ECVAM Prevalidation of Three Cell Transformation Assays

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Summary

A prevalidation study on the cell transformation assays in SHE cells at pH 6.7, SHE cells at pH 7.0 and Balb/c 3T3 cell line was coordinated by ECVAM focussing on issues of standardisation of protocols, within-laboratory reproducibility, test method transferability and between-laboratory reproducibility. The Validation Management Team concluded that standardised protocols are now available that should be the basis for future use. The SHE pH 6.7, and the SHE pH 7.0 protocols and the assays system themselves are transferable between laboratories, and are reproducible within- and between-laboratories. For the Balb/c 3T3 method, some clarifications and modifications to the protocol were needed to obtain reproducible results. Overall, three methods have shown to be valuable to detect rodent carcinogens.

Keywords: validation, carcinogenicity, cell transformation assay, bioassay, regulatory toxicology

Overview of the prevalidation study

Development and ultimate utilisation of new chemical compounds requires, among other prerequisites, the assessment of human safety. One of the main endpoints in this assessment process is the determination of potential carcinogenicity. Historically, such an evaluation has necessitated the conduct of lifetime carcinogenicity bioassays in rats and/or mice. These studies take around 4 years (experimental phase and analysis of the results), cost around 1 million Euro per chemical and use significant numbers of animals. The European 7th Amendment to the Cosmetic Directive (EU, 2003), the new European chemical legislation REACH (Regulation EC, 2006) and the revised regulation on pesticides and biocides (Regulation EC, in press), all limit the use of animal tests, triggering the need for alternative methods. In fact, the 7th Amendment to the Cosmetic Directive completely bans animal testing for cosmetic ingredients and finished products since March 2009. REACH, on the other hand, requires data on carcinogenicity for chemicals manufactured in volumes greater than 1000 tons per year which are classified as somatic mutagens and are widespread in the environment or for which there is evidence for long term human exposure. As a result, it is expected that in the coming years, a high number of carcinogenicity studies will have to be carried out to fulfil the REACH requirements. Therefore there is a fundamental and critical need for the availability and implementation of validated alternative test models for carcinogenicity testing of chemicals, which can be used to reduce animal usage, refine current *in vivo* test systems and replace animals that would otherwise be employed for such assays. Among the *in vitro* alternatives developed, the cell transformation assays (CTAs) are the most widely used. Despite their broad usage over decades of time, neither the identification of the ideal test method nor method standardisation of any of the available test methods have been fully resolved (Maurici et al., 2005). Nevertheless, the appeal offered by these assays is that they have been shown to involve a multistage process that closely models some stages of *in vivo* carcinogenesis (LeBoeuf et al., 1999), and therefore they are presumed to be worthy potential surrogates for rodent carcinogenicity systems.

Regulatory agencies have been reluctant to adopt these assays in their safety testing schemes, one of the reasons being the lack of formal validation.

On the basis of a conclusion made in a detailed review paper (DRP) of the OECD on cell transformation assays for the detection of chemical carcinogens (OECD, 2007) which concluded

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that the performance of the Syrian hamster embryo (SHE) and Balb/c 3T3 CTAs were sufficiently adequate and should be developed into formal OECD test guidelines. Further, the same OECD DRP recommended that although considerable and sufficient data on the performance of the assays were available, there was a need to develop standardised protocols and to assess the reproducibility of CTA results. On the basis of these conclusions and on recommendations of two expert meetings on cell transformation held at the European Centre for the Validation of Alternative Methods (ECVAM) (Combes et al., 1999), a formal prevalidation study on the Syrian hamster embryo (SHE) and Balb/c 3T3 CTAs was set up to address issues of standardisation of protocols, within-laboratory reproducibility, test method transferability, and between-laboratory reproducibility. Three variants of the CTAs were assessed: the CTA using SHE cells at pH 6.7, SHE cells at pH 7.0, and the Balb/c 3T3. In order to evaluate whether the tests would meet the criteria requested by the ECVAM principles on test validity (Balls et al., 1995), the modular approach to validation (Hartung et al., 2004) was followed. The reported study focused on the following four modules: test definition (e.g. definition of the test's scientific purpose, definite test protocol compliant with Good Laboratory Practice, prediction model, etc.), within laboratory reproducibility, transferability, between laboratory reproducibility. In addition, the fifth module, i.e. predictive capacity of the assay to predict the reference standard (i.e. *in vivo* test results), was preliminarily addressed in a limited way since only six chemicals were tested to serve that purpose.

In order to ensure that all study participants were adequately trained and that the respective test procedures were appropriately optimised, a preliminary study was conducted to specifically address those issues. Furthermore, the initial phase I of the study looked at the test definition and assessed both the within-laboratory reproducibility and transferability of the assay protocols by testing a non-coded and a coded compound. Subsequently, the between-laboratory reproducibility was determined by testing five additional coded compounds.

Each *in vitro* assay was conducted following the same agreed-upon protocol in four different laboratories for the SHE assay at pH 7.0 and in three different laboratories for the SHE assay at pH 6.7 and the Balb/c 3T3 assay. The laboratories involved encompassed industry, academia, contract research laboratories (CROs) and government establishments located in the USA, Japan and Europe. The chemicals were selected (Tab. 1) using data from the OECD DRP31 document (version August 2004) and a publication of Kirkland et al. (2005). The same chemicals were used for the SHE pH 6.7 assay and SHE pH 7.0 assay. Where possible the same chemicals were selected for the Balb/c 3T3 assay.

The following criteria were used to select the chemicals: (1) positive both in Balb/c 3T3 and in SHE, (2) negative both in Balb/c 3T3 and in SHE, (3) at least two references for each test chemical (for both Balb/c 3T3 and SHE), (4) if possible, data available using the SHE pH 7.0 and pH 6.7 protocol, (5) clear classification as *in vivo* carcinogen or non-carcinogen. All of these criteria could not be met in all cases.

As part of this validation exercise, photo catalogues for each variant of the respective CTAs were produced by the participating laboratories with the aim of establishing consistency in assessing colony/focus morphology and for the scoring experiments (see Fig. 1 and 2). The morphological criteria used to identify transformed colonies and transformed foci were adopted from Berwald and Sachs (1963, 1965), Kakunaga (1973), Reznikoff et al. (1973) and Schechtman (1985a,b).

Details on the test procedures followed, assay acceptance criteria, assessment criteria and photo catalogues and the results of the study will be published in a special issue on CTAs in Mutation Research (in preparation).

The three CTA assays are now in the reporting phase. In the present validation study three optimised and standardised protocols for the CTA in SHE cells at pH 6.7, in SHE cells at pH 7.0 and in Balb/c 3T3 cells have been established and assessed for their reproducibility and reliability. Each variant of the assay showed good within-laboratory reproducibility in all

Tab. 1: Chemicals selected

Compound	*In vivo* carcinogenesis	SHE pH 6.7	SHE pH 7	Balb/c 3T3
Benzo[a]pyrene[a]	positive	X	X	X
2,4-Diaminotoluene	positive	X	X	
3-Methylcholanthrene[b]	positive	X	X	X
o-Toluidine HCl	positive	X	X	X
Anthracene	negative	X	X	X
Phthalic anhydride	negative	X	X	
2-Acetylaminofluorene	positive			X
Phenanthrene	negative			X

a: Positive control for the SHE pH 6.7 and pH 7.0 assay
b: Positive control for the Balb/c 3T3 assay

laboratories. The transferability of the assays was shown to be successful. Furthermore, some of the laboratories had no previous experience in working with such assays. This suggests that CTA test methods can be easily transferred to any laboratory that has experience in cell culture techniques. However, since scoring of transformed colonies is at the moment still done manually under the microscope, training is necessary to ensure a scoring which is as objective and consistent as possible. It also should be noted that the dose-level selection is a crucial step for the success of CTAs, since the right doses need to be hit in order to detect a significant number of transformed colonies when the cells are treated with transforming agents. The between-laboratory reproducibility was shown to be satisfactory for the three assays. The concordance between the CTAs and the carcinogenicity classification of the chemicals assessed was satisfactory. Unexpected results were produced with phthalic anhydride in SHE cells at pH 6.7 and with phenanthrene in Balb/c 3T3 cells.

The Validation Management Team concluded that standardised protocols are now available that should be the basis for future use of CTAs. The SHE pH 6.7, and the SHE pH 7.0 protocols and the assays system themselves are transferable between laboratories, and are reproducible within- and between-laboratories. For the Balb/c 3T3 method, an improved protocol has been developed, which allowed to obtain reproducible results. Further testing of this improved protocol is recommended in

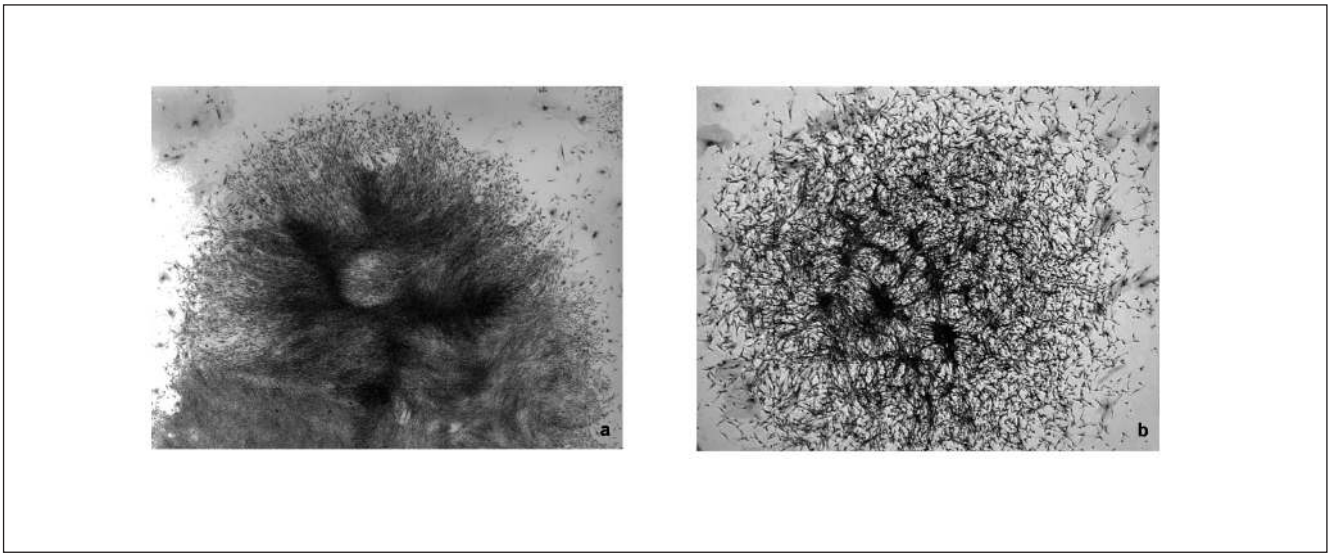

Fig. 1: A non transformed colony (a) and a transformed colony (b) produced upon treatment with benzo(a)pyrene in the SHE cell transformation assay.

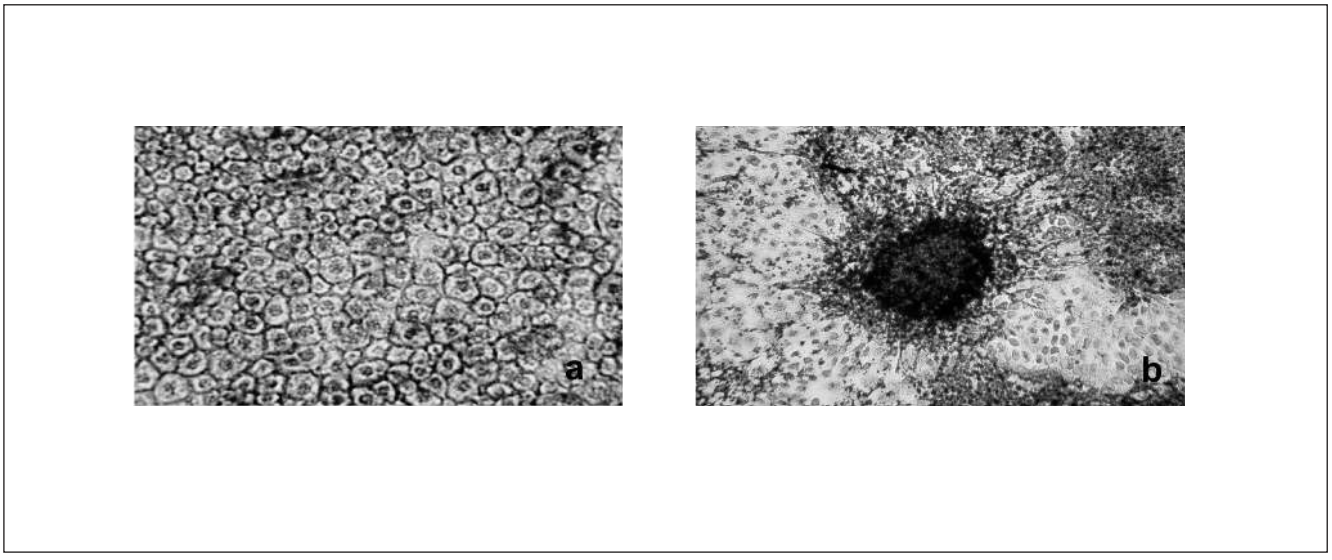

Fig. 2: Non transformed cells (a) and a transformed type III focus (b) in Balb/c 3T3 cells upon treatment with 3-methylcholanthrene.

order to confirm its robustness. Overall, these results in combination with the extensive database summarised in the OECD DRP31 (OECD, 2007) support the utility of *in vitro* CTAs for the assessment of carcinogenicity potential.

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