

Cancer Research Application Note No. 5

Improved Cellular Analysis for the Early Detection of Compound-Induced Hepatic Cytotoxicity to Reduce Animal Testing

Keywords: 3Rs concept, toxicology, hepatotoxicity, integrated workflow application, high-throughput cell analysis, biochemical assays, toxicogenomics, qRT-PCR, microarray.

1 Introduction

New innovative cell-based *in vitro* technology is required to fulfill the 3Rs concept of reducing, refining and replacing animal experimentation (1). Early identification of toxic compounds using *in vitro* assays in toxicology can reduce the number of laboratory animals used for *in vivo* testing of suboptimal compounds that ultimately fail at later stages of drug testing.

Most conventional cell-based *in vitro* assays for the assessment of cell viability and cytotoxicity are disruptive endpoint assays requiring cell lysis. A non-invasive and label-free way to continuously monitor cellular behavior can now be achieved using the xCELLigence System of Real-Time Cell Analyzers (RTCA) co-developed by Roche and ACEA Biosciences (2). xCELLigence RTCA SP, MP and DP Instruments measure electrical impedance generated when adherent cells contact the network of microelectrode sensors at the bottom of each well of the dedicated cell culture plate, E-Plate 16 (with 16 wells) and E-Plate 96 (with 96 wells). This technology allows long-term dynamic monitoring of cellular events, such as proliferation, cell death, adhesion, spreading and other morphological alterations, in real-time.

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Experimentally combining the continuous monitoring of cellular responses to a given treatment with cellular endpoint assays and other downstream applications, such as proteomics and toxicogenomics (3), is a powerful way to carry out high throughput predictive toxicological assessment, significantly bolstering the future role of *in vitro* testing.

In the present study, we used primary rat hepatocytes as our *in vitro* model for liver toxicity to test whether the xCELLigence System can be used in a well-established toxicology workflow. Compound-induced hepatotoxicity was continuously monitored using the xCELLigence RTCA MP Instrument (for six E-Plates 96 for a total of 576 wells), to produce continuous compound- and concentration-dependent cell impedance profiles, expressed as Cell Index values.

2 Materials and Methods

Hepatocyte isolation, cultivation and compound administration

Primary rat hepatocytes were isolated from 10 to 14 week old male HanBrl:WIST rats using a two-step collagenase (Roche) liver perfusion method, yielding approximately 2×10^8 cells per liver. Viability was assessed by trypan blue exclusion and ranged from 85% to 95%. Isolated cells were seeded into collagen-coated E-Plates 96 (Roche) or collagen-coated standard 96-well cell culture plates at a density of 1.5×10^4 cells/well and 2.0×10^4 cells/well, respectively.

Cells were plated in William's Medium E (WME), supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (0.1 mg/ml), insulin (100 nM), and dexamethasone (100 nM) and cultured at +37°C in a 5% CO₂ humidified atmosphere. After 4 hours, medium was replaced by FCS-free supplemented WME and cells were pre-cultured for 20 hours prior to compound treatment. Hepatocytes were treated with different concentrations of either tacrine, doxorubicin, or a novel kinase inhibitor (Roche Pharma), as well as the non-toxic compound 3-acetamidophenol (AMAP). For treatments, cell culture media were replaced with FCS-free WME working solution without insulin and dexamethasone, supplemented with the test compound.

Our findings show that continuous monitoring using the xCELLigence System provides detailed information about the cell culture conditions throughout the entire time course of the experiment, pinpointing appropriate times for compound treatments, and in parallel performance of downstream assays that address mechanistic questions. The comprehensive workflow used included:

- Continuous cell monitoring using the xCELLigence System.
- Functional biochemical endpoint assays to quantify cytotoxicity, cell viability, and cellular redox status.
- Gene expression profiling using NimbleGen microarrays and LightCycler® System's qRT-PCR.

This multifaceted workflow produced data that could be correlated, underscoring the added value achieved by the xCELLigence System in large-scale toxicology studies. Combining the xCELLigence System with well-established endpoint assays and gene expression profiling can, as shown here, significantly improve the quality of early drug and/or product safety evaluations.

Monitoring of cell growth using the RTCA MP Instrument

Cell growth behavior was continuously monitored for 72 hours using the xCELLigence RTCA MP Instrument (Roche). Background impedance was measured in 100 µl cell culture medium per well. The final volume was adjusted to 200 µl cell culture medium, including 1.5×10^4 hepatocytes per well. After plating, impedance was recorded in 15 min intervals. After compound administration, impedance was recorded in 2 min intervals for 6 hours, and 15 min intervals for the remaining time. All experiments were performed in triplicates. Cell Index (CI) values were normalized to the time point of compound administration (referred to as normalized CI).

Endpoint assays for the analysis of cytotoxicity, viability, and redox status

Biochemical endpoint assays were routinely performed 24 hours after compound administration using a multiplex approach in a 96-well format. Experiments were performed in triplicates. Acute cytotoxicity was determined by lactate dehydrogenase (LDH) release into the cell culture medium. LDH activity was determined spectrophotometrically using an ADVIA 1650 Autoanalyzer (Bayer Health Care). Enzyme activity in the media was expressed as the percentage of

total LDH activity present in the cells at the beginning of incubation. To assess the cellular redox status, intracellular reduced glutathione (GSH) levels were quantified using the fluorescent dye monochlorobimane (mClB). Hepatocytes were washed with Krebs-Henseleith-buffer (KHB). Background fluorescence was determined for KHB, using a Wallac Victor² multi-label counter (Perkin Elmer, exc=355 nm, em=460 nm). mClB was added to each well in a final concentration of 100 μ M and incubated for 15 minutes at +37°C. The mClB solution was replaced with KHB and fluorescence determined as described above. Cellular energy status was quantified using the ATP Bioluminescence Assay Kit HS II (Roche), which determines intracellular ATP levels. Cells were washed with prewarmed 0.9% NaCl, lysed, and processed according to the kit instructions. Luminescence measurement was performed using a Wallac Victor² multi-label counter (Perkin Elmer). ATP concentrations were calculated using a standard curve generated with ATP.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated 1, 6, and 24 hours after compound administration using the High Pure FFPE RNA Micro Kit (Roche) in combination with the MagNA Pure LC DNA Isolation Kit I Lysis/Binding Buffer (Roche). Cells were lysed using 100 μ l lysis buffer (75 μ l MagNA Pure LC DNA Isolation Kit I Lysis/Binding Buffer and 25 μ l ethanol) per well of an E-plate 96. Cell lysates were applied to High Pure FFPE RNA Micro Filter Tubes and processed according to the manufacturer's instructions. RNA amplification and cDNA synthesis was performed with 25 ng total RNA using the WT-Ovation Pico RNA Amplification System (NuGEN) according to the manufacturer's instructions. tRNA from brewer's yeast (Roche) was used as carrier. To remove excess nucleotides, cDNA was purified with the High Pure PCR Cleanup Kit (Roche), according to the protocol for the "removal of low molecular DNA". The quality and quantity of RNA and cDNA was assessed using the Agilent 2100 Bioanalyzer.

Microarray sample preparation and analysis

Microarray analysis of preamplified cDNA samples, was performed using NimbleGen Rat Gene Expression 12 x 135K arrays. Each slide contains 12 independent arrays, each with 135,000 probes covering 26,419 genes (5 probes/target gene). The cDNA (1 μ g) was Cy3-labeled according to the NimbleGen Arrays User's Guide for Gene Expression Analysis. After purification, Cy3-labeled cDNA was quantified using a NanoDrop spectrophotometer. Cy3-cDNA targets were hybridized to the arrays on NimbleGen Rat Gene Expression 12 x 135K slides, according to the recommended protocol. After washing, slides were scanned at 532 nm using the NimbleGen MS 200 Microarray Scanner at a 2 μ m resolution. Signals were extracted from scanned images using NimbleScan v2.5 software (Roche NimbleGen).

Data quality was assessed using signal uniformity, inter-array sample cross-contamination metrics, and reproducibility metrics. Quantile normalization was applied to arrays at 1, 6, and 24 hours after compound administration separately. The Robust Multichip Average (RMA) algorithm (4) was used to generate normalized gene expression values. Data analysis was performed using the Partek Genomics Suite (v6.5beta) and R software. Principal Components Analysis (PCA), and hierarchical agglomerative clustering using Euclidean distance and average linkage, were performed on samples at 1, 6, and 24 hours post-treatment. Statistical analysis using ANOVA models was performed to identify genes differentially expressed between treatment and control samples, for each compound/concentration combination at each time point. The ANOVA models including the factors time, compound/concentration combination and their interaction were built independently for each of the 26,419 genes present on the arrays, using log₂-transformed expression values. The significance of factor effects was tested using t-tests, and the analysis was performed using Partek software (v 6.5beta). Given the large number of statistical tests performed (one per gene), a step-up False Discovery rate (FDR) procedure was used for multiple test corrections. Genes with an FDR adjusted p-value less than 0.01, and an absolute fold change larger than 3, were identified as differentially expressed. Furthermore, the identified differentially expressed genes were used to calculate Gene Ontology (GO) enrichment scores against molecular function categories by applying Fisher Exact tests.

Real-Time qPCR and Data Analysis

PCR assays for the following genes were designed by the Universal ProbeLibrary Assay Design Center (Roche). Three reference genes (*Gapdh*, *Ppia*, *Tfrc*) and nine target genes (*Bcl2*, *Cyp1a1*, *Cyp3a18*, *Ddit1*, *Ddit3*, *Hmox1*, *Pcna*, *Serpib9*, *Slc2a1*) were evaluated. Assays were validated with rat cDNA, synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) from Rat Universal Reference Total RNA (ClonTech). The qRT-PCR was performed with

biological and technical duplicates using the LightCycler® 480 Probes Master (Roche), according to the manufacturer's instructions. The $\Delta\Delta C_p$ was calculated as the average sample C_p (C_p^{sample}), normalized to the average C_p^{ref} of 3 reference genes ($\Delta C_p = 2^{-\Delta(C_p^{\text{sample}} - C_p^{\text{ref}})}$) and calibrated to the gene expression level of the respective DMSO control sample ($\Delta\Delta C_p = \Delta C_p^{\text{sample}} / \Delta C_p^{\text{DMSO}}$).

Table 1: The list of validated rat PCR assays includes the respective Ensembl Gene ID, primer sequence, and probe number.

Gene	Ensembl Gene ID	Primer Sequence (5'-3')	Probe No.
Gapdh	ENSRNOT00000050443	left: aaagctgtggcgtgatgg right: ttcagctctgggatgacctt	26
Ppia	ENSRNOT00000009407	left: tgctggaccaaacacaaatg right: ctcccaagaccacatgct	42
Tfrc	ENSRNOT00000002407	left: gaggctcactgacatcatcaagca right: tccagcctcacgaggagtat	129
Bcl2	ENSRNOT00000003768	left: tagtcagaagcggcactgg right: aagactggatcattcgggaagaa	68
Cyp1a1	ENSRNOT00000026473	left: gtccttctcacagccaaag right: aaggcagaatgtggtagc	5
Cyp3a18	ENSRNOT00000001285	left: ttggttcaaccatttaagagaag right: tcagttaatgttgggtctacct	109
Ddit1/Gadd45a	ENSRNOT00000007698	left: agccaagctgctcaacgta right: cccggctgcatcttcat	40
Ddit3/Gadd153	ENSRNOT00000008941	left: caccacacctgaaagcagaa right: agctggacactgtctcaaagg	13
Hmox1	ENSRNOG00000014117	left: gtcagggtccagggaagg right: ctctccagggccgtataga	9
Pcna	ENSRNOT00000028887	left: tgaacttttcacaaaagccact right: tgcacctgtcagcaattta	94
Serpib9	ENSRNOT00000059949	left: gataatgacggggacctcag right: gtgttctcataaagctgggttg	64
Slc2a1	ENSRNOT00000047556	left: cccacagctctctgtgga right: tggagtctaagccgaacacc	105

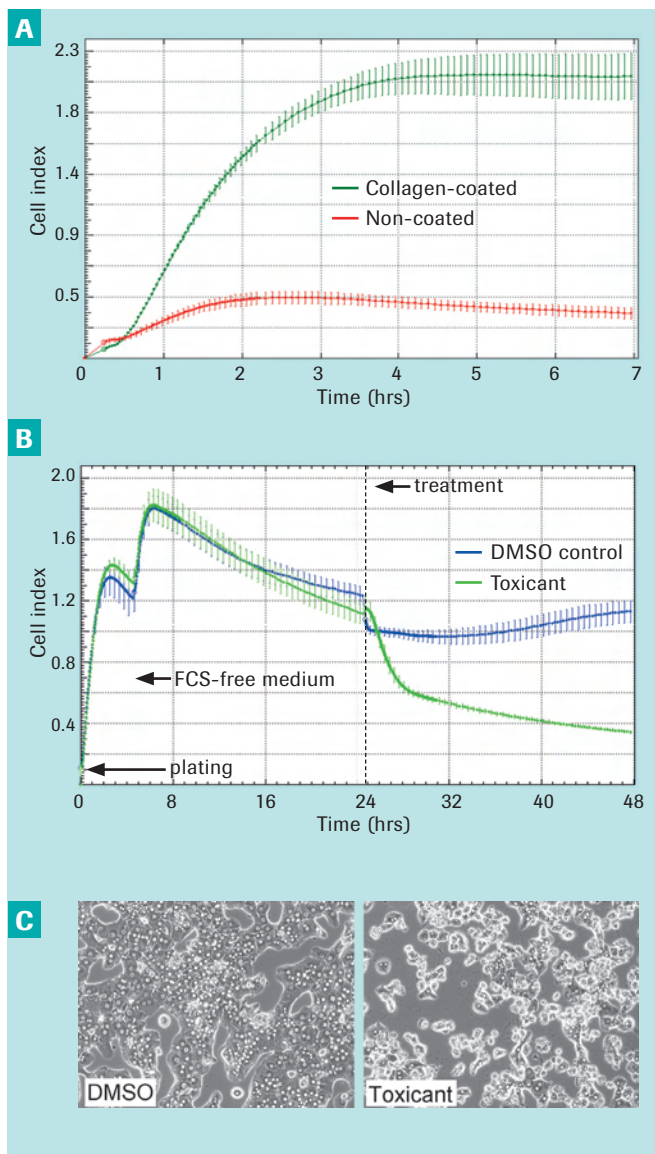
3 Results

Optimization of cell culture conditions

Primary rat hepatocytes were used as an *in vitro* model to assess liver-specific cytotoxic effects using the xCELLigence System. Different coating materials and cell densities were tested to identify the optimal cell culture conditions on the E-Plate 96 well format. Best results were obtained plating 1.5×10^4 hepatocytes on collagen-coated wells (see Figure 1A). As shown in Figure 1B, the Cell Index (CI) profile reflects the biological status of cultured cells throughout the course of the experiment. Cells attached during the first 4 hours of culture, reaching a CI value of 1.4. Cell culture media was replaced 4 hours post-plating, leading to a transient increase in the CI value. Cells were allowed to adapt to serum-free cell culture conditions during a pre-cultivation phase of 20 hours, reflected by a stabilization of the CI value. The administration of compounds at 24 hours post-plating caused a rapid decrease of CI values, whereas CI values of control cells stabilized at a value of approximately 1.0. The effects of the compound treatment on cell morphology are documented using light microscopy (see Figure 1C).

Figure 1: Optimal hepatocyte cell culture conditions on E-Plates 96.

- (A)** Plating of 1.5×10^4 primary hepatocytes/well was found to be the optimal cell density. Cells adhered best to collagen-coated plates.
- (B)** Experimental monitoring was done using the Cell Index (CI) profiles obtained using the xCELLigence System, measuring (i) cell adhesion, (ii) cell culture adaptation, and (iii) compound treatment. Addition of toxic compounds caused a rapid decrease in CI values, whereas control-treated cells showed unchanged CI levels.
- (C)** Photomicrographic images of treated hepatocytes showing morphological changes induced by the toxicant treatments.



Dynamic monitoring of drug-induced toxicity in liver cells

After cell culture optimization, the RTCA MP Instrument was used to analyze the hepatotoxic effects of doxorubicin, tacrine, and a kinase inhibitor. Primary rat hepatocytes were treated with different concentrations of the test compounds and CI values were recorded for at least 48 hours post-treatment (see Figure 2). Doxorubicin is an anthracycline antitumor drug which can also promote cytotoxicity in heart and liver (5). The acetylcholinesterase inhibitor tacrine has also been reported to induce hepatic injury in individuals (6). The widely used analgesic and antipyretic compound acetamidophenol (AMAP), a non-toxic regioisomer of acetaminophen, was used as control. Cell Index profiling revealed a characteristic cellular response to all tested compounds.

Doxorubicin, tacrine, and the kinase inhibitor induced large-scale cell death, as reflected by a dose-dependent decrease of CI values. Importantly, the xCELLigence System revealed the rapid effects on hepatocyte viability during the first 6 hours, by the early and steady decline of CI values. AMAP showed toxic effects only at very high doses (1 mM and higher). Interestingly, a very transient increase in CI values immediately after treatment could only be observed after tacrine administration, possibly reflecting a tacrine-specific cellular effect. Cytotoxicity was further quantified using the xCELLigence Software to calculate IC_{50} values for the time point 24 hours post-treatment, yielding a toxicity hierarchy of the compounds tested (doxorubicin > kinase inhibitor > tacrine > AMAP).

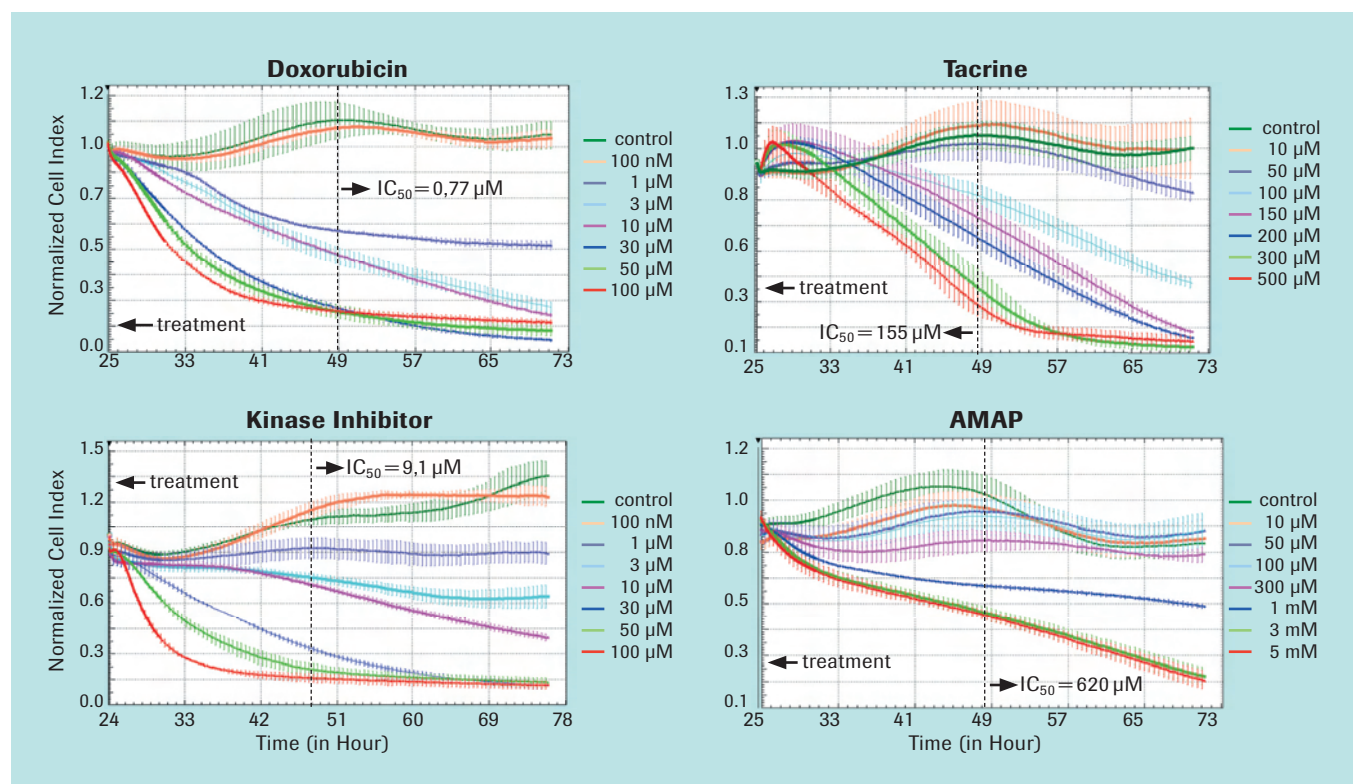


Figure 2: Compound specific impedance-based toxicity profiles.

Hepatotoxic effects of four compounds were continuously monitored using the xCELLigence System for 48 hours post-treatment. Cell Index (CI) values are normalized to the time point of compound administration, showing a dose- and time-dependent hepatotoxic effect, quantifiable

by IC_{50} values. Onset of cell death was detected at higher compound concentrations, as a rapid decline in CI values. Cell death was also detected at lower doses at later time points. Doxorubicin, tacrine and the kinase inhibitor produced hepatotoxicity, whereas the control substance, AMAP, caused cell death only at extremely high concentrations.

Biochemical endpoint assays

To gain further insight into the molecular mechanisms of compound-induced hepatotoxicity, xCELLigence System profiling and conventional biochemical assays were performed in parallel. LDH release, GSH depletion, and intracellular ATP content were assayed 24 hours post-treatment and compared to the corresponding CI values (see Figure 3). A clear increase in LDH release was observed in all test compounds except AMAP, indicating significant compound-induced cell damage in primary rat hepatocytes. Interestingly, the recorded CI values appear to show higher sensitivity when using the xCELLigence System compared to the LDH release assay, at 24 hours post-treatment. Similarly,

quantification of intracellular ATP and GSH levels revealed a compound-induced disruption of homeostasis. The reduction of energy and redox status, however, was only detected at higher doses of the tested compounds. Functional biochemical assays tended to reveal an all-or-nothing response, such as the ATP quantification in tacrine-treated cells, which did not allow the calculation of IC_{50} values. This restriction of some of the endpoint assays underscores the benefits of continuous impedance-based cell monitoring using the xCELLigence System, to pinpoint times for more detailed data analysis.

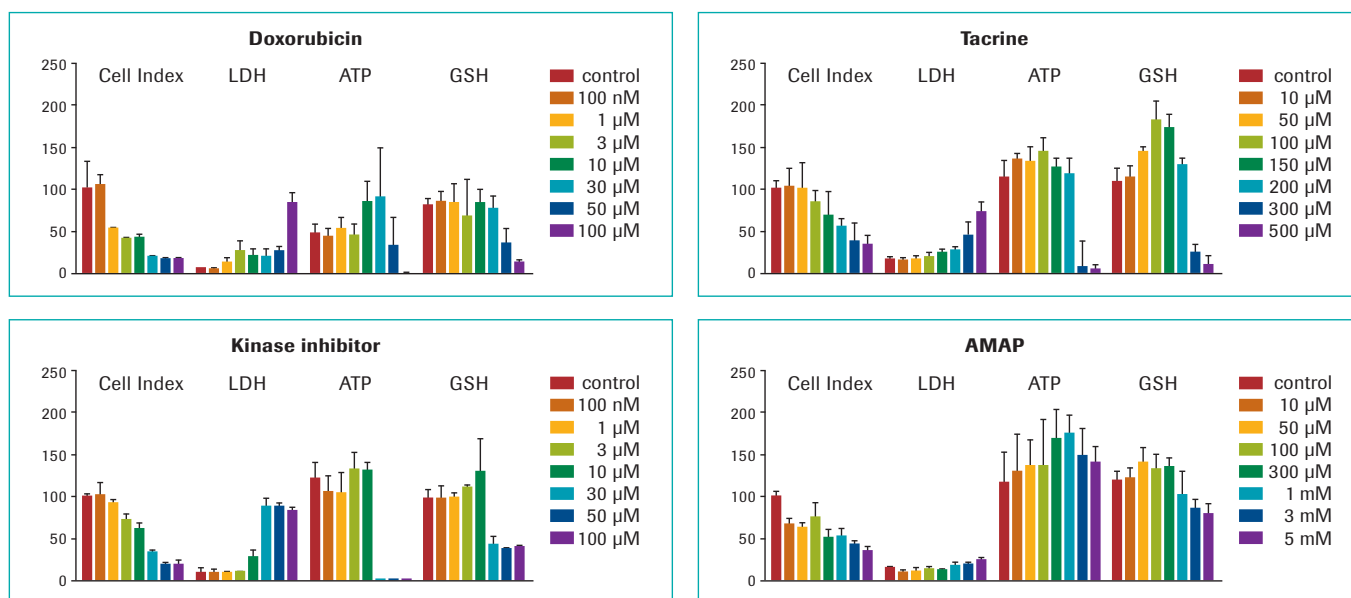


Figure 3: Biochemical endpoint analysis. Primary rat hepatocytes were cultured in conventional 96-well plates and treated with the indicated compounds in a concentration-dependent manner. Biochemical assays were performed 24 hours post-treatment. Results are from ATP, GSH, and LDH release assays, comparable to the CI values at 24 hours

post-treatment. In contrast to complete dose-dependent CI responses measured by the xCELLigence System, biochemical assays did not permit IC_{50} calculations at 24 hours post-treatment, underscoring the advantage of a continuous recording.

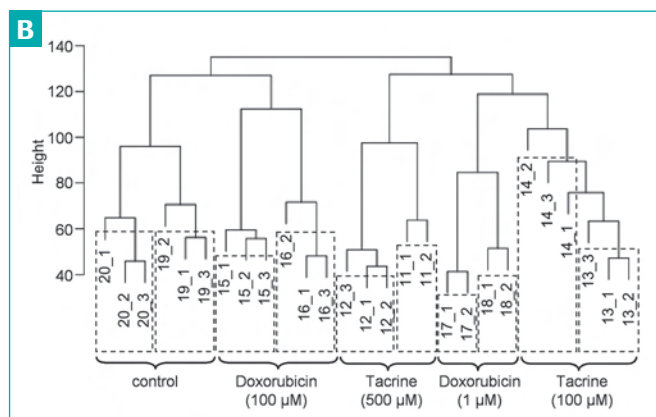
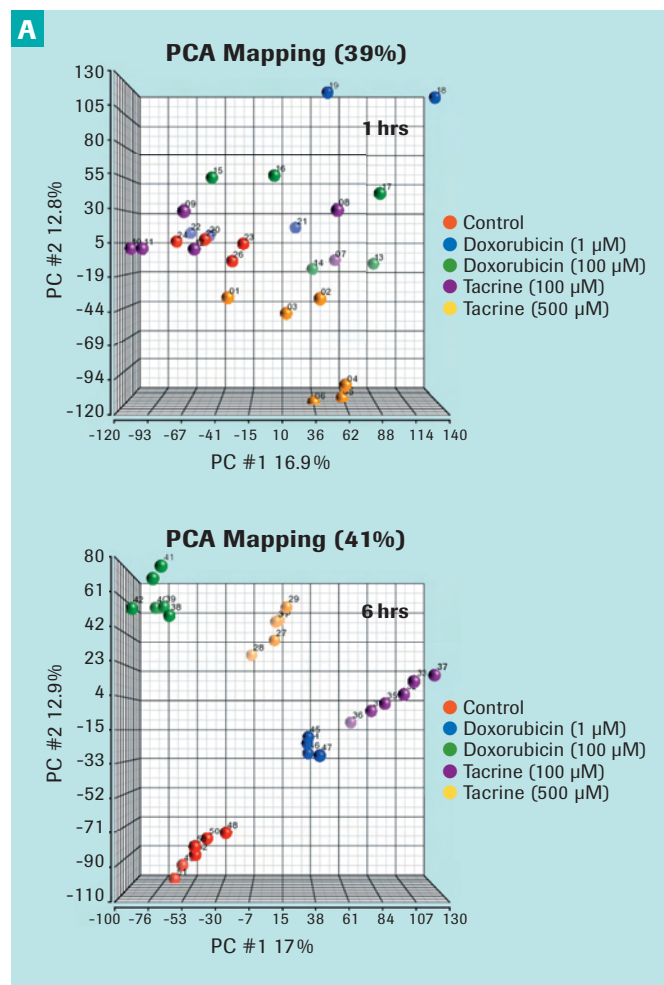
Gene expression analysis

Whole genome expression analysis was performed on cell cultures treated with doxorubicin (1 μM , 100 μM), tacrine (100 μM , 500 μM) and control-treated hepatocytes at 1, 6, and 24 hours after compound administration. Principle component analysis and hierarchical clustering revealed separation of data by sample type at 6 hours post-treatment, indicating that the samples are clearly distinguishable at that early time point (see Figure 4A and B). In contrast, at one hour post-treatment, clustering of biological replicates by sample type was not significant. ANOVA models were used to identify genes differentially expressed between treatment and control samples at 1, 6, and 24 hours post-treatment. Figure 4C shows the numbers of genes differentially expressed.

The number of identified differentially expressed (DE) genes increased with the duration of exposure to the compound. Several hundred DE genes were identified at the 6 h time point indicating that valuable biological information may be obtained by assaying cells after short durations of

compound exposure. Genes differentially expressed at 6 hours after treatment compared to control samples showed statistically significant GO enrichment scores for molecular functions involved in xenobiotic detoxification, such as transporter activity (enrichment scores of 4.5 and 5.2 for the 100 μM doxorubicin and 500 μM tacrine treatments, respectively), further underscoring the specificity of gene regulation at 6 hours post-treatment.

The expression levels of Bcl2, Cyp1a1, Cyp2c13, Ddit1, Ddit3, Hmox1, Pcna, Serpinb9, and Slc2a1 were further analyzed using qRT-PCR, for pre-amplified cDNA samples of tacrine (100 μM , 500 μM) and doxorubicin (1 μM , 100 μM) treated primary hepatocytes. Relative gene expression



Time	Sample	DE		
		(FC>3)	(FDR 1%)	(FC>3 + FDR<1%)
1 h	Doxorubicin (1 μM)	142	2195	122
	Doxorubicin (100 μM)	187	1879	141
	Tacrine (100 μM)	116	1160	91
	Tacrine (500 μM)	312	2307	260
6 h	Doxorubicin (1 μM)	984	4924	966
	Doxorubicin (100 μM)	746	5371	742
	Tacrine (100 μM)	1041	6982	1028
	Tacrine (500 μM)	3797	5672	1004
24 h	Doxorubicin (1 μM)	1337	6949	1308
	Tacrine (100 μM)	1597	7189	1583

Figure 4: Whole genome expression microarray analysis.

(A) PCA mapping of the cell cultures reveals a good correlation of the tested replicates at 6 hours, but not one hour post-treatment.

(B) Hierarchical clustering of the array data from samples 6 hours post-treatment shows a high correlation of replicates. Technical and biological replicates are highlighted by dashed boxes and braces, respectively.

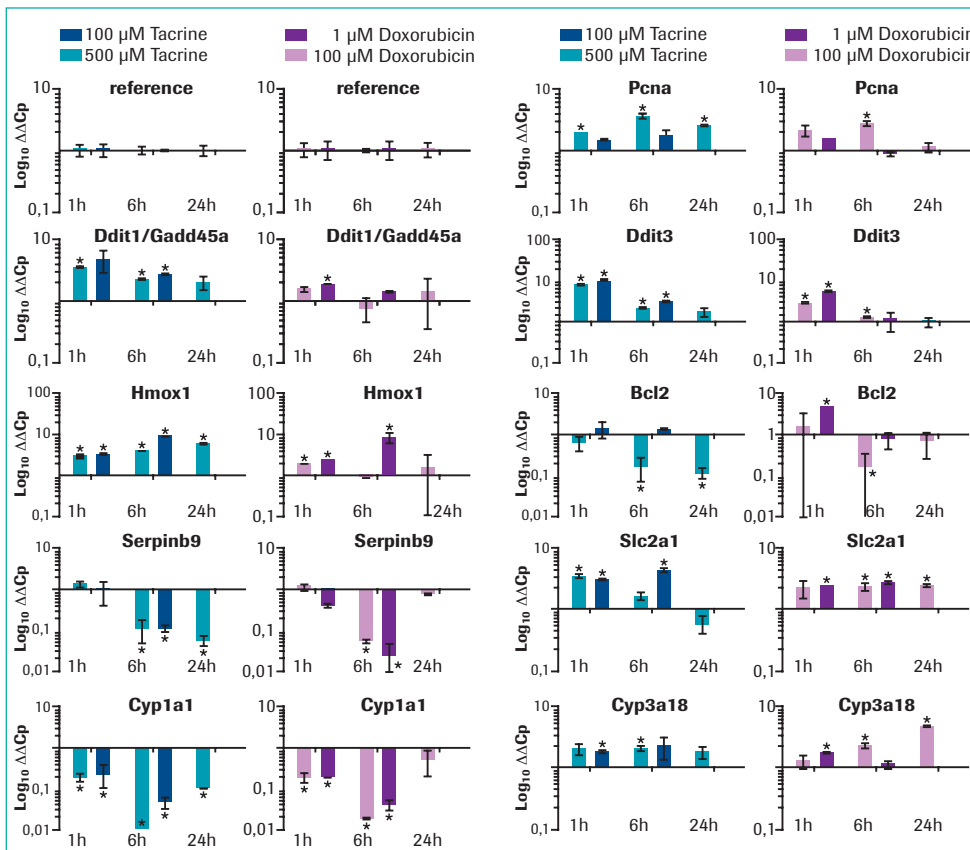
(C) Numbers of differentially expressed (DE) genes, compound-treated cell cultures compared to DMSO-treated control cultures, according to an FDR adjusted p-value of less than 0.01 and/or a fold change (FC) of more than 3 are listed.

levels ($\Delta\Delta\text{Cp}$) of the above nine target genes and the average of three different reference genes at 1, 6, and 24 hours post-treatment are summarized in Figure 5. As expected, the expression levels of the reference genes were unaffected by compound treatment and duration. The expression levels of the cell cycle regulators *Ddit1* and *Ddit3* were significantly upregulated one hour after compound administration, declined after 6 hours and reached control levels at 24 hours, indicating a rapid cellular response to induce cell cycle arrest.

Transcript levels of *Hmox1* and *Pcna*, both involved in DNA damage response and repair, were significantly elevated by tacrine and doxorubicin, peaking at 6 hours post-treatment. Interestingly, no significant change in *Hmox1* and *Pcna* expression was observed 24 hours after doxorubicin treatment, reflecting a transient stress response which is overcome when hepatocytes undergo large-scale cell death. Coinciding with the onset of cell death, the anti-apoptotic molecules *Bcl2* and *Serpib9* were strongly downregulated at 6 and 24 hours, most prominent in the tacrine-treated samples. Doxorubicin treatment, however, caused a down-regulation of *Bcl2* and *Serpib9* at 6 hours, whereas no effect could be observed after 24 hours.

The *Slc2a1* xenobiotic transporter was consistently up-regulated by doxorubicin from 1 to 24 hours, whereas tacrine caused *Slc2a1* upregulation only at 1 and 6 hours post-treatment, reflecting a cellular response to support xenobiotic detoxification by elevating transporter activity. Importantly, the cytochrome P450 family members *Cyp1a1* and *Cyp3a18*, which are known to be involved in drug metabolism, were inversely regulated. Whereas *Cyp1a1* was rapidly down-regulated from 1 to 24 hours post-treatment, *Cyp3a18* was significantly upregulated by doxorubicin and tacrine.

Figure 5: Gene expression analysis by qRT-PCR. Depicted is the relative gene expression ($\Delta\Delta\text{Cp}$) of 9 selected genes. Samples were harvested 1, 6 and 24 hours post-treatment with tacrine (100 μM and 500 μM) and doxorubicin (1 μM and 100 μM), and analyzed with Universal ProbeLibrary Assays on the LightCycler® 480 System. The $\Delta\Delta\text{Cp}$ reflects the relative gene expression level of the compound-treated cells compared to DMSO-treated control cells. As expected, gene expression levels of the reference genes were unaffected by compound treatment over time. Gene expression levels of all 9 selected genes were significantly changed dependent on compound, concentration and time. Importantly, most genes showed a strong change in expression by 6 hours post-treatment, the time point coinciding with the onset of cell death, identified by continuous monitoring using the xCELLigence System. Asterisk (*) indicates a significant difference compared to control, $P < 0.05$.



4 Discussion

The 3Rs concept was developed by W.M.S. Russell and R.L. Burch in their book “The Principles of Humane Experimental Technique” more than 50 years ago. The 3Rs elaborate ways to reduce and ultimately eliminate experiments on non-human vertebrates (1). In toxicology, cell-based *in vitro* assays are an essential tool for risk assessment to humans. Early identification of toxic compounds using *in vitro* assays could eventually reduce *in vivo* testing of compounds that go on to fail at a later stage of drug testing. In the present study, we describe a comprehensive workflow of *in vitro* techniques, including real-time cell analysis, biochemical assays, and gene expression analyses, for hepatotoxicity assessment. Compound-treated primary rat hepatocytes were continuously monitored using the xCELLigence System, revealing information in real-time about the status of the cells for the entire time course of the experiment. Optimal time points can be identified using this system for performing downstream proteomic and genomic analyses.

Hepatotoxic effects of the tested compounds were easily monitored using the xCELLigence System, to generate Cell Index (CI) profiles that quantitate the onset and progress of compound-induced cell death. Compared to routinely used biochemical endpoint assays, the xCELLigence System showed a higher sensitivity 24 hours post-treatment. Resulting kinetic profiles monitored toxicity from the beginning to end of the experiment. Similar detailed profiles are not possible using endpoint assays. Continuous CI recording quantifies cytotoxicity identifying the optimal

time point for IC₅₀ calculations. Interestingly, CI profiles revealed dose-dependent hepatocyte responses to the tested compounds as early as 6 hours post-treatment. This finding prompted us to investigate hepatotoxic effects by gene expression analysis at these early time points. Whole genome microarray analysis revealed a significant reproducible change in gene expression 6 hours post-treatment, coinciding with the onset of cell death found in the xCELLigence System data. Subsequent quantitative RT-PCR for a subset of selected genes revealed a significant change in gene expression 6 hours after compound administration, indicating early onset of gene regulation within the first hours post-treatment.

Taken together, these findings emphasize the added value of this comprehensive workflow: Real-time cell analysis, biochemical endpoint assays, and gene expression analyses. Continuous monitoring using the xCELLigence System easily identified modest cellular effects, providing a versatile way for pinpointing times for downstream proteomic and genomic analyses. Appropriately-timed molecular analysis using NimbleGen microarrays assures meaningful and reproducible whole genome expression data for identifying new target genes and possible biomarkers. Gene expression analyses using Universal ProbeLibrary Assays are also now available as pre-tested Real Time ready Assays. Combining the xCELLigence System with both well-established Roche endpoint assays and this new generation of Roche gene expression assays significantly improves the predictive quality of early safety evaluations that may go on to reduce current levels of animal testing in the future.

References

1. Russell, W. M. (1995) The development of the three Rs concept. *Altern Lab Anim* 23, 298-304
2. Xi, B., Yu, N., Wang, X., Xu, X., and Abassi, Y. A. (2008) The application of cell-based label-free technology in drug discovery. *Biotechnol J* 3, 484-495
3. Suter, L., Babiss, L. E., and Wheeldon, E. B. (2004) Toxicogenomics in predictive toxicology in drug development. *Chem Biol* 11, 161-171
4. Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U., and Speed, T. P. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4, 249-264
5. Carvalho, C., Santos, R. X., Cardoso, S., Correia, S., Oliveira, P. J., Santos, M. S., and Moreira, P. I. (2009) Doxorubicin: the good, the bad and the ugly effect. *Curr Med Chem* 16, 3267-3285
6. Lagadic-Gossmann, D., Rissel, M., Le Bot, M. A., and Guillouzo, A. (1998) Toxic effects of tacrine on primary hepatocytes and liver epithelial cells in culture. *Cell Biol Toxicol* 14, 361-373

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Ordering Information

Product	Catalog Number	Pack Size
Collagenase B	11 088 807 001	100 mg
	11 088 815 001	500 mg
	11 088 831 001	2.5 g
Collagen	11 179 179 001	30 mg
E-Plate	05 232 368 001	6 plates
	05 232 376 001	6x6 plates
RTCA MP Station	05 331 625 001	1 instrument
ATP Bioluminescence Assay Kit HS II	11 699 709 001	1,000 assays (microplate), 500 assays (tube)
High Pure FFPE RNA Micro Kit	04 823 125 001	Up to 50 isolations
MagNA Pure LC DNA Isolation Kit I Lysis/ Binding Buffer - Refill	03 246 752 001	100 ml
tRNA from brewer's yeast	10 109 517 001	100 mg
	10 109 525 001	500 mg
High Pure PCR Cleanup Micro Kit	04 983 955 001	Up to 50 purifications
	04 983 912 001	Up to 200 purifications
NimbleGen Rat Gene Expression 12x135K Array	05 543 827 001	1 slide
NimbleGen MS 200 Microarray Scanner	05 394 341 001	1 instrument
NimbleScan Software	05 933 315 001	1 - User license
Transcriptor First Strand cDNA Synthesis Kit	04 379 012 001	Up to 50 reactions incl. 10 control reactions
	04 896 866 001	Up to 100 reactions
	04 897 030 001	Up to 200 reactions
LightCycler® 480 Probes Master	04 707 494 001	5 x 1 ml
	04 887 301 001	10 x 5 ml
	04 902 343 001	1 x 50 ml

For detailed information on Universal ProbeLibrary Assays, please go to www.universalprobelibrary.com

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