Supplementary figures

Evaluation of a human neurite growth assay as specific screen for developmental neurotoxicants

Archives of Toxicology

Anne K. Krug, Nina V. Balmer, Florian Matt, Felix Schönenberger, Dorit Merhof, Marcel Leist

Overview:

Fig. S1  Toxicity curves of two positive compounds, vincristine and nocodazole, and of two negative compounds, etoposide and BSO
– Page (P.) - 1

Fig. S2  Separation of specific neurite growth modulators (individual experiments) from unspecific cytotoxicants
– P. 2

Fig. S3  EC50 values of neuronal precursor cells of neurite area and resazurin reduction compared to mature neurons
– P. 3

Fig. S4  EC50 values of neuronal precursor cells and mature neurons of resazurin reduction compared to data from non-neuronal cell types
– P. 4

Correspondence to be sent to:
Anne K. Krug
Doerenkamp-Zbinden Chair for In Vitro Toxicology and Biomedicine
University of Konstanz
78457 Konstanz/Germany
Tel: +49 (0) 7531 88 5331
Email: anne.krug@uni-konstanz.de
Fig. S1 Toxicity curves of two positive compounds, vincristine and nocodazole, and of two negative compounds, etoposide and BSO.

Cells were replated at day 2 (d2) and compounds were added in dilution series in triplicates. 24h later cells were stained with 1 µM calcein-AM and 1 µg/ml H-33342 for 30 min at 37°C. 

a) mean curve of vincristine toxicity of three biological replicates. 
b) mean curve of nocodazole toxicity of four biological replicates. 
c) single curves of viability and neurite area of three independent experiments of etoposide. 
d) single curves of viability and neurite area of three independent experiments of BSO.
Fig. S2 Separation of specific neurite growth modulators (individual experiments) from unspecific cytotoxicants.
Cells were treated on d2 as displayed in Fig. 1a, and 24 h later neurite area and viability were automatically quantified. Compounds were tested at several concentrations, and their EC50 values for effects on neurite area and cell viability were determined by a non-linear regression sigmoidal concentration-response curve fit, and EC50 values of neurite area were plotted against the determined EC50 values of cell viability. A reference control group of 9 unspecific toxicants comprised buthionine sulfoximine (BSO), carbonylcyanide-3-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (2,4-DNP), etoposide, bisbenzimide H (H-33352), potassium chromate (K2CrO4), tert-butyl hydroperoxide (tBuOOH), tween-20 and sodium dodecyl sulfate (SDS) (dots in grey, names are underlined). The solid line indicates an EC50 ratio of 1 for viability to neurite area. The dashed line indicates an EC50 ratio of 4.0 used as specificity cut-off here. Data for 40 compounds were classified according to this threshold value. Orange colour indicates substances classified to act unspecific on neurite growth: acrylamide, antimycin A, chlorpyrifos, chlorpyrifos oxon, cisplatin, cytochalasin, fipronil, haloperidol, hoorkiol, IPA-3, menadione, methamphetamine (METH), mevastatin, 1H-[1,2,4]oxadiazolo-[4,3-
"a"]quinoxalin-1-one (ODQ), okadaic acid, oligomycin, piericidin, protein tyrosine phosphatase inhibitor IV (PTP IV), puromycin, simvastatin and SP600125. Light blue: substances classified as specific neurite growth inhibitors, EC50 values of three individual experiments are displayed: Bisindolylmaleimide I (Bis1), brefeldin A, colchicine, cycloheximide, diquat, flavopiridol, methylmercury (II) chloride (MeHg), sodium orthovanadate (Na3VO4), narciclasine, nocodazole, paraquat, rotenone, U0126 and vincristine. Dark blue: substances with an augmenting effect on neurite area: blebbistatin, HA-1077, H1152, thiazovivin and Y-27632. Neurite area EC50s of these compounds were determined as response halfway between the baseline (100%) and maximum. Grey dashed lines encircle the individual EC50 values determined for one compound.
Fig. S3 EC50 values of neuronal precursor cells of neurite area and resazurin reduction compared to mature neurons.

Cells were replated at d2 and compounds were added to the culture medium in at least 5 distinct concentrations. For testing of mature neurons, cells were also replated at d2 and compounds were added in fresh medium at day 5 (d5). After 24 hours neurite area was quantified yielding concentration-response-curves. EC50 values were calculated, using the concentration-response-curves, as concentrations at 50% of neurite area were detected, respectively. All data are means of 3 to 4 independent experiments. Dotted lines mark equality of x-axis values to y-axis values.

a) Comparison of EC50 values of neurite area of developing (d3) and mature LUHMES cells (d6). The ratio of all d3 EC50 values to d6 is 11.43 ± 2.7.

b) Comparison of EC50 values of resazurin reduction of d3 and d6 cells. The ratio of all d3 EC50 values to d6 is 0.74 ± 0.79.
Fig. S4 EC50 values of neuronal precursor cells and mature neurons of resazurin reduction compared to data from non-neuronal cell types. Cells were replated at d2 and compounds were added to the culture medium in at least 5 distinct concentrations. For testing of mature neurons, cells were also replated at d2 and compounds were added in fresh medium at d5. After 24 hours resazurin reduction was quantified yielding concentration-response-curves. EC50 values were calculated, using the concentration-response-curves, as concentrations at 50% of resazurin reduction were detected, respectively. All data are means of 3 to 4 independent experiments. EC50 values were plotted against cytotoxicity data of non-neuronal cell lines derived from the Halle registry. Dotted lines mark the linear regression through the data points presented. a) and b) Comparison of EC50 values of resazurin reduction of d3 a) and d6 b) LUHMES cells with collected values of the Halle registry. The correlation of d3 to Halle registry is $R^2 = 0.87$ and of d6 to Halle registry is $R^2 = 0.85$. 