

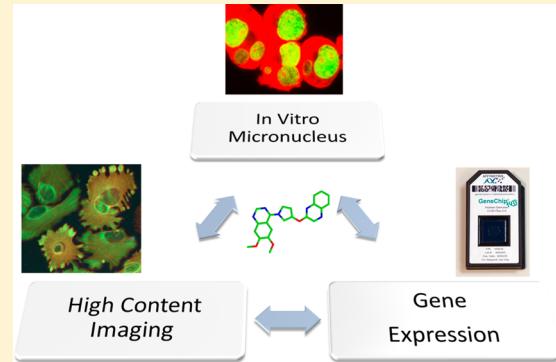
Integrating High-Dimensional Transcriptomics and Image Analysis Tools into Early Safety Screening: Proof of Concept for a New Early Drug Development Strategy

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Supporting Information

ABSTRACT: During drug discovery and development, the early identification of adverse effects is expected to reduce costly late-stage failures of candidate drugs. As risk/safety assessment takes place rather late during the development process and due to the limited ability of animal models to predict the human situation, modern unbiased high-dimensional biology readouts are sought, such as molecular signatures predictive for *in vivo* response using high-throughput cell-based assays. In this theoretical proof of concept, we provide findings of an in-depth exploration of a single chemical core structure. Via transcriptional profiling, we identified a subset of close analogues that commonly downregulate multiple tubulin genes across cellular contexts, suggesting possible spindle poison effects. Confirmation via a qualified toxicity assay (*in vitro* micronucleus test) and the identification of a characteristic aggregate-formation phenotype via exploratory high-content imaging validated the initial findings. SAR analysis triggered the synthesis of a new set of compounds and allowed us to extend the series showing the genotoxic effect. We demonstrate the potential to flag toxicity issues by utilizing data from exploratory experiments that are typically generated for target evaluation purposes during early drug discovery. We share our thoughts on how this approach may be incorporated into drug development strategies.



INTRODUCTION

The development of novel molecules that hold the greatest promise for being used as safe and effective therapies remains a continuous challenge to the pharmaceutical industry. Drug development is an increasingly long process, with an estimated cost of up to 2.6 billion U.S. dollars to bring a new drug to the market.¹ This cost is driven by the fact that 95% of the experimental medicines that are studied in humans fail to be both effective and safe. A recent analysis of Phase II and III attrition rates showed that safety issues remain a significant hurdle even in late stages of development.² Therefore, more and better predictive toxicology assays and models need to be implemented in earlier phases of the pharmaceutical R&D value chain.³

The molecular properties of a drug candidate are finalized at the interface between discovery and development. This means that properties causing mechanism (target)-related toxicity, off-target side effects, and compound-chemistry-related toxicity are all fixed at that point. A “fail early” strategy has been postulated to prevent drug candidates that will induce adverse effects in humans from entering clinical development. Alternatively, a SAR-directed approach may be used to direct the medicinal

chemistry to minimize the toxic liabilities of a chemical series. Hence, it would be better if target- and compound-related risk factors were addressed during the drug discovery phase, when discussions on novel drug targets take place and compound series are identified and optimized.³

Traditionally, the toxicological evaluation of pharmaceuticals is performed at rather late stages of development and has largely relied on animal models that have been developed to evaluate regulatory-required toxicological end points. However, besides the high cost of *in vivo* testing, low-throughput, and animal usage, *in vivo* test systems have a limited predictivity of human adverse effects.⁴ Therefore, several major initiatives have begun to utilize *in vitro* methods and a variety of new technologies to develop *in vitro* signatures of *in vivo* response. The Toxicology in the 21st Century (Tox-21c) movement, initiated with a report of the National Research Council,^{5,6} has stirred the toxicological community^{7–10} and initiated a paradigm shift in toxicity testing. Moving from traditional *in vivo* tests to less expensive and higher-throughput cell-based

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Table 1. Overview of the Different Compounds Tested and Their Results for the Different Assays: MNT (*in Vitro* Micronucleus Test), High-Content Imaging, and Gene Expression Profiling^a

Test compound	MNT 24h	High Content Imaging			Gene expression Tubulin down- regulation 8h, 10μM
		Aggregates (b) 8h	μM	Cell Count (c)	
DMSO control	-	-1.9	0	1.0	0.0
2858	NA	-1.3	3	1.0 (c)	0.2
7886	NA	-0.4	25	0.6 (c)	0.0
4808	NA	-0.2	10	0.4	-0.3
0558	-	-1.3	3	0.2	0.0
3273	NA	-0.9	10	0.2	0.0
9660	NA	-0.6	25	-0.2	0.1
7912	NA	1.0	25	0.1	-1.7
4782	+	1.4	25	-0.1	-1.7
5035	NA	1.7	25	0.0	-1.8
8148	+	1.7	25	0.3	-2.2
4735	NA	2.1	10	0.2	0.2
Griseofulvin	+	-1.3	3	1.1 (c)	0.2
Taxotere	+	-0.7	10	0.2	0.3 (d)
Nocodazole	+	2.2	3	0.3	-1.4
Vinblastine	+	2.1	1	0.2	-0.1
Colchicine	+	2.6	3	0.3	0.3 / -1.0 (e)

^aReadouts colored in green suggest no micronucleus induction, tubulin aggregates, and tubulin downregulation, whereas the opposite is true of those shaded red. Five reference aneugens were also included (griseofulvin, taxotere, nocodazole, vinblastine, and colchicine). NA: not available.

^bMicrotubule aggregate scores indicating the presence (>0) or absence (<0) of tubulin aggregates. ^cDecrease in cell number used as measure for exposure; compounds with cell count >50% were probably tested at concentrations that were too low. ^dTaxotere is a known aneugen and induces tubulin downregulation, but it does so only after 36 h. ^eTwo measurements are available, and both are given.

assays will allow the identification of key pathways and proteins linked with toxicity end points and thus will lead to a better understanding of the underlying mechanisms of toxicity.¹¹ Within the science of toxicology, strategic concepts such as pathways of toxicity¹⁰ and adverse outcome pathways^{12,13} should be considered. Both are conceptual constructs of linkages between a direct molecular initiating event and adverse outcomes at different levels of biological organization and are therefore highly relevant to risk assessment. Emerging technologies and techniques like the so-called omics technologies, high-content screening and imaging approaches, bioinformatics, and computational biology^{14–16} enable high-throughput analysis of treatment-related changes at the molecular level. This may contribute to the development of integrated testing strategies that can be implemented during the early drug discovery phase.

High-content imaging can simultaneously quantify multiple phenotypic and/or functional parameters in biological systems and is considered to be a promising technology to address the challenges for the toxicity testing in the 21st century approach.¹⁷

The use of toxicogenomics approaches^{18–20} requires the evaluation of the biological relevance of gene expression changes. Therefore, connecting the expression changes to phenotypic end points is crucial. This phenotypic anchoring

allows expression changes to be associated with biologically relevant observations. Especially in early discovery phases, many *in vitro* assays are typically run on many competing compound scaffolds, and correlating these phenotypic end points to gene expression markers and chemical structures may provide important information that may influence compound prioritization, also within the context of safety evaluation. To investigate this concept, a pilot project named QSTAR²¹ (quantitative structure–transcription–assay relationships) was undertaken at Janssen Research & Development. The goal of this project was to integrate high-throughput gene expression profiling data, chemical information, and bioassay data within the lead optimization phase in eight pharmaceutical discovery projects across four disease areas (oncology, virology, neuroscience, and metabolic diseases).

The data described in this article report on the outcome of this exercise performed on 76 compounds belonging to a single chemical backbone targeting PDE10A. PDE10A has been the target of a drug discovery research project at Janssen with the aim of modulating the activity of PDE10A via a small molecule.²² While the efficacy of the investigated compounds was high, we picked up changes in tubulin gene expression for a small subset of compounds when analyzing the gene expression profiles. Since it is known that perturbations in tubulin polymerization may contribute significantly to the induction

of micronuclei,^{23,24} this genotoxicity end point was selected as an early phenotypic biomarker for the identification of spindle poisons. Subsequent micronucleus assays on a select number of compounds showing downregulation of tubulin gene expression confirm that these compounds selectively induce micronuclei *in vitro*. In addition, high-content imaging confirmed the effects on tubulin fibers, thereby offering the opportunity to eventually implement a high-throughput assay at earlier stages in the drug development process.

MATERIALS AND METHODS

Test Compounds. In addition to 11 compounds targeting PDE10A, a number of reference aneugens (griseofulvin, taxotere, nocodazole, vinblastine, and colchicine) were tested in the different platforms (Table 1). Additionally, 65 compounds targeting PDE10A were tested in the gene expression platform only. Compounds were synthesized as described in WO 2006072828.

Cell Lines. HEK293. Human embryonal kidney cells (HEK293, ATCC CRL-1573) stable transfected with mouse PDE10A were cultured using modified Dulbecco's modified Eagle's medium, GIBCO 11960-044 (composition: 500 mL of DMEM, 57 mL of FBS (heat inactivated), 10 mL of Sol A, 4 mL of geneticin selective antibiotic G418 (100 mg/mL)). Cells were grown in 175 cm² culture flasks at 37 °C under 5% CO₂ and split at 80% confluence every 3 to 4 days. One day before the experiment, cells were detached with phosphate buffered saline containing 0.04% ethylenediaminetetraacetic acid (EDTA) and 0.005% trypsin and collected by centrifugation.

HepG2. Hepatocarcinoma cells (HepG2, ATCC HB-8065) were cultured using modified minimum essential medium, GIBCO 31095 (composition: 500 mL of MEM (GIBCO 31095), 57 mL of FBS (heat inactivated), 5.7 mL of L-glutamine, 5.7 mL of Na-pyruvate, 5.7 mL of NEAA (100X), 10 mL of NaHCO₃ 7.5%, 0.57 mL of gentamycine (50 mg/mL)). Cells were grown in 175 cm² culture flasks at 37 °C under 5% CO₂ and split at 80% confluence every 3 to 4 days. One day before the experiment, cells were detached with phosphate buffered saline containing 0.04% EDTA and 0.005% trypsin and collected by centrifugation.

LNCaP. Humane prostate cancer cells (LNCaP, ATCC CRL-1740) were cultured using modified ATCC-formulated RPMI-1640 medium, catalog no. 30-2001 (composition: 500 mL of RPMI 1640, 5.7 mL of L-glutamine 2 mM, 57 mL of FBS (not heat inactivated), 5.7 mL of gentamycine (50 µg/mL)). Cells were grown in 175 cm² culture flasks at 37 °C under 5% CO₂ and split at 80% confluence every 3 to 4 days. One day before the experiment, cells were detached with phosphate buffered saline containing 0.04% EDTA and 0.005% trypsin and collected by centrifugation.

SK-N-BE(2). Neuroblastoma cells (SK-N-BE(2), ATCC CRL-2271) were cultured using a modified 1:1 mixture of Dulbecco's modified Eagle's medium and F12 medium, DMEM/HAMF12 (composition: 500 mL of DMEM/HAMF12 (31331), 28 mL of FBS (heat inactivated, New Zealand origin), 5 mL of SOL B, 5.7 mL of NEAA, 8.5 mL of HEPES, 5.7 mL of L-glutamine). Cells were grown in 175 cm² culture flasks at 37 °C under 5% CO₂ and split at 80% confluence every 3 to 4 days. One day before the experiment, cells were detached with phosphate buffered saline containing 0.04% EDTA and 0.005% trypsin and collected by centrifugation.

TK-6. Human lymphoblastoid cells (TK6, ATCC CRL-8015) were cultured in RPMI 1640 (Life Technologies; catalog no. 72400-021) complemented with 10% horse serum (Life Technologies; catalog no. 26050-088). Cells were grown in culture flasks at 37 °C under 5% CO₂ and split at 15 000 cells/mL every 3 days.

U2OS. Human osteosarcoma cells with endogenously fluorescently labeled proteins were purchased from Sigma-Aldrich (U2OS, catalog no. CLL1218) and cultured in McCoy's 5A medium (Gibco 26600) supplemented with 10% FCS (Hyclone SV30160.03) and solution A (in-house prepared mixture of penicillin G 3.145g/L, streptomycin 5 g/L, L-glutamine 14.6 g/L, and sodiumpyruvate 5.5 g/L) at 37 °C under 5% CO₂. In these osteosarcoma cells, three genomic loci,

LaminB1, TUBA1B, and ACTB, have been endogenously tagged with fluorescent protein genes for blue fluorescent protein (BFP), green fluorescent protein (GFP), and red fluorescent protein (RFP), respectively.

Gene Expression Profiling. Cells (HEK293, LNCaP, HepG2, SK-N-BE(2)) were seeded in a 96-well plate, cultured for 8 h before treatment, and treated with a number of compounds from the PDE10A project at a concentration of 10 µM in 0.1% DMSO for 8 h. The basis of the single concentration used for gene expression profiling was the Connectivity Map publication.²⁵ However, instead of profiling at 6 h, as was done in Connectivity Map, we have chosen to profile our compounds at 8 h, based on internal experience, resulting in a better balance between primary effects versus later effects. Cells were lysed, and mRNA was extracted using the RNeasy96 kit (Qiagen) with DNase treatment on the column. For whole-transcript (WT) arrays, 200 ng of total RNA was used as input material. The respective protocols were followed according to the manufacturer's instructions. Microarray hybridizations and preprocessing were done using Human Gene ST 1.1 array plates according to the manufacturer's instructions. Gene expression raw intensities were log-transformed, quantile-normalized, and subsequently summarized using FARMS²⁶ and Entrez gene customCDF annotations.²⁷ Uninformative probe sets were removed using I/NI filtering,^{28,29} retaining only the probe sets that varied more among the compounds than among the DMSO controls. Exploratory analysis of the transcriptional data by spectral maps³⁰ was performed to assess the data quality, to identify outliers in early data analysis, and to find the few strongest transcriptional effects. Testing for differential expression was performed using LIMMA.³¹ The data are available via GEO (GSE70617).

In Vitro Micronucleus Assay. For the micronucleus test (MNT), a dose-response assay was performed in TK6 cells. The cells were seeded in a 96-well plate and cultured overnight before addition of compounds. TK6 cells are often used in the *in vitro* micronucleus test because of their human origin and p53 wild-type status.³² After treatment for 24 h (in the absence of metabolic activation), cells were centrifuged (cytospin) onto glass slides, fixed in methanol, and stained with acridine orange. Microscopic analysis (of blind coded cytosides) was performed manually to determine the number of micronucleated cells (MNC). The presence of micronuclei was evaluated in at least 2000 cells per test condition (at least 1000 cells per culture; 2 cultures per concentration). The frequency of micronucleated cells induced by the different test item concentrations was compared with those of the concurrent vehicle controls and scored according to a standardized protocol.^{33,34} A positive response was obtained when the test item induced a biologically significant (2-fold or higher) and concentration-related increase in the frequency of MNC above the concurrent vehicle control value.

High-Content Imaging. Human U2OS cells were seeded at 1000 cells/well in RPMI medium without phenol red (GIBCO 11835-063) supplemented with 10% FCS and solution A in a PDL coated CellCarrier 384-well plate (PerkinElmer 6007558) and cultured overnight at 37 °C under 5% CO₂ before addition of compounds. Compounds were added at different doses (from 1 nM to 25 µM) to cover a broad concentration range, and the cells were monitored over a period of 24 h at 37 °C under 5% CO₂. Every hour, cells were imaged at 20× magnification (Opera system), and image analysis was performed with DCILabs³⁵ and Phaedra.³⁶

From images of all wells, 661 features (related to the cells' intensity, shape, texture, geometry, etc.) were extracted. Feature values were measured at the cell level, but they were averaged across cells to obtain well-level aggregates. Aiming to find a small subset of features able to highly distinguish images showing microtubule aggregates from images showing other phenotypes, the discriminating power of the features was evaluated by means of *t*-tests comparing these two image groups. For this, both the significance of the difference in average feature values between both groups and the magnitude of the difference were taken into account. Three features were finally selected; all three texture features derived from the cellular regions of the tubulin-GFP channel and thus directly represent tubulin characteristics. To obtain a single microtubule aggregate score for each image, linear discriminant

analysis (LDA) was used to summarize the three selected features as a linear combination of the original feature values. On the basis of this microtubule aggregate score, images can be classified in two groups. Images of wells with a positive score show microtubule aggregates, whereas images not showing microtubule aggregates correspond to negative scores. Note that three features were considered to compute the score, as adding more no longer resulted in a significant reduction of the LDA misclassification error.

RESULTS

A novel series of 1,3-alkoxy-substituted pyrrolidines, substituted with different heteroaromatic ring systems (Figure 1), was

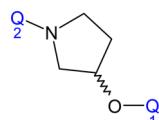


Figure 1. Synthesized chemotype consisting of 3-hydroxypyrrrolidines substituted with different heteroaromatic ring systems Q. Q1 is either (substituted) benzofuran, phenyl, quinoxaline, pyridine, quinoline, or pyrazine. Q2 is either (substituted) quinazoline, quinoline, imidazo-pyridazine, or triazolopyridine.

synthesized to inhibit the phosphodiesterase PDE10A, which is almost exclusively expressed in the striatum and is considered to be a novel therapeutic avenue in the discovery of antipsychotics.^{37,38} Fifty eight compounds of this series were transcriptionally profiled as part of the drug discovery process²¹ to search for potential signs of polypharmacological effects.

Search for Potential Polypharmacology through Gene Expression Profiling. Gene expression profiling was done on HEK293 cells transfected with the mouse homologue of PDE10A. Unsupervised analysis, using spectral map analysis²⁸ (Figure 2a) of the induced transcriptional effects of all quality filtered informative genes,²⁶ revealed that a subset of four compounds (8148, 4782, 5035, 7912) within this chemical

series showed strong downregulation on all informative tubulin genes (Figure 2b). Downregulation of tubulin genes suggests a possible genotoxic effect via interference with microtubule-based chromosome segregation.^{39,40}

To explore the relevance and biological reproducibility of the tubulin signature in other cellular backgrounds, the compound that showed most tubulin downregulation, compound 8148, was tested in three other human cellular backgrounds available at the time, namely, LNCaP (prostate, epithelial), HepG2 (liver hepatocellular carcinoma, epithelial), and SK-N-BE(2) (brain, neuroblastoma). Box plots of the fold changes of the informative genes are plotted in Figure 3, with the tubulin

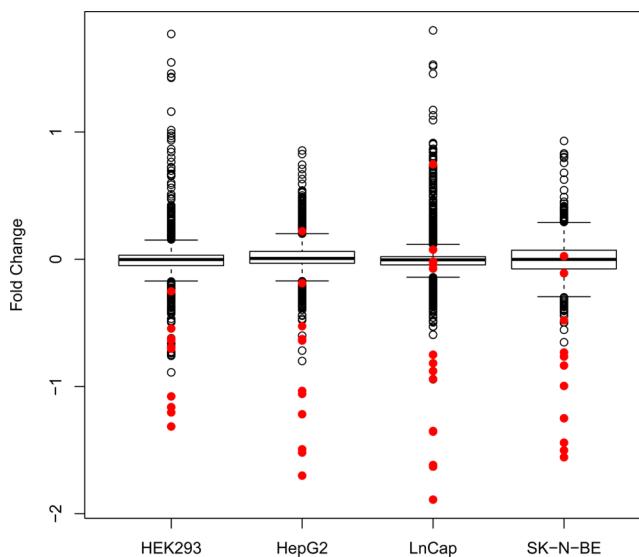


Figure 3. Box plot of the fold changes of all informative genes for compound 8148 in four different cell lines, where the tubulin genes are colored red.

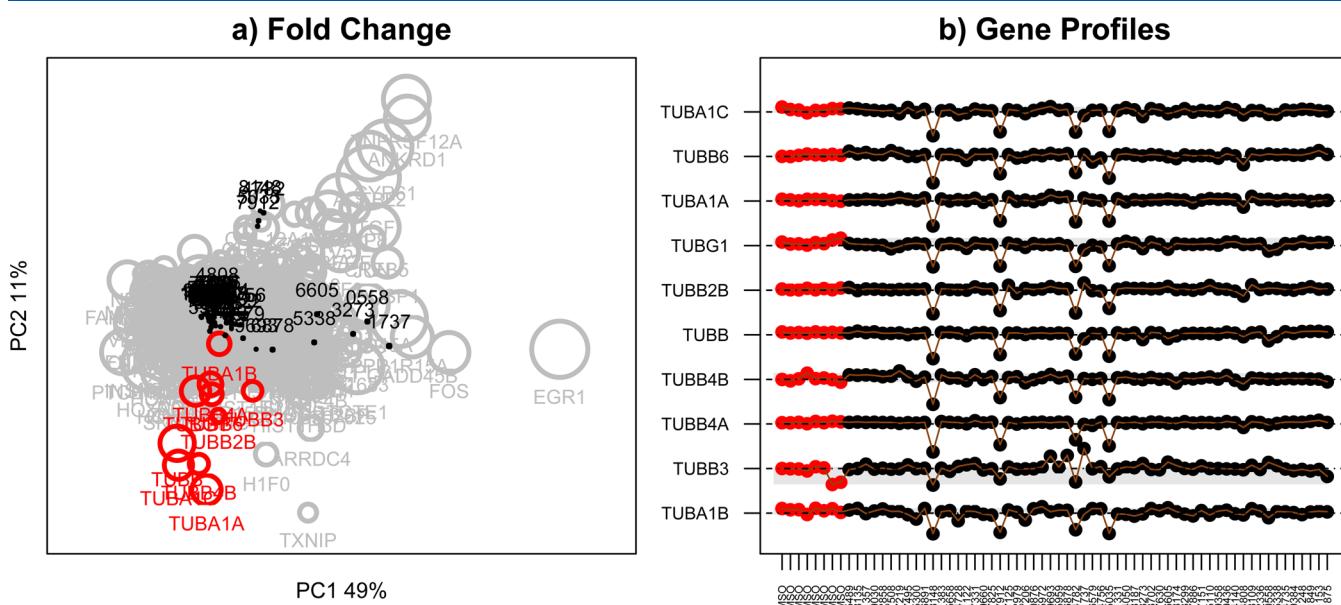


Figure 2. Exploration of gene expression profiling performed in the PDE10A project to search for potential polypharmacology for a subset of 58 compounds synthesized in this project. (a) Spectral map analysis, where the second component, displayed on the y-axis, distinguishes four compounds (on top) within the chemotype based on a set of tubulin genes (colored red). (b) Gene profile plot of all informative-called tubulin genes showing a significant downregulation for these four compounds. DMSO is indicated in red.

genes colored red. In general, the tubulin genes are among the most downregulated genes, suggesting that the observed effect is largely cell line-independent and not linked to PDE10A. Using the known annotation/gene function of tubulins in the context of chromosome segregation, it was hypothesized that these compounds may induce a genotoxic (aneugenic) effect, which should be verifiable via a specific assay that captures the phenotype of such compounds.

Confirmation of the Hypothesized Genotoxicity with the *in Vitro* Micronucleus Assay. The micronucleus test⁴¹ (MNT) detects the formation of micronuclei in the cytoplasm of interphase cells. Compounds 8148 and 4782, showing clear downregulation of the tubulin genes, were profiled in the MNT with human TK6 cells. Clear formation of micronuclei (Figure 4b) could be observed for both of them. When cells were

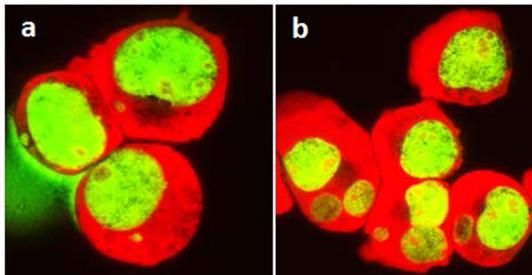


Figure 4. Images of micronucleated TK6 cells induced by (a) the clastogen mitomycin C and (b) compound 8148 (large sized MN).

treated for 24 h, both compounds induced a biologically significant (>10-fold) and concentration-related increase in the frequency of micronucleated cells. Large sized micronuclei and an increased number of bi- and polynucleated cells were observed, which is typical for spindle poisons and suggests aneuploidy.^{42,43} On the other hand, one compound not showing downregulation of the tubulin genes did not result in micronucleus formation. In a structurally related series, two compounds being negative in the MNT also did not show tubulin downregulation (data not shown). The positive MNT results indeed suggest that tubulin downregulation has an effect on microtubule-based chromosome segregation.

Use of High-Content Imaging Data To Visualize Effects on Tubulin Proteins. To further explore this finding, another phenotypic assay, based on high-content imaging, was used. In this experiment, compounds were tested on human osteosarcoma cells that express an endogenous fluorescent tubulin protein. Hence, the effect on the tubulin proteins can be followed by imaging. Eleven compounds, including 8148, together with five well-known aneugenic (MNT-positive) reference compounds were added to these cells at different concentrations (1 nM to 25 μ M) and followed over time (1–24 h). The MNT-positive compounds show, in contrast to the DMSO controls (Figure 5b), a characteristic phenotype with microtubule aggregates at the edges of the cell and around the nucleus (Figure 5a). The four compounds that downregulate the tubulin genes show similar profiles to the MNT-positive compounds at certain concentrations and time points, one of which is shown in Figure 5c. The compounds, within the same chemotype, that do not show downregulation of the tubulin signature have images across different concentrations and time points similar to those of the DMSO controls (Figure 5d). Hence, the presence of these microtubule aggregates might be correlated with tubulin downregulation and genotoxicity. To

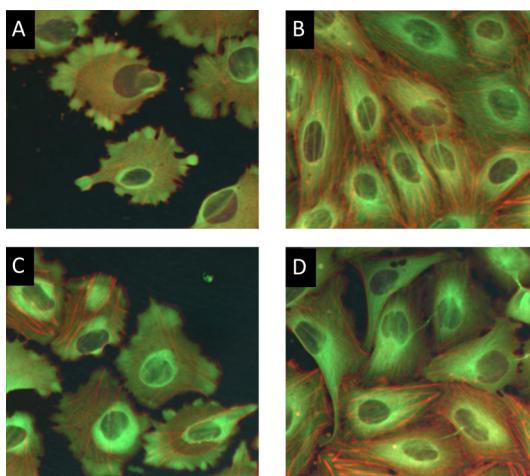


Figure 5. High-content images of U2OS cells where TUBA1B is tagged with green fluorescent protein after 8 h. Concentrations were chosen accordingly to select those pictures where the phenotype was most pronounced. (a) Nocodazole, an aneugenic MNT-positive compound, shows a particular phenotype that looks similar to the formation of microtubule aggregates (picture at 25 μ M). (b) DMSO controls do not show this phenotype. (c) Compound 8148, inducing tubulin downregulation, shows the microtubule aggregates (picture at 10 μ M). (d) Compound 0558, within the same chemotype as 8148 but not showing tubulin downregulation, has similar images as that for the DMSO control (picture at 30 nM).

quantify this phenotypic effect, a signature was defined consisting of a linear combination of three tubulin image features. This signature score discriminates the images showing cells with microtubule aggregates from images showing other phenotypes, with a positive score corresponding to the former and a negative score, the latter. The maximum signature score observed across all concentrations at time point 8 h, the same time point as that used for gene expression profiling, is plotted against the summarizing score²⁴ of the fold changes in the tubulin genes for the 11 compounds and the 5 reference aneugenic compounds (Figure 6). A positive score represents the presence of the microtubule aggregates. The reference compounds were transcriptionally profiled under similar conditions (HEK293 cells transfected with the mouse homologue of PDE10A at 8 h and 10 μ M). Compounds that show a downregulation of the tubulin genes (y-axis) have microtubule aggregates present in their images (x-axis). Among these compounds, the ones that were tested in the MNT show micronucleus formation, colored red in Figure 6. An overview of the results for the different assays, for the compounds tested, is given in Table 1. In the upper left corner of the plot are compounds that show neither tubulin downregulation nor microtubule aggregates, and they are negative in the MNT if tested. There are, however, a few exceptions observed, especially in the aneugenic reference compounds where no microtubule aggregates were observed. Here, it might be important to assess if the cells were exposed to high enough concentrations to induce the phenotypic changes; more specifically, we quantified the relative increase in cell count using image analysis over 24 h (Figure 8). This revealed that the compounds griseofulvin, 2858, and 7886 were probably not dosed high enough to draw conclusions regarding their toxicity. Only the aneugenic compounds vinblastine, colchicine, and taxotere remained for which no correlation among tubulin gene expression, microtubule aggregates, and micronucleus forma-

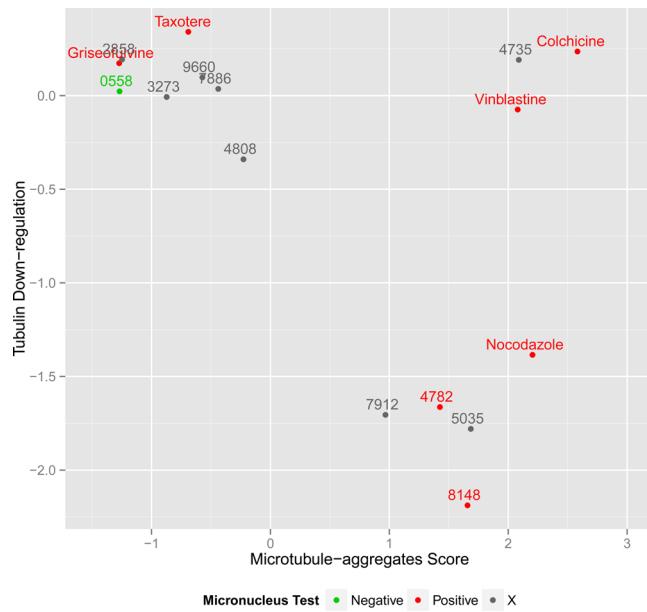


Figure 6. Scatterplot of the maximum microtubule aggregate score observed across all concentrations at time point 8 h versus summarizing score of the tubulin genes. Compounds indicated in red show micronuclei formation, as confirmed by MNT, whereas the compound in green does not show the formation of micronuclei. The gray compounds are not tested in MNT.

tion could be observed. Taxotere does not show an effect on tubulin gene expression levels or on tubulin aggregation. However, taxotere is a known aneugen, and there is some evidence in the literature that it induces tubulin downregulation, but it does so only after 36 h.⁴⁴ Furthermore, vinblastine, colchicine, and 4735 show tubulin aggregation, but they do not show downregulation of the tubulin genes at 8 h and 10 μ M. Therefore, we investigated the formation of microtubule aggregates for different concentrations at 8 h. In Figure 7, the signature score based on the preselected image

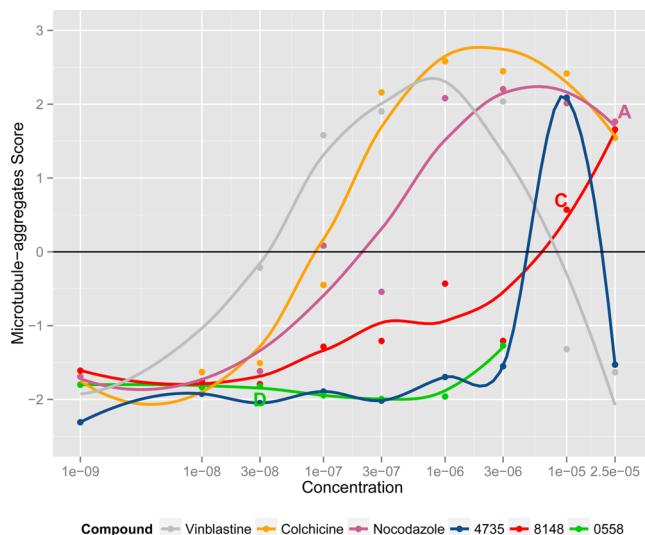


Figure 7. Microtubule aggregate score plotted as a function of concentration for multiple compounds (vinblastine, colchicine, nocodazole, 4735, 8148, and 0558) indicated with different colors. The concentration at which the image is shown in Figure 5 is indicated with the corresponding letter.

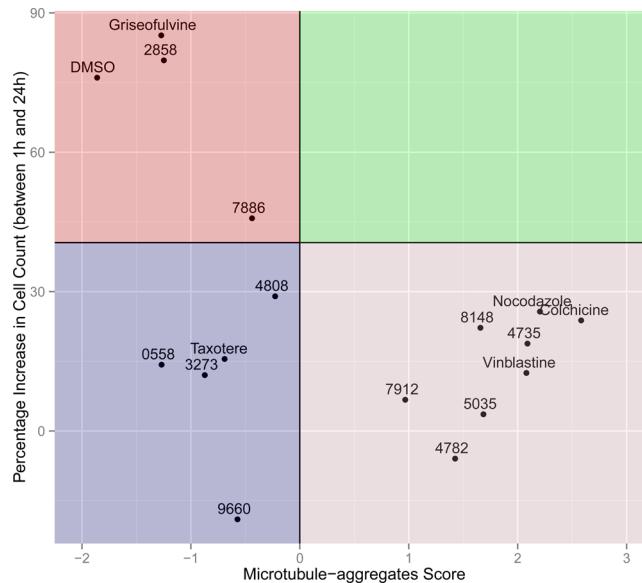


Figure 8. Maximum microtubule aggregate score observed across all concentrations at 8 h plotted as a function of the percentage increase in cell count over 23 h at the corresponding concentration. The horizontal line indicates the minimum increase in cell count over the replicates of the DMSO control, whereas the average increase in cell count is plotted for DMSO. Compounds at the right of the vertical line have a positive microtubule aggregate score.

features is plotted against the different concentrations tested in the imaging experiment. These concentration profile plots are shown for the compounds vinblastine, colchicine, and 4735 together with three compounds for which correlations between the different data sources were clear, namely, nocodazole, 8148, and 0558. Nocodazole and 8148 show a positive effect in the MNT, whereas 0558 does not. Nocodazole, colchicine, and 8148 start to form microtubule aggregates from a certain concentration onward, whereby their concentration determines their cytotoxicity: more cytotoxic compounds result in aggregate formation at lower concentrations. Compound 0558 does not show microtubule aggregates over the full range of concentrations tested. Compound 4735 shows aggregates for one concentration only (10 μ M), which is the same one as that used in the gene expression experiment. However, 4735 does not show aggregates at the highest concentration, and cell death did not occur. So, either there is a technical issue (only one plate well shows the aggregates) or we have a small window where the compound is genotoxic. Vinblastine shows a bell-shaped curve: microtubule aggregates start to form, but they disappear again since, at the highest concentrations, another phenotype, tubulin paracrystals,⁴⁵ is seen (see Supporting Information Figure S1). This means that at the second highest concentration, the concentration used in the gene expression experiment, no aggregates are present, which might correspond to the absence of tubulin downregulation in the gene expression experiment. Given the concentration profile plot, tubulin downregulation is expected for vinblastine at concentrations lower than 10 μ M.

Exploration of Aneugenic Reference Compounds in Connectivity Map. We investigated the aneugen reference compounds further using the Connectivity Map²⁵ approach (CMAP) in the search for other gene expression profiles at different concentrations. CMAP is a collection of genome-wide transcriptional expression data from cultured human cells

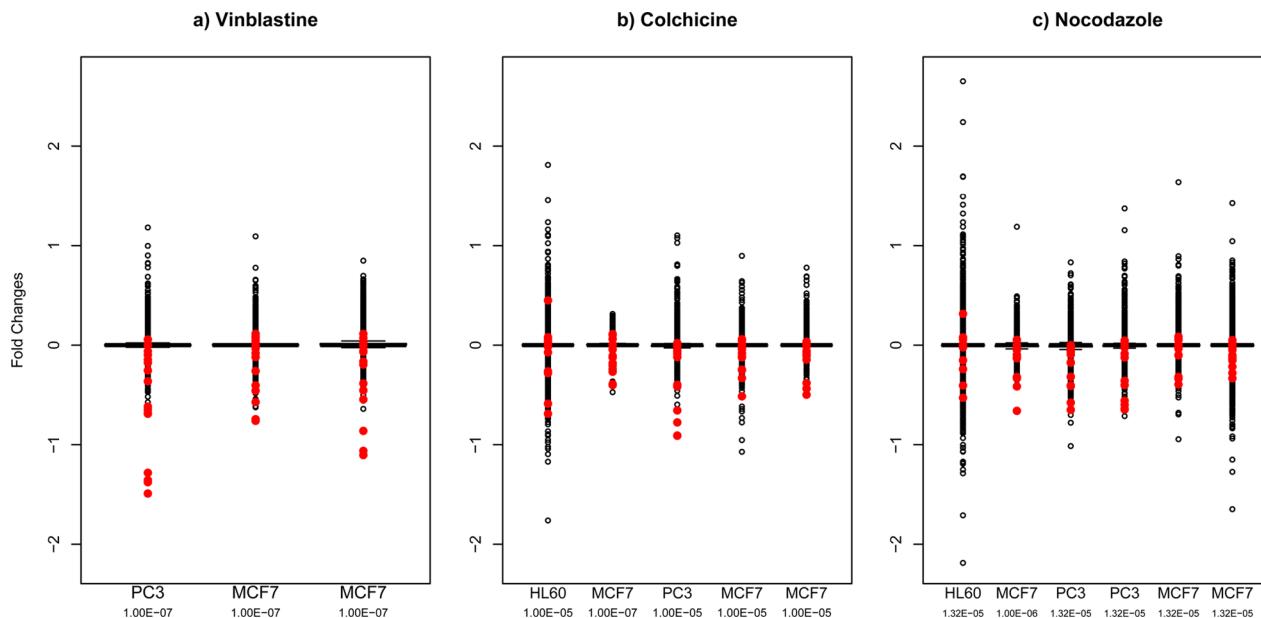


Figure 9. Exploration of aneugenic reference compounds in Connectivity Map (CMAP). Box plots are shown of the fold changes of all informative genes, where the tubulin genes are colored red. (a) Vinblastine is profiled in CMAP at 0.1 μ M in two different cell lines, PC3 and MCF7. (b) Colchicine is profiled in CMAP at 10 or 0.1 μ M in three different cell lines, HL60, PC3, and MCF7. (c) Nocodazole is profiled in CMAP at 1 or 13 μ M in three different cell lines, HL60, PC3, and MCF7.

treated with bioactive small molecules. Three of the five aneugenic reference compounds are profiled in CMAP, namely, colchicine, vinblastine, and nocodazole.

Vinblastine is profiled at 0.1 μ M at 6 h on two different cell lines, PC3 and MCF7. At this concentration, the tubulin genes are among the most downregulated genes in both cell lines (Figure 9a), which is consistent with the concentration profile plot, where, indeed, a positive score is found for this concentration (Figure 7). Colchicine is profiled at 10 μ M in cell lines PC3, MCF7, and HL60 at 6 h and at 0.1 μ M in MCF7 at 6 h. From the concentration profile plot of colchicine (Figure 7), a downregulation of tubulin genes is expected at 10 μ M at 6 h but not for 0.1 μ M. Investigation of the distribution of the fold changes and the position of the tubulin genes (Figure 9b) reveals that at 0.1 μ M no clear downregulation can be observed, whereas at 10 μ M, there is a clear downregulation in the MCF7 cell line (one technical replicate), but this is less pronounced in the other cell lines. A repetition of our own experiment showed a downregulation of tubulin genes for colchicine in the HEK293 cell line (Figure 11). Nocodazole is profiled at two concentrations, 1 and 13 μ M, at 6 h in cell lines PC3, MCF7, and HL60 (Figure 9c). In some cell lines, tubulin genes are among the most downregulated genes, as observed in our experiment as well.

Furthermore, CMAP is particularly built to search for compounds within the database with common gene expression changes to be able to discover the functionality of the gene expression signature. Hence, tubulin downregulation was defined as a signature and subsequently used to query the database using the method of Zhang and Gant,⁴⁶ which checks for compounds in the CMAP database having a similar ranking of the tubulin genes. The average rank of the informative tubulin genes from the profiling in the HEK293 cell lines across the four compounds was taken as a signature. The top-scoring CMAP compound with the most similar ranks for the tubulin genes is the aneugenic reference compound vinblastine, with a high score of 0.98.

Structure Activity–Relationship Analysis. Finally, structure–activity relationship (SAR) analysis was performed since only a few compounds within the same chemotype show strong tubulin downregulation, namely, those where Q2 (Figure 1) is a quinazoline and those where Q1 is either a quinoline or quinoxaline (Figure 10). The substitution pattern on these

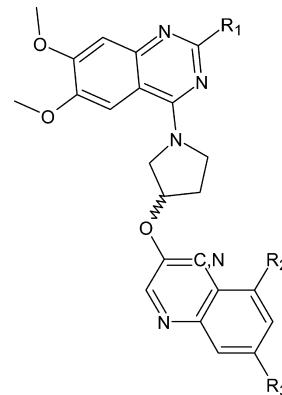


Figure 10. New compounds were synthesized in the quinazoline series, where we varied mainly position R₁. Substitution at R₂ and R₃ on the quinolone or quinoxalines reduces genotoxicity.

heteroaromatic ring systems is rather limited. Compounds with electron-donating groups as R₁ do not show tubulin downregulation. Additionally, substitutions in R₂ and R₃ are not allowed. On the basis of these observations, a set of 13 compounds with different R₁ groups, either electron-withdrawing or very small, both for the quinoline and the quinoxaline series was synthesized. It was hypothesized that these compounds have a high chance of being genotoxic. This set of compounds was transcriptionally profiled on HEK293 cell lines at 10 μ M together with the four compounds downregulating the tubulin signature, four negative compounds, and the reference compounds, vinblastine, colchicine

Gene Profiles

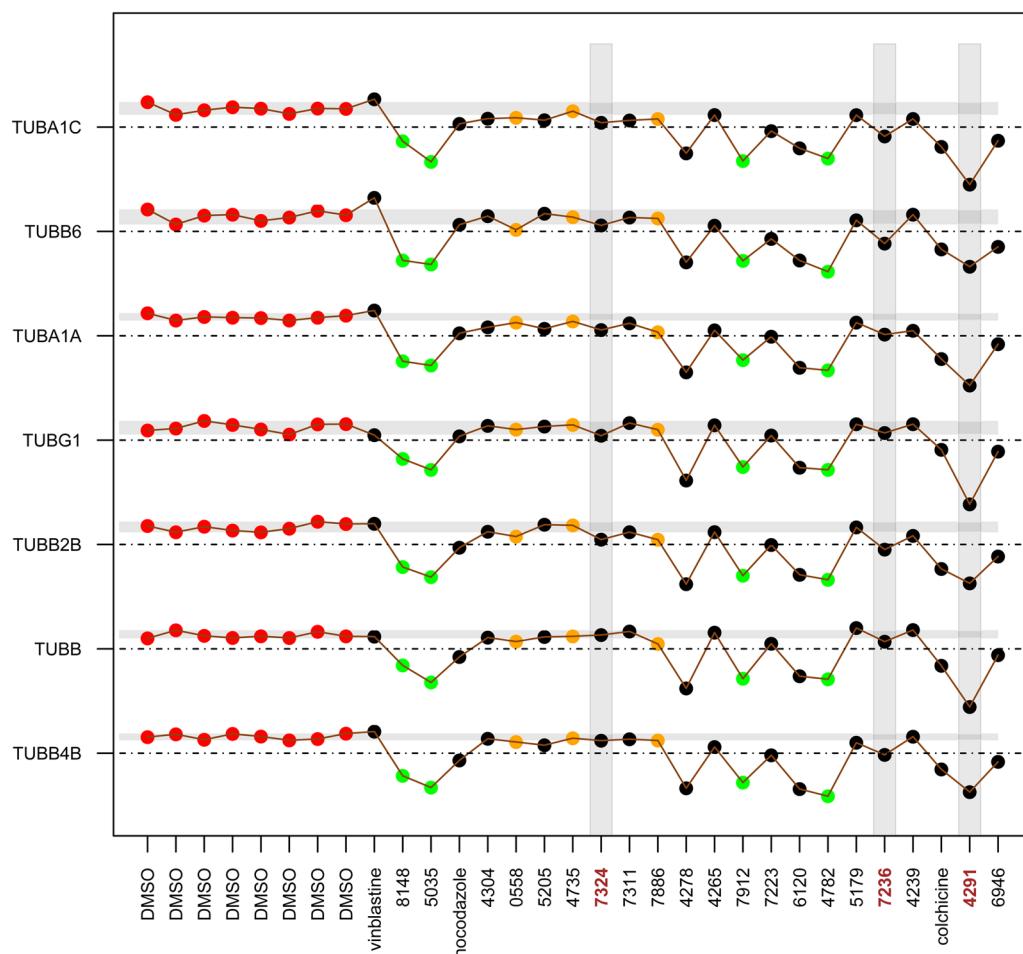


Figure 11. Gene expression profile plot of a transcription profiling experiment with the newly synthesized compounds, reference compounds (nocodazole, vinblastine, and colchicine), and some positives (colored green) and negatives (colored orange) from the earlier profiling experiment. DMSO is indicated in red, and its variation is shown with gray horizontal bars. Three compounds, with varying tubulin downregulating activity, were identified (vertical gray bars) and subsequently tested in MNT.

and nocodazole. Figure 11 shows the gene expression profile plot of the tubulin genes across the compounds. Both the positives and negatives of the earlier experiment could be confirmed. Vinblastine again shows no tubulin downregulation at this concentration, which was expected from the concentration profile plot, whereas nocodazole as well as colchicine show tubulin downregulation (Figure 7). From the set of newly synthesized compounds, three compounds (4291, 6120, and 4278) show a clear downregulation, whereas some others demonstrate a downregulation similar to that with the two reference compounds, nocodazole and colchicine. Hence, it is expected that from the set of synthesized compounds a fair amount again show genotoxicity. Three compounds (7324, 7236, and 4291) were tested in the MNT, indicated with gray bars in Figure 11. These compounds were chosen since they cover the full range of tubulin downregulation. Compound 7324 shows no tubulin downregulation, whereas compound 4291 shows the strongest downregulation. All three compounds show micronucleus formation; even compound 7324 does, which does not show downregulation of the tubulin genes at all. However, these compounds show clear differences in the concentration at which they reach 50% relative population

doubling (RPD), which is 2.71, 4.17, and 16.0 μM for 4291, 7236, and 7324, respectively. Again, the gene expression profiling at 10 μM is probably too low in concentration to reach the exposure levels at which significant gene expression changes and genotoxicity occur for compound 7324.

■ DISCUSSION

The drug discovery process in the pharmaceutical industry can be divided in four distinct phases: early discovery, late discovery, preclinical, and clinical phases. Despite extensive testing during early stages, too many new molecular entities (NME) are dropped from development during the preclinical and clinical stages, and many of those attritions are due to toxicity issues.^{47,2} Avoiding these attritions would save an enormous amount of time and resources.

Current practice at Janssen involves testing biological activities in *in vivo* and *in vitro* models during early drug discovery; however, toxicity testing is mainly performed at relatively late stages after moving the compound into preclinical development. Ideally, however, information on potential toxicities should be collected early during the drug development pipeline, preferably in very close contact with medicinal

chemists so that very early on the chemistry can be guided away from potential harmful structures and toward biologically active but safe structures. An approach incorporating extensive screening for biological activity, pharmacokinetics, pharmacodynamics, and toxicity early in development can guide the drug development process and potentially reduce the attrition of compounds that occurs late in development when extensive resources have already been spent. Given the recent advancements in technologies available for screening (e.g., high-content imaging, several omics technologies, chemogenomics, etc.), the wealth of human-relevant *in vitro* models (3D models, cocultures, organs-on-a-chip, etc.), the huge amount of mechanistic (biological and toxicological) and chemical information freely available through the Internet (e.g., CMAP, Toxcast, etc.), and the advances in computational capacities, the time is right to redirect the drug development paradigm toward new early screening approaches.

In this article, we presented a proof of concept for how to improve risk/safety assessment early in drug discovery by linking data from modern high-dimensional biology technologies such as high-content imaging and transcriptional profiling with early discovery of a compound's effects. The data described here are part of the QSTAR program, which has been published recently and in which more information can be found.²¹

For this study, we have utilized various data sources: existing data (CMAP), data that has been generated in an exploratory drug discovery setting, and data from qualified toxicity assays. It was not the intention of this study to introduce a complete platform but to describe the most important lessons learned so that it will become possible to utilize modern high-dimensional biology technologies as broad screening tools to flag potential toxicity issues early on during the drug discovery process. This is of relevance to the pharmaceutical industry, as transcriptional profiling and high-content profiling are done earlier in the drug discovery pipeline compared to toxicology profiling, thereby offering the potential to prevent failures of selected compounds in later stages. Using the information gained from these broad screens, medicinal chemistry can be guided to work toward less or even nontoxic chemotypes, and informed decisions can be made on how to develop (or drop) a chemical series.⁴⁸ In this context, it is important to note that in this study the effects of the compounds on tubulin downregulation have been observed across different cell lines *in vitro*. This indicates that the concept of toxicity screening can be implemented during early screens that are initially aimed at identifying biological activity in selected cell lines. This allows an economy of strategy in which information and data from *in vitro* screens can serve multiple purposes. However, it also indicates the need to involve the relevant scientists and (molecular) toxicologists during early discovery candidate selection scenarios and to allow them to regularly review data produced by discovery teams.³

In this article, we have shown how to combine the use of high-dimensional biology technologies (a gene expression-based tubulin signature and a high-content imaging-based microtubule aggregate signature) together with an established toxicity assay such as the *in vitro* micronucleus test (MNT). The *in vitro* MNT is part of the genotoxicity test battery in preclinical development⁴⁹ and is performed to identify clastogens and/or aneugens, compounds that induce structural (chromosome breaks) and numerical (spindle poisons) chromosome mutations, respectively. Microtubules are extremely important in the process of mitosis, during which the

duplicated chromosomes of a cell are separated into two identical sets before cleavage of the cell into two daughter cells. Binding to microtubules or interfering with microtubule dynamics causes mitotic arrest and cell death in different tumor cells.³⁹ We have explored active compounds in various cellular backgrounds as well as connected the signatures to a phenotypic end point via an experimentally independent imaging-based genotoxicity assay.

In this article, we have shown that the observed downregulation of tubulin gene expression is associated with micronucleus formation for several PDE10A compounds. However, we have not been able to show the involvement of tubulin downregulation in micronucleus formation for all compounds. Several compounds are known that interact with microtubules, and drugs that disrupt microtubule/tubulin dynamics are widely used in cancer chemotherapy.⁵⁰ There are several different binding regions of drugs within tubulin that are known. Taxanes, including paclitaxel, promote tubulin stabilization, thereby interfering with tubulin dynamics. Vinca alkaloids, including vinblastine, promote depolymerization of microtubules by preventing self-association of tubulin by interacting at the interface between two $\alpha\beta$ -tubulin heterodimers. Another group of microtubule-interfering agents is represented by colchicine, which induces microtubule depolymerization by introducing a steric clash between colchicine and α -tubulin, thereby inhibiting microtubule assembly. For colchicine, it is known that it increases the intracellular pool of tubulin monomers, which modulates the stability (triggers the degradation) of microtubulin mRNAs.⁵¹ Our data suggest that the PDE10A compounds that show tubulin mRNA downregulation may act according to a similar mechanism as that of colchicine.⁵² Vinblastine did not show tubulin mRNA downregulation, possibly because of the formation of paracrystals at the concentration at which it was tested in the gene expression analyses. However, Cleveland et al.⁵² also failed to show a reduction in tubulin synthesis for vinblastine, possibly indicating another working mechanism of this microtubule-depolymerizing compound.

Aneuploidy is recognized as a common hallmark of cancer cells, and it has been suggested to play a role in carcinogenesis, aberrant embryonic development, aging, and development of neurodegenerative disease.⁵³ The identification of an aneugenic mode of action is important, as it allows the application of threshold-based risk assessment to define a safety window and thus derisk flagged drug candidates.⁵⁴

In order to gain better insight into the underlying mechanisms when positive *in vitro* genotoxicity data are obtained, transcriptomic analyses can be helpful.⁵⁵ To improve the translation of an *in vitro* hazard into human risk assessment, toxicogenomic profiling allows a weight-of-evidence approach to be used.^{56–58} Typically, efforts such as these are integrated at later stages during development, after an alert for genotoxicity has been observed and the compound needs to be rescued. Ideally, this derisking strategy should be avoided where possible, preferably by incorporating toxicity screening early in discovery and moving only clean/safer compounds to later stages in the drug development pipeline.

Our findings demonstrate the complementarity of mechanistically agreeing data from two independent high-dimensional biology technologies that jointly create biological insights beyond the classification provided by the micronucleus assay. Further confirmation of the findings presented in this article could, therefore, result in the development of an early marker

for aggregate formation linked to compounds that downregulate the expression of tubulin genes, interfere with correct microtubule formation, and, as a consequence, result in aneuploidy. The tools that have been used in this article demonstrate the capacity of implementing this approach early in the pipeline to identify potential risk and to allow for a fail-early approach. However, the mechanistic information that has been gained also allows us to develop a mitigation strategy for further and timely investigative toxicology studies.

However, as these technologies are currently primarily used in a drug discovery setting, the experimenters are frequently not knowledgeable about aspects that are of relevance for the experimental design of toxicity studies. We hope that this article has demonstrated the applicability of data integration and the necessity to ensure that sufficiently high concentrations of a compound are tested. Compounds showing no toxicity-related effects can, indeed, be classified as negative for the experimental setting and do not need to be considered as false negatives due to too little compound exposure.

We have observed that it is possible to miss the flag for genotoxicity in these experiments if transcriptional and/or high-content profiling is done under only one condition. However, when detecting both microtubule aggregates as well as downregulation of tubulin gene expression, it leads to genotoxicity under these particular conditions. This is an important property for early screening in development.

We have shown the potential value of gene annotation for generating hypotheses regarding the underlying molecular mechanisms of toxicity by connecting the gene expression signature to an assay assessing the formation of micronuclei. However, in general, limitations exist in the interpretability of transcriptional signatures, as the current state of gene annotation does not always provide sufficient insights to formulate hypotheses on underlying mechanisms of toxicity. Care needs to be taken not to overinterpret gene expression data that is obtained, especially in simple *in vitro* models that are typically employed in early drug discovery settings. Gene expression data reflect several biochemical processes that are simultaneously occurring in culture, including cell division processes, potential on-target effects of a drug, and off-target effects, some of which may be associated with adverse outcomes at the cellular level. Overinterpretation of this myriad gene expression fluctuations would likely result in false positive findings. A more appropriate implementation would be to scan the gene expression profiles for flags that may be investigated further using other technologies (e.g., high-content imaging technologies) and trying to anchor them to established assays (e.g., the micronucleus assays in this article). In this article, we observed downregulation of several tubulin mRNAs, which aroused our curiosity for further investigation to identify the link to micronucleus formation. Other pathways that might warrant further investigations could be, for example, oxidative stress-related gene expression signatures, which might indicate the potential of a drug to generate ROS. Several compounds that generate ROS are known to be liver toxicants.²⁰ Therefore, if specific expression signatures can improve the prediction of drug-induced liver injuries (DILI), this could have a significant impact, as hepatotoxicity is one of the main causes of drug attrition and drug withdrawal.⁵⁹ As implementation of gene expression and other technologies will increase, additional pathways (of toxicity) and more robust interpretation of gene expression data may become feasible, thereby justifying their use in early drug discovery.

Similarly, high-content imaging features are typically not directly interpretable but, rather, require optimized experimental conditions in combination with reference treatments (such as compounds or RNA knockdown) that provide landmark signatures to relate observed phenotypic effects of compounds with unknown mechanisms of toxicity. Furthermore, not all liabilities can be captured via imaging or transcriptional profiling, and the use of a cell line cannot capture effects that emerge from mechanisms within a whole organ or body.

Still, identifying and exploiting gene expression signatures and implementation of novel and broad technologies like high-content imaging, combined with computational sciences and data mining, can be a suitable approach to reduce costly late-stage failures during clinical trials by impacting the ranking and selection of those compounds that are most suitable for further development. Current research aims to increase the throughput of gene expression profiling while reducing the cost and ease of use in the laboratory. For example, the L1000 platform⁶⁰ is currently used within the National Institutes of Health Library of Integrated Network-based Cellular Signatures (LINCS)⁶¹ program to generate hundreds of thousands of transcriptional profiles for thousands of perturbagens across many different cellular backgrounds. We believe that such initiatives represent a significant step toward aiding medicinal chemistry projects by pushing polypharmacological compound characterization of desired and undesired effects, as much as possible, to the front of the drug discovery process. We believe that by using these approaches the efficacy of the drug development pipeline can be increased and attrition rates can be lowered.

CONCLUSIONS

We discovered a transcriptional signature, consisting of downregulation of tubulin genes, that is predictive of a genotoxic effect (specifically, aneugenic). This shows that transcriptional data, generated during the drug discovery process, can be used to flag compounds for genotoxicity. However, since transcriptional profiling at these stages is often done at a single concentration, it is possible that there might be some false negatives where the signature was not observed although the compound might be genotoxic. Similarly, in the high-content imaging experiment, formation of microtubule aggregates can be predictive. Hence, both techniques can be used to check if a flag is present for genotoxicity. In the next step, we performed a SAR analysis and synthesized a new set of compounds that were expected to be genotoxic, and, indeed, the subset of compounds within one chemotype could be extended.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.chemrestox.5b00103](https://doi.org/10.1021/acs.chemrestox.5b00103).

Image of U2OS cells treated with 25 μ M vinblastine showing paracrystal formation ([PDF](#)).

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Notes

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ABBREVIATIONS

SAR, structure–activity relationship; R&D, research and development; QSTAR, quantitative structure–transcription assay relationships; PDE10A, phosphodiesterase 10; DMSO, dimethyl sulfoxide; MNT, micronucleus test; CMAP, Connectivity Map; LINCS, library of integrated network-based cellular signatures; NME, new molecular entity; RPD, relative population doubling

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