Assessment of Chemical-Induced Impairment of Human Neurite Outgrowth by Multiparametric Live Cell Imaging in High-Density Cultures

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Chemicals that specifically alter human neurite outgrowth pose a hazard for the development of the nervous system. The identification of such compounds remains a major challenge, especially in a human test system. To address this issue, we developed an imaging-based procedure in LUHMES human neuronal precursor cells to quantify neurite growth of unfixed cultures. Live imaging allowed the simultaneous evaluation of cell viability and neurite outgrowth within one culture dish. The procedure was used to test the hypothesis that inhibitors of specific pathways can impair neurite outgrowth without affecting cell viability. Although the cells were grown at high density to allow extensive networking, overall neurite growth in this complex culture was quantified with a signal-to-noise ratio of > 50. Compounds such as U0126 slowed the extension of neuronal processes at concentrations > 4 times lower than those causing cell death. High numbers of individual viable cells without neurites were identified under such conditions, and neurite outgrowth recovered after washout of the chemical. Also an extension-promoting compound, Y-27632, was identified by this unique multiparametric imaging approach. Finally, the actions of unspecific cytotoxicants such as menadione, cadmium chloride, and sodium dodecyl sulfate were tested to evaluate the specificity of the new assay. We always found a ratio of EC50 (cell death)/EC50 (neurites) < 4 for such chemicals. The described novel test system may thus be used both for high-throughput screens to identify neuritotoxic agents and for their closer characterization concerning mode of action, compound interactions, or the reversibility of their effects.

Key Words: neurite outgrowth; live imaging; LUHMES; neurotoxicity; developmental toxicity.

Developmental neurotoxicity (DNT) is caused by exposure to toxicants during sensitive periods of the formation of the central nervous system (Grandjean and Landrigan, 2006). It usually manifests in neurobehavioral or cognitive deficits, which may persist long after removal of the original stimulus. This has been unequivocally demonstrated in humans for lead, arsenic, and mercury compounds, as well as for polychlorinated biphenyls and some organic solvents (Grandjean and Landrigan, 2006). A further outcome of DNT may be the increased susceptibility to neurological and psychiatric disease later in life, as suggested by some human data and animal experiments (Grandjean, 2008).

Although epidemiological studies yield highly relevant information for humans, they are limited by their very low detection power. Moreover, they can identify problems only after exposure of large numbers of people has occurred. Therefore, animal test systems have been developed in order to predict potential human hazard. DNT is currently tested in vivo according to test guidelines published by the OECD or US Environmental Protection Agency (US EPA, 1998). Most information is available from such studies on pesticides, whereas regulatory testing for industrial chemicals is very rarely done (Makris et al., 2009). Frequently, these studies yield little or no mechanistic information, and the extrapolation of rodent data to the human situation is a huge challenge when the mode of action remains unknown.

The current mechanistic concepts of DNT have been developed on the basis of data for a very small number of model compounds and by reference to the knowledge on pivotal processes in developmental neurobiology (Kuegler et al., 2010; Lein et al., 2007). Accordingly, two classes of DNT toxicants may be distinguished. The first comprises certain antimitotic compounds, such as the DNA-alkylating agent methylazoxymethanol (Goldey et al., 1994). The second class of compounds is assumed to interfere with cellular signaling processes that are relatively specific for the developing nervous system. They may affect migration of neural progenitors, neuronal maturation, the balance between gliogenesis and neurogenesis or neuronal subtype specification, as well as synaptogenesis and neurite outgrowth (Kuegler et al., 2010). For instance, cyclopamine causes holoprosencephaly by inhibiting the sonic hedgehog pathway required for pairing of anterior forebrain structures (Cooper et al., 1998). Also, overstimulation of pathways can be detrimental, as shown by the neuroteratogenic effects of...
retinoic acid, a strong agonist of the nuclear RXR receptor (Zimmer et al., 2011).

Especially the inhibition of axon and dendrite extensions has been considered an important mode of action for specific developmental neurotoxicants (Lein et al., 2007; Radio et al., 2008; Radio and Mundy, 2008). The correct wiring of the brain depends on neurite outgrowth during development. Connections appear to be disturbed in schizophrenia, anxiety disorders, or mental retardation and specific self-destruction programs have evolved for axonal pruning in developing neurons (Saxena and Caroni, 2007). Even mature neurite structures are known to be a particularly sensitive target for a wide range of chemicals, including many chemotherapeutics. The outgrowth of neurites is controlled by additional pathways that may be affected by toxicants. For instance, the signaling of rho pathway, which has evolved for axonal pruning in developing neurons, or mental retardation and specific self-destruction programs appear to be disturbed in schizophrenia, anxiety disorders, and autism spectrum disorders (ASD). The correct wiring of the brain in these conditions would be desirable that measure outgrowth in high-density cultures. Unspecific cytotoxicity is very hard to distinguish from neurite effects independent of unspecific cytotoxicity. In the present study, we explored the usefulness of LUHMES human neuronal precursor cells (Lotharius et al., 2005; Schildknecht et al., 2009) for neurite outgrowth assays. For this culture system, we developed new methods for unbiased quantification of the neurite growth in high-density cultures. We used live cell stains that avoid processing artifacts and allow the simultaneous acquisition of viability parameters. The new test system allowed us to investigate the question, whether a set of diverse compounds could be identified that genuinely inhibited human neurite outgrowth without affecting the overall cellular viability.

**MATERIALS AND METHODS**

**Materials and chemicals.** Calcein-AM, Hoechst bisbenzimide H-33342, resazurin sodium salt, methylmercury (II) chloride, cytochrome c assay kit, calphostin C, and mitomycin C were from Sigma (Steinheim, Germany). Recombinant human FGF-2 and recombinant human GDNF were from R&D Systems (Minneapolis), Bisindolylmaleimide I, U0126, and brefeldin A were from Calbiochem (Darmstadt, Germany); Y-27632 was from Tocris Bioscience (Bristol, UK), tween-20 was from Roth (Karlsruhe, Germany), DMSO from Merck (Darmstadt, Germany), and metamphetamine was obtained from Lipomed (Arlesheim, Switzerland). All culture reagents were from Gibco unless otherwise specified.

**Cell culture.** LUHMES cells (ATCC CRL-2927) were derived from female human fetal (8 weeks) brain by clonal selection of conditionally immortalized (tetracycline-controlled v-myc) ventral mesencephalic cells as previously described in detail (Lotharius et al., 2005; Schildknecht et al., 2009). The cells were propagated in a 5% CO₂/95% air atmosphere at 37°C in proliferation medium, consisting of advanced DMEM/F12 containing 2 mM L-glutamine, 1 × N2 (Invitrogen), and 40 ng/ml FGF-2. In the proliferation state, the cells expressed v-myc and had a doubling time of 24 h. Differentiation followed a 3-step procedure: For preparation of the differentiation, 8 million cells of passage 5–15 were seeded in a Nunclon T175 flask and were grown for 24 h in proliferation medium. In a second step, the differentiation process was initiated by changing the medium to differentiation medium I consisting of advanced DMEM/F12 supplemented with 2 mM L-glutamine, 1 × N2 (Invitrogen), and 40 ng/ml FGF-2. In the proliferation state, the cells expressed v-myc and had a doubling time of 24 h. Differentiation followed a 3-step procedure: For preparation of the differentiation, 8 million cells of passage 5–15 were seeded in a Nunclon T175 flask and were grown for 24 h in proliferation medium. In a second step, the differentiation process was initiated by changing the medium to differentiation medium I consisting of advanced DMEM/F12 supplemented with 2 mM L-glutamine, 1 × N2, 2.25 μM tetracycline, 1 mM dibutyryl-cAMP, and 2 ng/ml recombinant human GDNF. Under these conditions, v-myc was switched off rapidly and cells became postmitotic. In the third step, LUHMES cells differentiated for 2 days were trypsinized and seeded on dishes precoated with 50 μg/ml poly-L-ornithine (PLO) and 1 μg/ml fibronectin under the continued presence of tetracycline but without cAMP and GDNF (differentiation medium II = DM II).

**Standard experimental setup.** Cells were seeded at a density of 30,000 cells per well in 50 μl DM II on PLO/fibronectin coated 96-well dishes. Compounds were serially diluted in DM II, and 50 μl were added to the cells 1 h after seeding. Maximal test concentrations of the compounds were based on pilot experiments or published data as shown in Table 1. The maximum DMSO concentration used was 0.33% and had no influence on cell viability or neurite outgrowth. To investigate the reversibility of effects on neurite outgrowth, their medium was removed on d3 and 100 μl DM II was added to the cells. All analyses were performed 24 h after initiation of the treatment or after 48 h for reversibility studies. The acceptance criterion for all neurite outgrowth assays included in the data analysis for the results section was that the positive control compound U0126 (25 μM), run along in each assay, decreased neurite area to at least 70% of the control without a significant difference in viable cell number.

**Resazurin measurement.** Cell viability was detected by a resazurin metabolism assay (Schildknecht et al., 2009). Briefly, 10 μl resazurin solution were added to the cell culture medium to obtain a final concentration of 10 μg/ml. After incubation for 30 min at 37°C, the fluorescence signal was measured at an excitation wavelength of 530 nm, using a 590 nm long-pass filter to record the emission. Fluorescence values were normalized by setting fluorescence values of untreated wells as 100% and the values from wells containing less than 5% calcin-positive cells as 0%.

**Quantification of neurite outgrowth.** Neurite outgrowth was defined in this study on a population basis as the difference of total neurite area per unit area over time. In brief, all cellular components not belonging to the...
somata were quantified and regarded as the “neurite area.” In detail, cells were
stained with 1 μM calcein-AM and 1 μg/ml H-33342 for 30 min at 37°C. An automated microplate reading microscope (Array-ScanII HCS Reader, Cellomics, PA) equipped with a Hamamatsu ORCA-ER camera (resolution 1024 × 1024; run at 2 × 2 binning) was used for image acquisition (Schindlmeier et al., 2009). Ten fields per well were imaged. Images were recorded in 2 channels using a 20x objective and excitation/emission wavelengths of 365 ± 50/355 ± 45 to detect H-33342 in channel 1 and 474 ± 40/355 ± 45 to detect calcein in channel 2. In both channels, a fixed exposure time and an intensity histogram-derived threshold were used for object identification. Neurite pixels were identified using the following image analysis algorithm: nuclei were identified as objects in channel 1 according to their size, area, shape, and intensity which were predefined on untreated cells using a machine-based learning algorithm, and manual selection of nuclei to be classified as intact. The nuclear outlines were expanded by 3.2 μm in each direction, to define a virtual cell soma area (VCSA) based on the following procedure: The average width of the cytoplasmic ring (distance nucleus – cell membrane) of LUHMES cells was experimentally determined to be 2.3 μm. Size irregularities were not always due to growing neurites, as determined by combined F-actin/tubulin beta-III staining. To avoid scoring of false positive neurite areas, the exclusion ring (VCSA) was made bigger than the average cell size. Then, we used two control compounds (U0126 and bisindolylmaleimide I) to vary the expanded outlines from 0.6 to 4 μm. We found 3.2 μm to be optimal both to detect neurite growth over time and to identify reduced neurite growth with high sensitivity. All calcein-positive pixels of the field (beyond a given intensity threshold) were defined as viable cellular structures (VCSs). The threshold was dynamically determined for each field after flat field and background correction and intensity normalization to 512 gray values and was set to 12% of the maximal brightness (channel 63 of 512). The VCS defines the sum of all somata and neurites without their assignment to individual cells.

In an automatic calculation, the VCSAs, defined in the H-33342 channel, were used as filter in the calcein channel and subtracted from the VCS. The remaining pixels (VCS – VCSA) in the calcein channel were defined as neurite area. Apoptotic cells were not specifically excluded in the neurite area determination, but dead cells did not give confounding signals, as they were calcein-negative. In our laboratory, this procedure was performed using Cellomics Bioapplication SpotDetector.V2 on the Array-ScanII HCS Reader. This software automatically performs a segmentation of the image field into areas belonging to one cell (by virtually inflating each cell outline until it meets with the inflated neighboring cell outline). This process is not necessary for the actual calculation of the neurite area, but we used it to correct for edge effects. Cells that were only partially in the image field were excluded, together with a corresponding part of neurites lying in this cell’s segmentation area. Corrections for cell numbers per field were not performed, as our cell counting data showed that cell numbers/field were highly reproducible. Some distortions may occur under situations of cell death. The necessary information is always displayed, as the number of viable cells and the neurite area are plotted in the same figures. However, other corrections were not performed, as the method was mainly designed to gain information for concentrations not triggering cell death (identification of specific neurite outgrowth inhibitors).

Quantification of individual viable cells by imaging. For a quantitative assessment of viable cells, the same images used to assess neurite area were analyzed using another image analysis algorithm: nuclei were identified in channel 1 as objects according to their size, area, shape, and intensity. Nuclei of apoptotic cells with increased fluorescence were excluded. A VCSA was defined around each nucleus by expanding it by 0.3 μm in each direction. Calcein-AM staining, labeling live cells, was detected in channel 2. The algorithm quantified the calcein intensity in the VCSA areas. Cells having an average calcein signal

### TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell types&lt;sup&gt;a&lt;/sup&gt;</th>
<th>References</th>
<th>Concentration range (μM)</th>
<th>Reported&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Found&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Maximal&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Main target&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>U0126</td>
<td>PC12</td>
<td>Das et al. (2004)</td>
<td>10–30</td>
<td>4 (12.5)</td>
<td>&gt;50</td>
<td>MEK1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CRC</td>
<td>Radio et al. (2010)</td>
<td>3–100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BisI</td>
<td>PC12</td>
<td>Radio et al. (2010)</td>
<td>0.1–10</td>
<td>100 (1.25)</td>
<td>&gt;20</td>
<td>PKC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CRC</td>
<td>Das et al. (2004)</td>
<td>1–10</td>
<td>100 (1.25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MeHg</td>
<td>CRC</td>
<td>Radio et al. (2010)</td>
<td>0.003–10</td>
<td>0.05 (0.04)</td>
<td>0.26 n.k.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>hN2</td>
<td>Parran et al. (2001)</td>
<td>0.03–3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brefeldin</td>
<td>hN2</td>
<td>Harrill et al. (2010)</td>
<td>0.1–1</td>
<td>1.3 (4)</td>
<td>&gt;90 Membrane transports</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>DRG</td>
<td>Roche et al. (2009)</td>
<td>20</td>
<td>0.3 (0.5)</td>
<td>&gt;30 Protein synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CRC</td>
<td>Lein and Higgins (1991)</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>PC12</td>
<td>Parker et al. (2000)</td>
<td>0.1–1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.02 (0.016)</td>
<td>&gt;1 CDK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na&lt;sub&gt;3&lt;/sub&gt;VO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>hN2</td>
<td>Harrill et al. (2010)</td>
<td>3–100</td>
<td>4.7 (25)</td>
<td>&gt;200 Tyrosine phosphatase</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>hippoc.N.</td>
<td>Mandell and Banker (1998)</td>
<td>30–100&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.9 (2)</td>
<td>&gt;100 ROCK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y-27623</td>
<td>ONE</td>
<td>Ichikawa et al. (2008)</td>
<td>30–100&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<sup>a</sup>Type of cells or culture system used to study the effect of chemicals on neurite outgrowth as described in the adjacent references. CGC, rodent primary cerebellar granule cells; hNT, human teratocarcinaoma NeuroD2-derived neurons; DRG, rat dorsal root ganglion cells; ERSN, embryonic rat sympathetic neurons; hippoc.N., rat hippocampal primary neurons; ONE, rat optic nerve explants.

<sup>b</sup>Range of concentrations reported in references to affect neurite outgrowth.

<sup>c</sup>EC50 for neurite effects found here. In brackets: data point with first significantly inhibiting concentration.

<sup>d</sup>MEK 1, mitogen-activated protein kinase; PKC, protein kinase C; n.k., not known; ROCK, rho kinase; CDK, cyclin-dependent kinases.

<sup>e</sup>Data for positive effects on neurites.
intensity in the VCSAs below a predefined threshold were classified by the program as “not viable.” Valid nuclei with a positive calcein signal in their cognate VCSA were counted as viable cells, as illustrated in Figure 2B. A positive calcein signal was based on measurements of the average intensity (normal cells: 1300 ± 115, threshold: < 50) and the total integrated intensity (normal cells: 186,000 ± 23,600, threshold < 1000) of cells. The thresholds had been determined to correlate with the resazurin readout.

Quantification of viable individually identified neurons without neurites. Three fields were randomly chosen from different wells of 2 to 3 independent experiments. The number of “cells with neurites” was determined by visual examination of the images and manual counting of cells that had at least one neurite longer than the diameter of the cell body. For the determination of “cells without neurites,” H-33342 positive nuclei that showed staining in the calcein channel but had no extension longer than the diameter of the cell body were counted. Cell numbers were expressed as a percentage of total cell number per image and averaged over different fields. This morphometric analysis was performed for all compounds in the concentration range that resulted in the maximum number of cells without neurites. This concentration range was determined before the analysis by a population-based automatic quantification of the neurite area.

Western blot analysis. Cells were lysed in RIPA-buffer (50mM Tris-base, 150mM NaCl, 1mM EDTA, 0.25% sodium deoxycholate, 1% NP40, pH 7.5) containing 1× protease inhibitor complete (Roche; Mannheim, Germany). A BCA protein assay kit (Pierce; Thermo Scientific, Rockford) was used to quantify protein concentrations. Twenty-five microgram of total protein were loaded onto 12% SDS gels and separated electrophoretically. Proteins were transferred onto nitrocellulose membranes (Amersham; Buckinghamshire, UK). Membranes were blocked with 5% milk in TBS containing 0.1% Tween (TBS-T) for 1 h at RT. Membranes were incubated with primary syntaxin-1A antibody (Synaptic Systems, Göttingen, Germany) (mouse, 1:10,000) over night at 4°C. Following washing steps with TBS-T, membranes were incubated with anti-mouse HRP (1:1000, Jackson Immuno Research) for 1 h at RT. For visualization, ECL Western blotting substrate (Pierce) was used. For loading control, membranes were stripped and stained again for beta-III tubulin (Tuj1) was obtained from Covance (mouse, 1:10,000) by incubation with 4% paraformaldehyde, 2% sucrose in PBS for 15 min in 24-well cell culture plates (Nunclon). Cells were fixed at indicated time points by incubation with 4% paraformaldehyde, 2% sucrose in PBS for 15 min at RT. After washing in PBS, cells were blocked in PBS containing 10% FCS (Gibco) for 1 h at RT, and primary antibodies in PBS containing 2% FCS were incubated at 4°C overnight in a wet chamber. Following washing in PBS containing 0.05% Tween (PBS-T), secondary antibodies were added for 1 h at RT. DNA was stained with H-33342. Coverslips were mounted with FluorSave Reagent (Calbiochem, Merck). An Olympus IX 81 microscope (Hamburg, Germany) equipped with a F-view CCD camera was used for visualization. The antibody against VMAT (rabbit, 1:200) was from Millipore (Billerica, MA), the one against neuronal class III beta tubulin (Tuj1) was obtained from Covance (mouse IgG2A, 1:1000). The Cy3 labeled secondary anti-rabbit and anti-IgG2A (Molecular Probes, Invitrogen) antibodies were used in a dilution of 1:1000.

Statistics. Individual experiments were always performed with freshly prepared chemical dilutions. Multiple technical replicates were run in each experiment. These were usually 3 for treatment data, 6 for controls, and 9–15 for basic system data (Fig. 2). Three independent biological experiments (different cell preparations) were run, and the means of the technical replicates of each experiment were used to calculate all the data shown in the figures (means ± variance of the different biological replicates). The curves in Figures 3–6 were calculated by a 4 parameter nonlinear logistic regression based on the averaged data displayed. EC50 values and their variance were determined by logistic regression based on the entire set of data using GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA). Statistical analysis was performed on the latter program, using one-way ANOVA followed by Dunnett’s post hoc test or, for comparison of neurite area to cell viability, by Newman–Keuls post hoc test. Significances were calculated compared with the lowest concentration of serially diluted test compounds, after pilot experiments had established this concentration as ineffective. The procedure was used to allow a stringent mathematical data treatment after the percentage of control transformation that eliminates the standard deviation of the untreated control. Hence, the lowest concentration used is referred to as control. The signal-to-noise ratio was calculated using the following term: S/N = MeanSignal – MeanBackground. To further characterize the assay quality, the mean of signal and background as well as their standard deviation were used to calculate the variance factor corresponding to the width of their separation band as described earlier (Leist et al., 2010).

RESULTS

Growth Factor-Independent Maturation of LUHMES Neuronal Precursors to Neurons

Differentiation of LUHMES cells to neurons with an elaborate neurite network is well established under conditions of cAMP and GDNF addition to the medium (Schildknecht et al., 2009). We were interested whether assays could also be run in a simplified medium less likely to interfere with toxicant effects. To address this, LUHMES cells were predifferentiated according to established procedures. Then cells were detached, separated into two pools, and replated in medium either containing cAMP/GDNF or not. This procedure not only allowed for a standardized comparison of media supplements but also improved the synchronization of the cells with respect to neurite outgrowth (Fig. 1). The cells were stained for neuronal beta-III-tubulin (Tuj1 antigen) or vesicular monoamine transporter-2 (VMAT) at different days of differentiation. Beta-III-tubulin was already detectable in undifferentiated cells (d0) and the intensity of the stain increased strongly on day 3 (d3) and d6 (Fig. 1B). Neither the staining intensity nor the morphological features of the differentiated cells were affected by the presence of cAMP/GDNF (Fig. 1B). VMAT was not expressed on d0, was expressed slightly on d3, and more strongly on d6. Again, the presence of CAMP/GDNF made no difference (Fig. 1B). To add a further endpoint and a more quantifiable analytical technique, we used Western blot to investigate syntaxin 1A levels. The strong increase from d0 over d3 to d6 was similar as described for VMAT and also independent of the presence of CAMP/GDNF (Fig. 1C). These initial experiments showed that LUHMES can be differentiated sufficiently well for neurite outgrowth assays without a need for the addition of CAMP/GDNF during the assay phase.
Development of Observer-Independent Methods to Quantify Neurite Outgrowth and Cell Viability without Need for Cell Fixation

To establish an algorithm for neurite growth, cells were differentiated in medium lacking cAMP and GDNF during the neurite growth phase (Fig. 1A) and stained at different times by addition of calcein-AM and H-33342 to the medium. The algorithm for quantification of overall neurite growth is based on the measurement of the difference in the total area of the entire neurite network on different time points. This allowed for the quantification also under conditions of extensive neurite intersections and network formation and did not necessitate exclusion of high proportions of cells, as is commonly the case with single neurite detection algorithms (Fig. 2A). The method is based on the strategy to measure the entire area (amount of pixels) occupied by cell bodies “plus” neurites in each field. Then, cell bodies are identified and subtracted from this area. The result is regarded as the neurite area. The use of calcein for neurite labeling not only avoided fixation and staining artifacts compared with immunocytochemical methods used earlier (Lotharius et al., 2005), but it also allowed for a direct viability assay within the same fields used for neurite analysis. The algorithm that identifies viability on the single-cell level is based on the assumption that viable cells have a nucleus with normal chromatin structure and that they accumulate calcein. By applying object identification based on H-33342 staining and by combination of the information from two fluorescent channels for nucleus-calcein colocalization, identified cells were classified as viable (calcein-positive) or nonviable (Fig. 2B). This algorithm enables a simultaneous evaluation of compound effects on neurite outgrowth and on cell viability under identical experimental conditions.

Basic Assay Characterization

Using the above algorithm and cell differentiation system, the dynamics of neurite outgrowth were analyzed in LÜHMES cells after predifferentiation and replating. In a typical experiment, cells had a low neurite area of about 500 pixels/field at 2 h after plating, which increased to about 3900 pixels/field after 24 h and to roughly 4800 after 48 h (Fig. 3A). The neurite area further increased over time, independent of the presence of growth factors (Fig. 3B). A saturation value was reached after approximately 4 days, due to neurites growing over cell somata and over one another. This potential confound has been taken into account for the selection of the optimal cell number and timing. We found that the neurite area/field was strongly dependent on the cell number (Fig. 2C).

For all subsequent assays, 30,000 cells/well were used, and measurements showed that the number of cells plated initially remained on the same level throughout this time period (Fig. 3B). We chose the time period between 2 h and 24 h after replating for all further analysis to evaluate the neurite growth during the initial period of vigorous outgrowth.
signal-to-noise ratio for these conditions was > 50 and the \( z' \)-factor was 0.6. The signal refers to the neurite area 24 h after replating the LUHMES cells. The neurite area 2 h after replating is regarded as background, as neurites barely have emerged at that time point.

As test for the robustness of the system, the effect of putatively neutral compounds was examined. Different concentrations of acetylsalicylic acid (ASS) or D-mannitol were added to the medium 1 h after replating. Neither compound affected neurite area or cell viability significantly, when these were measured after 24 or 48 h (Figs. 3C and 3D).

Specific Reduction of Neurite Outgrowth by Inhibition of Pivotal Signaling Kinases

In the next step of assay characterization, the sensitivity of the human neurite outgrowth test system to inhibitors of pivotal signaling pathways was tested. LUHMES cells were treated with different concentrations of the MAPK signaling inhibitor U0126 or with the broad PKC inhibitor bisindolylmaleimide I. U0126 decreased the neurite area at concentrations of 12.5 \( \mu \)M and higher in the absence of significant effects on cell viability (as assessed by Newman–Keuls post hoc test) (Fig. 4A). Notably, the neurite area was reduced at concentrations that did not cause unspecific cytotoxicity, and the calcein stain clearly showed viable cells lacking their neurites (Fig. 4A).

Bisindolylmaleimide I affected neurite outgrowth already at concentrations of 1.25 \( \mu \)M, whereas the number of viable cells was reduced only when the compound levels were above 10 \( \mu \)M (Fig. 4B). These data suggest that the new test system is able to detect specific effects on neurite outgrowth in the absence of general cytotoxicity. This also held true, when compounds like U0126 and Bisindolylmaleimide I were combined. In this situation, the effects on neurite growth were additive, whereas cell viability was not affected (Fig. 4C). Using U0126, we took two additional approaches to test whether cytotoxicity and effects on neurites were indeed separate effects in our test system. First, the incubation time was doubled. This resulted in a relatively larger decrease of neurite outgrowth but not in significant cytotoxicity (Fig. 4D). Alternatively, we examined damaged phenotypes. They were stained with H-33342 and calcein-AM and imaged after 30 min. (i) H-33342 staining; (ii) automatic identification of cell nuclei, displayed with a color-coded outline of their shape (blue for normal nuclei). Nuclei with an intensity above a fixed threshold were excluded by the algorithm (orange outline and arrow). A VCSA was defined around each nucleus. (iii) Live cell labeling by calcein. (iv) The algorithm quantified the calcein intensity in the VCSA areas (pink). Cells (VCSAs) without calcein staining were classified by the program as not viable (green arrows). The width of the micrographs shown is 110 \( \mu \)m. (C) LUHMES cells were seeded at different densities. After 24 h, the neurite area was assessed as described above. The red box highlights the cell number that was used for all other figures of this study. All data are means ± SEM of three experiments.

FIG. 2. Description of the algorithms quantifying neurite area and viable cells. (A) Untreated LUHMES cells were stained with H-33342 and calcein-AM on d3 and imaged. (i) H-33342 staining; (ii) nuclei-based object identification (green outline); (iii) calcein staining; (iv) all pixels beyond a given intensity threshold were defined as viable cellular structures (VCS) = somata and neurites, without distinction of individual cells. A VCSA was defined by expanding the nuclear outlines by 3.2 \( \mu \)m in each direction (orange outline). In an automated calculation, the VCSAs defined in the H-33342 channel were used as filter in the calcein channel and subtracted from the VCS. The remaining pixels in the calcein channel (marked in red) were defined as neurite area. The field was representative for the average cell density in all experiments, which was 8 ± 1.4 cells per 88 \( \mu \)m field (width of the displayed figure). (B) LUHMES (30,000 cells per well) were treated on d2 for 24 h with 300nM MeHg to generate a mixed population of cells with healthy and damaged phenotypes. They were stained with H-33342 and calcein-AM and imaged after 30 min. (i) H-33342 staining; (ii) automatic identification of cell nuclei, displayed with a color-coded outline of their shape (blue for normal nuclei). Nuclei with an intensity above a fixed threshold were excluded by the algorithm (orange outline and arrow). A VCSA was defined around each nucleus. (iii) Live cell labeling by calcein. (iv) The algorithm quantified the calcein intensity in the VCSA areas (pink). Cells (VCSAs) without calcein staining were classified by the program as not viable (green arrows). The width of the micrographs shown is 110 \( \mu \)m. (C) LUHMES cells were seeded at different densities. After 24 h, the neurite area was assessed as described above. The red box highlights the cell number that was used for all other figures of this study. All data are means ± SEM of three experiments.
reversibility of the effects observed at 24 h. Washout of 6μM U0126 resulted in a complete reversion of the neurite effect. Washout of 12μM still resulted in 80% reversion (Fig. 4D).

**Evaluation of the Dynamic Range of the Test System**

After assay characterization by using mechanistic pathway inhibitors, we chose a set of more broadly acting and putatively neurite-affecting (Table 1) compounds to further assess the dynamic range of the test system. Brefeldin A strongly decreased the neurite outgrowth by 95% at concentrations ≥ 4μM (Fig. 5A). At the same time, cell viability decreased by about 45%. Resazurin reduction was significantly affected at all concentrations that impaired neurite outgrowth, whereas the number of viable cells at 4μM was only slightly decreased (Fig. 5A). At concentrations ranging from 4 to 100μM, neurite area was significantly stronger decreased than the viability parameters. Treatment with flavopiridol decreased the neurite outgrowth at concentrations as low as 16nM and by about 85% at 125–1000nM, whereas the number of viable cells was decreased at these concentrations by about 40% (Fig. 5B). Both, the effect on the neurites and the difference of reductions in neurite outgrowth versus cell viability were statistically significant. Particularly interesting results were obtained with Y-27632, which has been described as inhibitor of rho kinases. This compound increased neurite outgrowth at concentrations as low as 16nM and by up to about 100% without effects on the number of viable cells or on resazurin reduction (Fig. 5C). These data show that significant reductions and increases are detectable by our test system and that the dynamic range was well chosen to allow the quantification of outgrowth-enhancing chemicals. Also this compound was tolerated well over 48 h. At this time, neurite outgrowth was still significantly enhanced, but we did not observe a significant washout effect (Fig. 5D). Possibly the dynamic range and sensitivity would need to be optimized for in depth studies of neurite-prolonging compounds.

**FIG. 3.** The dynamics of neurite outgrowth in LUHMES cells after replating. After 48 h of cultivation in differentiation medium cells were replated on d2 at a density of 30,000/well and stained with H-33342 and calcine-AM 24 h later. Ten fields per well were automatically imaged, and neurite area as well as cell number were measured. (A) Representative images and the average neurite area/field ± SEM from one of four independent experiments are shown for LUHMES cells 2, 24, and 48 h after replating. (B) Neurite outgrowth and the number of nuclei (as measure for the cell number) per field are shown for different time points and medium devoid of cAMP/GDNF (solid line). Data are means from 3 (0–24 h) to 5 (48–144 h) independent experiments. Neurite area was also measured over 72 h in the presence of cAMP/GDNF (dashed line). (C) Acetylsalicylic acid (ASS) or (D) D-mannitol were added to LUHMES cells after replating. After 47 h, resazurin was added to the medium and the fluorescence signal was determined 30 min later. Subsequently, cells were incubated with H-33342 and calcine-AM for 30 min, images were taken and the average neurite area and number of viable cells per field were measured. All data are means from six wells, measured in two independent experiments and are displayed as percentage of control values. The width of the micrographs shown is 110 μm.
In contrast to this, the system seemed to be well suited to study the effects of interacting compounds and of the interference of different signaling pathways. U0126 concentration dependently attenuated the effects of Y27632. In particular, the reversal of the negative effect of U0126 on neurite outgrowth by Y-27632 further corroborates the notion that the neurite inhibitory effect was not caused by cytotoxicity of the compound (Figs. 5E and 5F). For more mechanistic studies on toxicity pathways, it may be desirable to modify certain targets in LUHMES cells and to use the cultures for repeated measures. To test the feasibility of such approaches, cells were transfected with green fluorescent protein (GFP) and used for neurite outgrowth assays. The effects of U0126 and Y-27632 were now measured without calcein addition, using GFP as viability and neurite marker, and the compounds’ effects were similar to the data obtained with calcein (Supplementary fig. 1). The system was not optimized, as this was not the aim of our study, but the pilot data suggest that repeated measures and genetic cell modifications are feasible in this new model system.

Identification of Neurotoxicants Preferentially Targeting Neurites

Metamphetamine was chosen as a test compound that is well established to target the neurites of fully developed dopamine-containing LUHMES but should not specifically affect the neurite outgrowth of the still tyrosine hydroxylase-negative immature cells (Schildknecht et al., 2009). Indeed, mM concentrations were required for cytotoxic effects, and these were accompanied by a reduction of the neurite area, which did not display a statistically significant specificity (Fig. 6A).

Another cytotoxicant, the broad-spectrum tyrosine phosphatase inhibitor Na3VO4 has been suggested to act preferentially on neurites (Table 1). We found that concentrations of ≥ 25 μM...

FIG. 4. Reduction of neurite area by inhibition of kinases related to neurite growth. LUHMES cells were replated on d2, and compounds were added to the culture medium at the concentrations indicated. After 23 h, resazurin was added to the medium and the fluorescence signal was determined 30 min later. Right afterward H-33342 and calcein-AM were added, and cells were imaged to determine the average neurite area and number of viable cells per field. In representative images, the red arrow points out long neurites in unaffected cells and red rings show viable cells without neurites. (A) U0126. (B) Bisindolylmaleimide I. (C) A fixed concentration of bisindolylmaleimide I (2.5 μM) with increasing concentrations of U0126. Data are normalized to untreated controls (untr). *p < 0.05 versus bisindolylmaleimide I alone. The width of the micrographs, showing representative images, is 110 μm. (D) Cells were exposed to U0126 for different times, and the neurite area was measured and normalized (= 100%) to the value of untreated cells after 24 h. Cells treated with U0126 (solid line) were compared with untreated cells (dotted line). In a variation of the experiments, the chemical was washed out after 24 h, and cells were left with control medium for further 24 h (dashed line). All data points are means ± SEM from three independent experiments. The number of viable cells was determined for all conditions and was in no case significantly different from 100%.

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decreased the neurite area without affecting the general viability parameters significantly (Fig. 6B). This suggests that the LUHMES assay has a high specificity for toxicants affecting neurite outgrowth, as such a clear discrimination is not observed in other test systems (Harrill et al., 2010).

The known developmental neurotoxicant methylmercury has been frequently used in tests for inhibition of neurite outgrowth. Due to its pleiotropic mode of action, specific effects have only been observed under some experimental conditions, and it was therefore interesting to evaluate the biological activity in the LUHMES system. The compound strongly impaired neurite growth (about 50%) starting at concentrations as low as 40nM. At this low concentration range (40–80nM), the effect on neurites appeared to be specific, as general viability was not affected. At higher concentrations, the generally cytotoxic effect prevailed (Fig. 6C).

FIG. 5. Effects of negative and positive modifiers of neurite outgrowth. LUHMES cells were replated on d2, and compounds were added to the culture medium at the concentrations indicated. After 23 h, resazurin was added to the medium, and the fluorescence signal was determined 30 min later. Right afterward H-33342 and calcein-AM were added, and cells were imaged 30 min later to determine the average neurite area and number of viable cells per field. (A) Brefeldin A data and sample images. (B) Flavopiridol data. (C) Y-27632 data and sample images. The width of the micrographs shown is 110 μm. (D) Cells were exposed to Y-27632 for different times, and the neurite area was measured and normalized (= 100%) to the value of untreated cells after 24 h. Cells treated with Y-27632 (solid line) were compared with untreated cells (dotted line). In a variation of the experiments, the chemical was washed out after 24 h, and cells were left with control medium for further 24 h (dashed line). All data points are means ± SEM from three independent experiments. The number of viable cells was determined for all conditions and was in no case significantly different from 100%. (E) A fixed concentration of Y-27632 was used with increasing concentrations of U0126. Data are normalized to untreated controls (untr). *p < 0.05 for comparison to Y-27632 alone. (F) A fixed concentration of U0126 was used with increasing concentrations of Y-27632. Data are normalized to untreated controls (untr). *p < 0.05 for comparison to U0126 alone. All data points shown are means ± SEM from three independent experiments.
window of specificity may explain why methylmercury is not detected as specific neurite toxicant in all assay systems (Radio et al., 2008). The data from the above compounds also show the advantage of our assay system, as it allows a direct comparison of the effects on the number of viable cells and on neurite outgrowth in each individual field and for every assay.

Characterization of the Relative Impact of General Cytotoxicants on Neurite Outgrowth and Cell Viability

Different concentration-response curves for inhibited neurite outgrowth and cell death may indicate that a compound has a specific effect on neurites. An alternative explanation would be that cell death without direct effects on neurites is triggered, but that the manifestations of cell demise are first seen on the thin and fragile neurite structures. Therefore, some compounds with known general cytotoxicity (=direct cell death induction) were analyzed here. The redox-cycler menadione is known to kill cells by generation of oxidative stress. This compound triggered a steep decrease in neurite growth and viability at concentrations \( \geq 8\mu M \) (Fig. 7A). The membrane-disrupting detergent sodium dodecyl sulfate caused a sharp decline in neurite outgrowth and the number of viable cells at concentrations \( \geq 33\mu M \) (Fig. 7B). The cytotoxic metal salt cadmium chloride reduced neurite area and viability at > 40\( \mu M \) (Fig. 7C). At 41\( \mu M \), \( \text{CdCl}_2 \) neurites were significantly more affected than cell viability. This suggests that nonspecific toxicants can inhibit cellular extensions, but that this occurs as a consequence of unspecified toxicity which needs to be measured in parallel to obtain a specific neurite outgrowth assay.

Analysis of Neurite Outgrowth Inhibition on the Single-Cell Level

During the course of the study, viable cells without neurites had been observed on some of the images recorded. As this information on the single-cell level provides additional information on the specificity of the test system, we decided to quantify these initially qualitative observations. Viable cells having at least one neurite longer than the diameter of their cell body, and viable cells without such neurites were counted for a set of test chemicals. A high (\( \geq 40\% \)) number of cells without neurites was observed for at least one concentration of U0126, sodium orthovanadate, flavopiridol, cycloheximide, indicated. After 23 h, resazurin was added to the medium and the fluorescence signal was determined 30 min later. Subsequently, H-33342 and calcein-AM were added, and cells were imaged 30 min later to assess neurite area and number of viable cells per field. (A) Methamphetamine (METH). (B) Sodium orthovanadate (Na3VO4). (C) Methyl mercury chloride (MeHg). All data points shown are means from 2 to 3 independent experiments. *p < 0.05 neurite area versus control of neurite area. #p < 0.05 neurite area versus number of viable cells. The width of the micrographs shown is 110 µm.
and brefeldin A (Fig. 8). This confirmed at the single-cell level that these compounds affect neurite outgrowth independent of the triggering of overall cell death. Under conditions of general cytotoxicity, the total percentage of viable cells decreased, e.g., for SDS, menadione, CdCl₂, methylmercury, and for higher concentrations of the neurite-selective compounds (Fig. 8). Notably, also some concentrations of unspecific cytotoxicants (e.g., 41 µM of CdCl₂) lead to the appearance of a certain proportion (up to 30%) of cells without neurites. These findings are important for considerations concerning the prediction model of assays based on single-cell analysis. Without additional information, the assay data in the below 30% range do not indicate whether a compound is inhibiting neurite outgrowth or just acts as cell death inducer.

**Overall Correlation of Chemical Effects on Neurite Area and Cell Viability**

In order to examine whether a specificity threshold can be suggested for the field-based quantification of the neurite network growth, we plotted the EC50s of the number of viable cells against the EC50s of neurite area reduction for all test compounds. Chemicals known to be generally cytotoxic plotted below an EC50 ratio of 4. Their average ratio was 2.5 with a 95% confidence band extending to 4.7 (Supplementary fig. 2). Chemicals that affected the neurites more than 4.7 times more potently than the overall viability may therefore be considered as specific neurite outgrowth toxicants (Fig. 9). The data were essentially similar, whether the intact cell number or resazurin reduction were used as viability parameters (Supplementary fig. 2). The only exception was methylmercury, a compound well known to affect metabolic functions, such as resazurin reduction without necessarily triggering cell death. Using calcein retention as cell death endpoint, methylmercury would be classified as neurite outgrowth inhibitor, with a small window of specificity (Supplementary fig. 2). This agrees well with the individual data over many concentrations analyzed in Figure 6C. All other compounds were clearly classified, and the threshold suggested above appears useful for future validation of the assay with a different set of compounds selected to contain “bona fide” developmental neurotoxicants, other neurotoxicants, and nonneurotoxic compounds.

**FIG. 7. Reduction of neurite outgrowth and viability by cytotoxic compounds.** LUHMES cells were replated on d2, and compounds were added to the culture medium at the concentrations indicated. After 23 h, resazurin was added to the medium and the fluorescence signal was determined 30 min later. Subsequently, H-33342 and calcein-AM were added, and cells were imaged 30 min later to quantify neurite area and number of viable cells per field. (A) Menadione. (B) Sodium dodecyl sulfate (SDS). (C) Cadmium (II) chloride (CdCl₂). All data points shown are means from 2 to 3 independent experiments. *p < 0.05 versus control. The width of the micrographs shown is 110 µm.
DISCUSSION

We developed an observer-independent and fully quantitative test method to detect toxicant effects on neurite outgrowth of human neuronal precursor cells growing in a high-density culture. The reaction to a panel of mechanistically diverse pharmacological and toxicological inhibitors indicated that the activity of multiple nonredundant pathways is required for neurite extension. Both, inhibition and acceleration of outgrowth by chemicals are therefore detectable by the described assay. Simultaneous measurement of cytotoxicity within the same experiment was found essential to eliminate unspecific cytotoxicants.

Screening systems to detect neurite outgrowth have been developed for a variety of cell types and have promoted our understanding of intrinsic modulators of neurite outgrowth (Larsson, 2006; O’Donnell et al., 2009) and of extrinsic compounds adversely influencing the growth of neurites (Radio and Mundy, 2008). Progress in the latter field faces a number of difficulties that have been addressed in our study: (1) distinction of general cytotoxic effects from specific effects on neurite outgrowth; (2) limited availability of human, non-transformed neuronal cells; (3) difficulties with quantitative image processing in high-density cultures; (4) limited availability of bona fide neurite outgrowth inhibitors during test establishment.

Under some circumstances, reciprocal interactions exist between adverse effects on neurites and overall cell death. For instance, neurite damage can trigger apoptosis (Berliocchi et al., 2005; Leist and Jaattela, 2001; Volbracht et al., 2001), and failure of outgrowth to a target is a common trigger of programmed cell death in developing neurons (Buss et al., 2006). Literature data on whether differentiating neurons tolerate a toxicant-induced prevention of neurite outgrowth over long time are relatively limited. Also, many toxicants may not only affect neurite growth but also other important cellular processes. The unspecific actions may result in adverse effects. To avoid such unspecific toxicity in a screening assay, it is of advantage if cells do not have to be exposed to toxicants for long periods of time, i.e., if altered neurite outgrowth can be measured with a good signal-to-noise ration within short time (e.g., 1–4 days). Extensive pilot experiments to our study showed that the chosen growth
interval of 24 h or 48 h for reversibility experiments results in the maximum neurite extension per time and that the extent of growth during this period allows for easy measurements of its disturbance. As chemicals may affect different targets and pathways, cell death can be triggered by such toxicants in parallel to or even completely independent of the inhibition of neurite outgrowth. Then, the disintegration of the projections would be an indirect and passive event. When neurite length is assessed on fixed cultures by immunostaining, it is hard to determine whether a cell was dead or alive before the fixation, i.e., whether it was a live neuron with shortened neurites or whether it was dead and had therefore also lost axons and/or dendrites (Harrill et al., 2010; Radio et al., 2008, 2010). Simultaneous measurement of viability and neurite effects in the same cultures adds valuable information to the test method, as neurite length determinations can be restricted to live cells. The measurement within the same well also avoids problems arising from the fact that the effects of chemicals on both cell viability and on neurites depend to a certain extent on the exact cell density and other culture conditions that show variations within culture wells, culture dish formats and from day to day. For instance, the free active concentration of a chemical in a culture dish is not only determined by the nominal amount added but also by the surface-volume ratio, by the lipid and protein content (i.e., the exact cell number) and the cellular physiological state (Blaauboer, 2010; Gulden et al., 2006, 2010). Some of these properties can be extremely dependent on the initial cellular concentration, as variations of as little as 10–20% of plated cells can already determine the type of differentiation occurring (Chambers et al., 2009; Ying and Smith, 2003) and thus modify the number and type of cells present at the start of the toxicity experiment. Additional variations may occur from well to well in substrate-dependent cultures, e.g., due to differences in coating and other handling steps, and this further suggests that measurement of multiple endpoints within one culture well has advantages over determinations in parallel cultures.

In the LUHMES neurite outgrowth assay, we attempted to examine different types of toxicants and their effects on cellular viability parameters. This requires some clarification of the terminology we used. We use the term “viability” to summarize different measures of cellular integrity and function. In particular, we assessed the capacity of the cells to grow neurites, to retain calcein (as measure of cell membrane integrity), and to reduce resazurin (as a measure of metabolic capacity). We found a high degree of correlation between the latter two parameters for all types of compounds and used the term “cytotoxicity” for the process measured by these parameters. This is an operational definition, which implies that we use the term cytotoxicity to signify cell death but not sublethal types of cell damage. In the context of test development, we considered it useful to define a group of compounds that are generally known to trigger cytotoxicity in many cell types, independent of their differentiation state and special physiological functions. As selection criterium for these compounds, we required that they are not known to be specifically neurotoxic or to affect neurodevelopment and that they kill other cell types in a similar concentration range as neurons by membrane disruption, unspecific oxidative stress, or interference with vital metabolic processes. These compounds were used as “unspecific controls” (Leist et al., 2010) to help defining the specificity range of the assay. Specificity is a measure of the false positives of a test, and its determination requires the presence of negative controls in the test sample. For a test with two endpoints, e.g., neurite growth and cytotoxicity, two types of negative controls are required: One group that has no effect at all on any parameter, and one group that is expected to affect both measures in the same way, i.e., being negative with relation to the difference of the endpoints. Data from such unspecific control compounds showed that a certain number of viable cells without neurites can also be found when direct cytotoxicity is triggered (Fig. 8), and the chemical has no direct effect on neurite outgrowth. These findings were used to suggest a threshold above which compounds are likely to be positive hits (i.e., primarily affecting neurite outgrowth).

Several successful test systems to screen for compounds affecting neurite outgrowth have been developed earlier. Some use rodent cells, such as PC-12, a subclone thereof (Neuroscreen-1) (Radio et al., 2008) or primary cerebellar granule cells (CGCs) (Radio et al., 2010). Others use the human neuroblastoma cell line (SH-SY5Y) (Frimat et al., 2010) or human embryonic stem cell derived neural cells (hN2) (Harrill et al., 2010). Each of these systems offers advantages and disadvantages depending on the available infrastructure and questions asked. Particular strengths of the system described here are the easy and free availability of all components, the human background and the particularly high detection power for specific neurite outgrowth inhibitors. However, there are also shortcomings. For instance, the neurites do not take distinct characteristics of axons and dendrites, as they are positive for the axon marker tau as well as for the dendrite marker microtubule associated protein-2 (data not shown). The toxicological implications of this mixed immature phenotype are not clear yet, but some chemical effects may be missed, as other studies have shown that compounds may specifically affect axons or dendrites, whereas they have no or even the opposite effect on the other type of neuronal extension (Abdu et al., 2010; Howard et al., 2005; Kim et al., 2009). Another disadvantage of the LUHMES test system is the relatively low complexity (branching) of the neurites and an inability of the algorithm to quantify parameters describing neurite complexity such as neurites/cell or the number of branch points. Moreover, it is not clear at the present stage, how differentiation and neurite growth are coupled in this model. Due to the replating of the cells, a kind of neurite regrowth phenomenon may occur. It also will require clarification in the future, whether inhibitory compounds may block neurite growth by blocking overall differentiation of the cells.
One outcome of the study is the definition of a set of test compounds that reproducibly affect neurite growth at concentrations below the ones affecting overall viability. All these compounds have been used earlier in other studies, which implies that they may have a general neurite-affecting activity, and not be specific for the LUHMES test system. To our knowledge, the list we compiled is the largest one at present that was compared within one assay and tested positive in a human neurite outgrowth system. The availability of a set of positive controls will be very helpful for further studies in this test system, addressing other potential neurite outgrowth inhibitors and especially screening unknown compounds for this potential adverse effect. As neurite outgrowth inhibition is not a parameter easily obtainable from existing animal data, a solid in vitro definition, taking unspecific toxicities into account is particularly important to form the basis, e.g., for high-throughput testing of unknown chemicals and for identification of potential common toxicity pathways.

Two tested chemicals (U0126 and bisindolylmaleimide I) affected known pathways of outgrowth control. The concentration range in which U0126 inhibited neurite outgrowth in LUHMES cells is comparable to other test systems (Table 1). In the case of bisindolylmaleimide I, inhibition of neurite outgrowth of LUHMES cells seems to be more sensitive than in hN2 and CGC and about similar to PC12 (Table 1). As both compounds impaired neurite outgrowth at concentration that did not affect cell viability, the importance of PKC and MAPK signaling in LUHMES cells was corroborated. In addition, we showed that these effects were additive, time dependent, and reversible. Rho kinase (ROCK) is known to regulate contractility of actomyosin and to be essential for growth cone collapse. Its inhibition has been suggested to favor or potentiate neurite outgrowth, and this was corroborated here with the specific ROCK inhibitor Y-27632. Flavopiridol inhibits cyclin-dependent kinases (cdk). Within this family, cdk5 takes an important role in neuronal growth cones and is not associated with cell cycle control. Its loss or inhibition in neurons resulted in altered neurite outgrowth (Hahn et al., 2005). More specific investigations of this pathway in human cells will be facilitated by the LUHMES system. Sodium orthovanadate is a broad-spectrum tyrosine phosphatase inhibitor shown to decrease axonal outgrowth for instance in hippocampal neurons or hN2 cells (Harrill et al., 2010; Mandell and Banker, 1998). In LUHMES, these effects were corroborated in a similar concentration range and with the advantage that the inhibition of outgrowth was clearly more pronounced than adverse effects on viability. Methylmercury is known as human developmental neurotoxicant (Castoldi et al., 2008). Animal data show that it has multiple effects on the developing nervous system that can lead to functional impairment without gross pathology or neuronal loss (Castoldi et al., 2008). On rodent cells, methylmercury shows a large variety of activities, in addition to pronounced effects on neurite development in PC-12 (Radio and Mundy, 2008). Our data (specific effect only in a very narrow concentration window) suggest that other modes of action may be more relevant for human DNT of methylmercury.

In summary, an assay was presented in which viability is determined simultaneously with neurite outgrowth. On this basis, a panel of mechanistically different chemicals has been found here to inhibit human neurite outgrowth in a new model system.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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