (Bohnenberger et al., in press; Maire et al., in press, c; Sasaki et al., in press, b), as well as the results of the study (Pant et al., in press; Maire et al., in press, a; Tanaka et al., in press) will be published in a Special Issue on CTAs in *Mutation Research*.

3. Peer review

ESAC peer review of the CTA prevalidation study results

Following completion of the CTA prevalidation study and finalization of the Study Reports, ECVAM requested its scientific advisory committee, the ESAC, in its meeting in October 2010, to conduct a scientific peer review on this study. The ESAC established an ESAC Working Group (WG) charged with preparing a detailed Working Group Report that should serve as a basis for the development of an ESAC opinion on the study. The review started in October 2010 and was concluded in February 2011.

The ESAC conclusions are summarized in Table 1. Generally, they were in line with those of the VMT regarding the satisfactory results of the SHE CTAs and the need for further refinement of the BALB/c 3T3 assay acceptance and assessment criteria. Important merits of the study include the avail-

Tab. 1: Summary of the ESAC peer review conclusions on the prevalidation study results of the SHE pH 6.7, SHE pH 7.0, and BALB/c 3T3 Cell Transformation Assays

Module assessment [#]	SHE pH 6.7 CTA	SHE pH 7.0 CTA	BALB/c 3T3 CTA
1. Test definition	√ Satisfactory	√ Satisfactory	Improved protocol available, further evaluation of the refined interpretation rules needed
2. Within-laboratory reproducibility	Only one chemical tested	Only one chemical tested	Only one chemical tested
3. Transferability	√ Satisfactory	√ Satisfactory	√ Satisfactory
4. Between-laboratory reproducibility	√ Satisfactory	√ Satisfactory	Promising but confirmation needed with further testing

[#]According to Hartung et al., 2004

ability of standardized protocols for the SHE CTA, an improved protocol for the BALB/c 3T3 CTA, and specific photo catalogues, all of which support the assay transferability and reproducibility.

The ESAC noted some shortcomings with regard to the study design and execution, but the shortcomings did not critically influence the outcome of the study. For instance, the data generated and evaluated did not allow for a proper assessment of within-laboratory reproducibility since only one compound, coded and non-coded, had been tested for each assay variant for this purpose. Moreover, the laboratories involved in the study had some experience with different variants of the CTA, even if training of all participants had been necessary preliminary to the study due to specific differences in the focus/colony aspect and scoring between the assay variants. This may somehow limit the information gathered about the challenges related to assay transferability to and reproducibility in inexperienced laboratories.

The ESAC further suggested that test performance, which was outside the scope of this study, should be characterized based on a larger set of chemicals to support standardized use of the CTA, including for regulatory purposes. However, considering the extensive body of information available, especially in the OECD DRP (OECD, 2007), the ESAC was of the opinion that existing data could be used for such a characterization. This applied in particular to the SHE CTA data, on the basis of the apparent robustness of the SHE assay as shown in the OECD DRP and the appreciable similarity between the protocols with which those data had been generated and the ones used in the prevalidation study.

Finally, the ESAC made detailed suggestions regarding the next necessary steps in view of a possible routine use of the CTA, which should start with the definition of the intended regulatory purpose of the assay to facilitate a detailed and targeted characterization of its performance.

ECVAM recommendation

Based on the above-mentioned documents (i.e., ESAC opinion, ESAC WG Report, Study Reports) and other relevant documents (mainly the OECD DRP (OECD, 2007) and the ECVAM Workshop Report on CTAs (Combes et al., 1999)), ECVAM has drafted a recommendation on the SHE pH 6.7, SHE pH 7.0, and BALB/c 3T3 CTAs, that aims to provide ECVAM's view on the validity of these test methods, in addition to advice on possible regulatory applicability, limitations, and proper scientific use of the test methods, as well as to suggest possible follow-up activities[1].

In particular, ECVAM's recommendation emphasizes the potentially significant impact of the CTAs on the 3Rs, since the use of CTA data can support partial replacement or reduction when used in a weight-of-evidence approach for hazard identification and risk assessment. ECVAM further recommends that a test guideline for the SHE CTA be developed. ECVAM also encourages the use of the BALB/c 3T3 CTA, which is considerably more appropriate from a 3R perspective, since it uses an established mouse fibroblast cell line and not primary embryonic hamster cells, as is the case for the SHE CTA.

Input from ECVAM's stakeholder forum (ESTAF), the network of Member State Regulators (Preliminary Assessment of Regulatory Relevance network PARERE), ECVAM's international partner organizations cooperating under the International Cooperation on Alternative Test Methods (ICATM) umbrella, as well as the general public, was considered prior to finalization of the recommendation.

4 Future steps

The current OECD work plan (OECD, 2010) foresees the development of test guidelines for the SHE and BALB/c 3T3 CTAs following submission of the relevant Standard Project Submission Forms (SPSFs) by France in 2003 (SHE CTAs) and Japan in 2007 (BALB/c 3T3 CTA). However, following the recommendation of the OECD DRP on CTAs (OECD, 2007) and in order to benefit from the outcome of the ongoing (at the time) ECVAM prevalidation study, these projects have been on hold up to now.

Prior to publication of the ECVAM Recommendation, ECVAM had shared with the OECD the Study Reports, the ESAC WG Report, and the ESAC Opinion to support discussion on the two pending test guideline projects. Using the documents provided by ECVAM, the OECD Working Group of National Coordinators of the Test Guidelines Programme (WNT) decided in April 2011 to convene an expert group to discuss the way forward for CTA test guidelines finalization. The first meeting of this expert group was held in December 2011.

5 Conclusion

The CTA has been in use for decades, serving as a possible alternative to the rodent cancer bioassay. However, the lack of standardized protocols and their formal assessment has limited its regulatory acceptance. The ECVAM-coordinated prevalidation study of the CTAs focused on protocol standardization, as well as assay transferability and reliability of the most promising assay variants (i.e., SHE pH 6.7, SHE pH 7.0 and BALB/c 3T3 CTAs) previously identified in the OECD DRP (OECD, 2007). The data generated were peer-reviewed by the ESAC and add to the existing body of knowledge supporting *in vitro* CTA utility for the assessment of carcinogenicity potential. The protocols and the photo catalogues developed during the study will support standardized conduct and widespread use of the assay, with the potential to significantly re-

[1] http://ihcp.jrc.ec.europa.eu/our_activities/alt-animal-testing/eurl-ecvam-recommendations/EURL-ECVAM%20-Recommendation.pdf Last accesssed March 22, 2012

duce the use of animals for chemical carcinogenicity assessment. It is expected that the ECVAM recommendation, the ESAC Opinion, and the VMT conclusions will support and facilitate discussions at the OECD with regard to a possible way forward concerning the development of test guidelines on *in vitro* CTAs.

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