Growth Inhibition Assay - Summary

Eye Irritation

The growth inhibition assay determines the inhibition of cell proliferation of established cells lines after exposure to an ocular irritant.

Objective & Application

The growth inhibition assay is a cytotoxicity test to investigate the effect induced by ocular irritants on cell proliferation. The assay has been proposed as an alternative to the Draize rabbit eye irritation test (OECD TG 405, 2012 and Method B.5 of Annex to Commission Regulation 440/2008/EC, EU 2008) to investigate ocular irritancy induced by chemicals (including surfactants, metal salts, alcohols, ketones, and acetates), cosmetic ingredients and consumer products (Reinhardt *et al.*, 1985; Bracher *et al.*, 1987; Reinhardt *et al.*, 1987; Kennah *et al.*, 1989; Horwath-Winter *et al.*, 2004).

Basis of the Method

The effect of an ocular irritant *in vitro* is assessed by counting the number of cells after treatment. Reductions in cell numbers in comparison to controls are either the result of a reduced cell division rate or/and cell death. These effects are considered indicative for cytotoxic effects on corneal epithelium of the eye.

The growth inhibition assay is used with long-term (48 h) (Reinhard *et al.*, 1985) or with short-term (30 min) exposure periods (Kennah *et al.*, 1989). The inhibition of cell growth is determined directly after the 48 h treatment by Reinhardt *et al.* (1985), while Kennah *et al.* (1989) detected the growth inhibition 24 h after treatment. Reinhardt *et al.* (1985) used fibroblastic cell lines of hamster (BHK) and human origin (Keller cells, MRC-5). Kennah *et al.* (1989) applied the assay using a murine fibroblast cell line (3T3). The concentration of a test substance resulting in a cell number reduced to 50% of that of controls is called GI-50 (Reinhardt *et al.*, 1987; Kennah *et al.*, 1989).

Experimental Description

Biological and Endpoint Measurement:

CELL NUMBER: is determined with an coulter counter

Endpoint Value:

GI-50: the concentration of a test substance (in mM, org/ml) reducing the cell number to 50% of that of controls

Experimental System:

3T3 FIBROBLASTS (MOUSE): commercially available murine fibroblastic cell line

BHK-21/C13: commercially available fibroblastic cell line derived from Baby Hamster Kidney (BHK)

KELLER CELLS: immortalized human diploid fibroblastic cell line derived from an arm biopsy

MRC-5 CELLS: commercially available human fibroblastic cell line derived from embryonic lung

Cells are seeded in culture plates and incubated for 1 - 2 days prior to exposure. Test substance dilutions are applied to the monolayer for 48 h for long-term exposure (Reinhardt *et al.*, 1985), respectively 30 min for short-term exposure (Kennah *et al.*, 1989). Untreated cultures serve as negative controls. Cells are counted with a coulter counter after trypsination of cells. Reinhardt *et al.* (1985) determined the lowest concentration of a test substance that induced a significant effect in the cell growth inhibition assay (GIlow, cell numbers at the end of the incubation period in controls and exposed samples were expressed as percentages of the cell number at the beginning of the incubation period, no further information on how these data were transformed to the GIlow was given by the authors).
Kennah *et al.* (1989) exposed the cells for 30 min and incubated the cells for another 24 h in fresh medium. The authors expressed differences between numbers of treated cells versus those of untreated controls according to the following formula (Kennah *et al.*, 1989):
% GI = (no. of cells of control - no of cells of sample) / (no of cells of control) x 100
The endpoint value GI-50 is determined by linear regression analysis from individual values for % GI, obtained at various test concentrations.

Data Analysis/Prediction Model

Kennah *et al.* (1989) developed a prediction model for the short-term exposure (30 min) of the growth inhibition assay. The authors performed a linear regression analysis correlating the log GI-50 values with the percentages of corneal swelling *in vivo*. The following equation was derived by Kennah *et al.* (1989):

% corneal swelling = -29.98 (log GI-50) + 135.57

Increasing percentages of corneal swelling *in vivo* were correlated with increasing severeness of eye irritation by the same authors in a previous paper (Kennah *et al.*, 1989) in the following way:

% corneal swelling *in vivo*	Irritancy classification
0 – 119	Mild
120 – 136	Slight
137 – 180	Moderate
181 – 232	Severe
> 232	Extreme

So far, no prediction model is available for the long-term exposure of the growth inhibition assay.

Test Compounds

Chemicals (including surfactants, metal salts, alcohols, ketones, and acetates), cosmetic ingredients, and consumer products.

Modifications

Horwath-Winter *et al.* (2004) used primary human conjunctival fibroblasts and exposed these cells for up to 72 h.

Discussion

Using the long-term exposure assay, Reinhardt *et al.* (1985) evaluated three cytotoxicity assays (cell detachment, cloning efficiency test, and growth inhibition test) using three different cell lines. According to the authors, the ranking order of the cytotoxicity of most chemicals was comparable for all three tests using the different cell lines. The authors reported that the *in vitro* results of the growth inhibition assay correlated well (correlation coefficient was 0.92) with human data (Reinhardt *et al.*, 1985). In another investigation of the same working group three cytotoxicity tests (cell detachment assay, growth inhibition test, membrane permeability assay) and the hen's egg test (HET-CAM, see method summary for more details) were used to classify 24 different tensides (Reinhardt *et al.*, 1987). According to the authors, the growth inhibition assay did not correlate with the *in vivo* data obtained from studies with guinea pigs, which were performed in analogy to the Draize rabbit eye test using guinea pigs as test animals.

Bracher *et al.* (1987) compared the performance of four *in vitro* cell toxicity assays (cell detachment assay, the growth inhibition test (long-term exposure), membrane permeability assay, NRU assay), when used with 26 cosmetic ingredients. From the comparison with *in vivo* data Bracher *et al.* (1987) concluded that the growth inhibition assay produced a large number of false predictions (especially false negatives) and was unable to distinguish minimally irritating substances from strong irritating, respectively from non-irritating ones.

According to Kennah *et al.* (1989) the short-term exposure growth inhibition assay showed a good correlation between *in vitro* and *in vivo* results for surfactants and alcohols. According to the authors, the correlation between *in vitro* and *in vivo* results was poor for substances of other chemical classes such as ketones, acetates, and aromatics (Kennah *et al.*, 1989).

Selling and Ekwall (1985) developed a MIT to determine the proliferation rate of HeLa cells in the presence of ocular irritants. The authors reported that they could determine cell proliferation, respectively growth inhibition by observing the colour change of the culture medium. After 7 days of culture an increase of cell number (proliferation) is characterized by colour change (indicator phenol red) from red into yellow/orange (pH 6.0 – 6-5), since acid metabolites accumulate in the supernatant of proliferating cells, thus altering the pH of the culture medium. Cultures which do not change the colour of the medium were regarded as inhibited, while a colour change into violet (pH 8.0) was regarded as

complete inhibition of cell proliferation by Selling and Ekwall (1985).

Status

So far, in the scientific literature, neither evaluation nor validation studies have been reported for the growth inhibition assay.

Abbreviations & Definitions

BHK: Baby Hamster Kidney
EC: European Commission
EU: European Union
GI: Growth Inhibition
HET-CAM: Hen's Egg Test on the Chorioallantoic Membrane
NRU: Neutral Red Uptake
MIT: Metabolic Inhibition Test
OECD: Organisation for Economic Co-operation and Development
TG: Test Guideline

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