WP4: New cell systems and new endpoints (P9, P11, P14 and P21)

Selection of reference chemicals with immunotoxic and hematotoxic potential

As controls of toxicity on immune and hematopoietic systems studied in WP4, the following substances were included: 5-fluorouracil, benzene, tert-butyl hydroperoxide, acrolein and cadmium II chloride pentahydrate.

Methods descriptions

The human whole blood assay measuring cytokine release

The multiplexed assay is based upon the use of a dedicated flow cytometer (Luminex system) and a mixture of fluorescent microspheres covered with capture antibodies that bind specific analytes, in this case, the inflammatory cytokines IFN-γ, TNF-α and IL-6, from the supernatants of cell cultures. Bound cytokines are in turn revealed by a second fluorescent antibody. In this way, the concentration of several cytokines (multiplexing) can be determined simultaneously when bead mixtures are run through the flow cytometer and a calibration curve constructed. The assay involves incubation of cultures of human whole blood for 24 hours with lipopolysaccharide (LPS), a monocyte activator, in the presence or absence of a range of concentrations test compounds or appropriate immunosuppresors. Endpoint measured is secretion of IFN-γ, TNF-α and IL-6 and the expression of the final results is given by IC_{50} (inhibition by test compound of LPS-induced cytokine secretion) or EC_{50} (enhancement by test compound of LPS-induced cytokine secretion).

The Colony forming unit-granulocytes/macrophage assay (CFU-GM)

Human umbilical cord blood cells were mixed in tubes containing cell culture mixture with linearity controls, vehicle controls, and eight concentrations of reference compounds for the dose-response curve. Each tube was used to prepare three culture dishes. All the toxicant dilutions were prepared at 200x the final dilution, in order to obtain the final fold dilutions of drug in the culture dish. The cultures were incubated at 37°C in air + 5% CO_2 under saturated humidity for 14 days. All dishes were scored for colony counts following a random fashion. The survival curves of CFU-GMs exposed to different doses of the compounds were obtained and IC values were calculated.

The Cytomics Toxicity Panel

This group of assays is based on the use of flow cytometry and endpoint-specific fluorescent probes for general cytotoxicity markers. By means of the multiparametric capability of cytomics, measurements are restricted to live cells, thus providing early biomarkers of cytotoxicity, evident before the cell is displaying an overt death program. The cell system used were human established cell lines of different (neuroblastoma SH-SY5Y, hepatoma HepG2 and kidney adenocarcinoma A704). Cells were exposed for 24 hours (SH-SY5Y and HepG2 cell lines) or 48 hours (A704 cells) and a range of 3 - 4 concentrations tested. The assay panel measures the following endpoints: Intracellular Ca^{2+}, plasma membrane potential, and mitochondrial membrane potential. The expression of the final results was done either as

IC_{50} (decreased intensity of endpoint-associated fluorescence) or EC_{50} (increased intensity of endpoint-associated fluorescence) values.

The Cytomics Oxidative Stress Panel

This group of assays is based on the use of flow cytometry and high-content analysis by bioimaging to quantify endpoint-specific fluorescent probes for oxidative stress markers. By means of the multiparametric capability of cytomics, measurements are restricted to live cells, thus providing early biomarkers of oxidative damage to cells, evident before the cell is displaying an overt death program. The cell system used were human established cell lines of different origin (neuroblastoma SH-SY5Y, hepatoma HepG2 and kidney adenocarcinoma A704). Cells were exposed for 24 hours (SH-SY5Y and HepG2 cell lines) or 48 hours (A704 cells) and a range of 3 - 4 concentrations tested. The assay panel measures the following endpoints: Mitochondrial superoxide anion and intracellular peroxidative activity by flow cytometry and the levels of oxidized DNA base 2-deoxy-8-guanine in genomic DNA and in mitochondrial DNA by high-content analysis by biomaging. The expression of the final results was done either as IC_{50} (decreased intensity of endpoint-associated fluorescence) or EC_{50} (increased intensity of endpoint-associated fluorescence) values.

Delayed cytotoxicity

The delayed cytotoxicity of the Multicentre Evaluation of *In vitro* Cytotoxicity (MEIC) reference chemicals was investigated in rat hepatoma-derived Fa32 cells. The cells were treated for 24 h with the test chemicals, and were than further cultured for 5 days in normal culture medium. The cytotoxicity was measured by the neutral red uptake inhibition, and the results were quantified by determining the NI50del. This is the concentration of test compound required to decrease the neutral red uptake with 50% compared with control cells. The results were compared with the acute NI50, the corresponding value measured immediately after 24 h treatment of the cells.