Roche

Cellular Analysis Application Note No. 5

Nutlin-Induced Apoptosis Analyzed using the xCELLigence System and qPCR on RealTime ready Focus Panels

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1 Introduction

The p53 tumor suppressor plays a central role in the cellular defense against cancer. It controls a complex signaling network, the p53 pathway, which can halt cell proliferation and induce apoptosis in response to diverse oncogenic stresses. MDM2, a specific E3 ubiquitin ligase, tightly controls the cellular level of p53. MDM2 binds p53 in its N-terminal region and blocks the transcriptional activity of p53. However, the cellular level of MDM2 depends on the transcriptional activity of p53. MDM2 and p53 mutually regulate their cellular levels through a negative feedback loop. p53 is a powerful growth suppressive and pro-apoptotic molecule and represents an attractive target for pharmacological intervention. One of the main approaches is to activate wild-type p53 in cancer cells by blocking MDM2 from binding to p53. Recently, small-molecule inhibitors of the p53/MDM2 interaction, the nutlins, have been identified. They represent valuable tools for studying p53 regulation and leads for development of cancer therapeutics (Carvajal et al., Tovar et al.).

In the present study, we describe a model system of the cellular effects of nutlin 3a (active enantiomer) and nutlin 3b (inactive enantiomer) using continuous cell recording with the xCELLigence System and qRT-PCR analysis using the LightCycler® 480 Real-Time PCR System. In addition, we provide insight into nutlin-induced cellular events by various endpoint assays for cell viability, proliferation and apoptosis/necrosis. This workflow proved to be a powerful model system for cellular analysis, pinpointing the optimal time points for functional endpoint assays and qPCR.

SJSA1 osteosarcoma cells, expressing high levels of MDM2 protein due to amplification of the *mdm2* gene (Xia et al.), were selected as a model system. It has been shown previously that these cells are highly sensitive to the apoptotic activity of nutlin-induced p53, likely due to MDM2 overexpression (Vassilev at al., Tovar et al.).

Exponentially growing SJSA1 cells were treated either with nutlin 3a, nutlin 3b or DMSO, and cell growth was monitored using the xCELLigence System. Time points for RNA collection and endpoint assays were selected according to the Cell Index (CI) profiles recorded using the xCELLigence System. Subsequently, high quality RNA was purified and cDNA was synthesized. The expression levels of 372 apoptosis related genes and 84 cell cycle related genes were compared using the LightCycler® 480 Instrument in combination with the RealTime ready Human Apoptosis Panel, 96 and 384 and the RealTime ready Human Cell Cycle Regulation Panel, 96.

Western blot analyses were carried out to demonstrate the presence of p53, p21, and MDM2 proteins.

2 Methods and Material

Cell Cultivation and Compound Treatment

The SJSA1 cell line (ATCC) was cultured in RPMI medium (RPMI, 10% FCS, 2 mM L-glutamine) without antibiotics.

For RNA isolation and generation of protein extracts, 6 x 10^5 cells were cultivated in T25 cell culture bottles. For analyses using the Cell Proliferation Reagent WST-1 (Roche), the Cell Proliferation ELISA, BrdU (chemiluminescent) (Roche), the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche), and Cell Death Detection ELISA^{PLUS} (Roche), cells were cultured in regular microtiter plates with 5000 cells/well. All cell cultures were incubated at +37°C and with constant supply of 5% CO_2.

For cell treatment, the compound nutlin 3a was added to a final concentration of 10 µM. To control cells, nutlin 3b, the inactive enantiomer of nutlin 3a, was added to a final concentration of 10 µM. Both compounds were dissolved in DMSO at a concentration of 10 mM. This results in a final DMSO concentration in the medium of 0.1%. A second set of control cells was treated with DMSO to a final concentration of 0.1%, and a third set of control cells was treated with medium only. All experiments were performed in triplicates.

Monitoring of cell growth with the RTCA MP Instrument

Cell growth was monitored in real time, using the RTCA MP Instrument (Roche). Experiments were done in triplicates. Intervals for data collection were 15 minutes prior to treatment and within the first six hours after treatment and one hour for the remaining experiment. For the monitoring of cell growth using the RTCA MP Instrument, 5000 cells/well were grown in E-Plates 96 (Roche).

Endpoint Assays for the Analysis of Cell Viability, Cell Proliferation, Cytotoxicity and Apoptosis

1. Cell Proliferation Reagent WST-1

For the analysis of cell viability, the Cell Proliferation Reagent WST-1 (Roche) was used following the manufacturer's protocol; 10 µl/well of the Cell Proliferation Reagent WST-1 were added at defined time points and incubated for one hour before absorption readout was performed at 450nm, using a reference wavelength of 600nm.

2. Cell Proliferation ELISA, BrdU (chemiluminescent)

Cell proliferation was quantified by BrdU uptake during active DNA synthesis using Cell Proliferation ELISA, BrdU (chemiluminescent) Kit (Roche). BrdU was added to a final concentration of 10 µM. BrdU incorporation for two hours, was followed by cell fixation and signal detection, according the manufacturer's protocol.

3. Cytotoxicity Detection Kit^{PLUS} (LDH)

To quantify cell lysis, the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche) was used following the manufacturer's protocol. For recommended high and low controls, two sets of control cells were analyzed. One of them was treated (high control) with supplied lysis buffer, the other remained untreated (low control). Absorption readout was performed at 490nm, using a reference wavelength of 600nm.

4. Cell Death Detection ELISA^{PLUS}

To identify apoptotic and necrotic cell death, the Cell Death Detection ELISA^{PLUS} (Roche) was used following the manufacturer's protocol. Absorption readout was performed at 405nm, using a reference wavelength of 490nm.

RNA Isolation and cDNA Synthesis

Samples for RNA isolation were collected after defined time points. Total cellular RNA was isolated using the High Pure RNA Isolation Kit (Roche). Cells were washed once with cold PBS before they were lysed by addition of lysis buffer directly to the bottom of the cell culture bottle. The total content of one cell culture bottle was then further processed on one spin column per sample, following the manufacturer's protocol.

The quality and quantity of RNA was determined using the NanoDrop Instrument and the Agilent Bioanalyzer. cDNA was synthesized using the LightCycler® RNA Pre-Amplification Kit (Roche) according to the manufacturer's recommendations.

Real-Time qPCR

cDNAs were first tested using a housekeeping gene (ß2M) to standardize amounts used for the RealTime ready Focus Panels.

RealTime ready Human Apoptosis Panels, 384 (Roche) were analyzed using the Light Cycler® 480 Probes Master (Roche). cDNAs were diluted according to the factor determined using the ß2M assay with a final reaction volume per well of 10 µl.

RealTime ready Human Cell Cycle Regulation Panels, 96 (Roche) and RealTime ready Human Apoptosis Panels, 96 (Roche), were analyzed as above in a final reaction volume of 20 µl.

LightCycler® 480 Software, Version 1.5 and the Roche provided macro for each panel, enabled easy sample setup and data analysis.

Individual qPCR Assays

Individual qPCR assays for p53, p21 and MDM2 were designed using Universal ProbeLibrary (UPL) ProbeFinder Software for PCR primers and matching UPL probes.

Immunoblotting

Samples for western blot analysis were collected at defined time points, 1, 2, 4, 8, 24 and 48 hours after treatment. Medium was aspirated and adherent cells were lysed with 250 µl cOmplete Lysis Buffer-M (Roche) directly added into the cell culture bottle. Total protein concentration was evaluated for each sample using the Quick Start Bradford Dye Reagent, 1x (BioRad); 5 µg of total protein were separated by SDS-PAGE using 4–12% Tris Glycin Gels (Invitrogen). After blotting onto PVDF Western Blotting Membranes (Roche), the following primary antibodies were applied: p53 (sc-263) and MDM2 (sc-965) from Santa Cruz Biotechnology, p21 (OP64) from Calbiochem, and beta-actin (AC-74) from Sigma.

For the detection of the primary antibodies, the Lumi-Light^{PLUS} Western Blotting Kit (Mouse/Rabbit) (Roche) was used following the manufacturer's protocol.

3 Results

1. Analysis of Cell Growth using the RTCA MP Instrument
The xCELLigence System enables continuous online monitoring of cell growth. We used the RTCA MP Instrument to record Cell Index (CI) profiles of the SJSA1 cell line (see Figure 2).

The compounds, the solvent alone, or medium only were added at the time recorded CI values reached one third of the maximum CI, approximately 20 hours after seeding, when cells were in early logarithmic growth phase.

Changes in the CI values were recorded already within the first 6 hours with nutlin 3a treated SJSA1 cells. A large decrease in CI was observed, reaching zero approximately 48 hours after treatment, when cells had detached from the microelectrode surface.

All control cells, whether treated with the inactive enantiomer nutlin 3b, DMSO or medium, showed nearly identical CI profiles until the end of data collection, indicating similar growth behavior.

Different curves for nutlin 3a and nutlin 3b immediately after compound addition may reflect receptor and compound transportation events and need to be further evaluated.

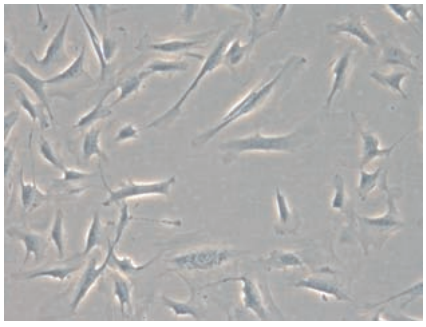

Figure 1: Light microscopic photomicrograph showing the SJSA1 osteosarcoma cells (400x magnification).

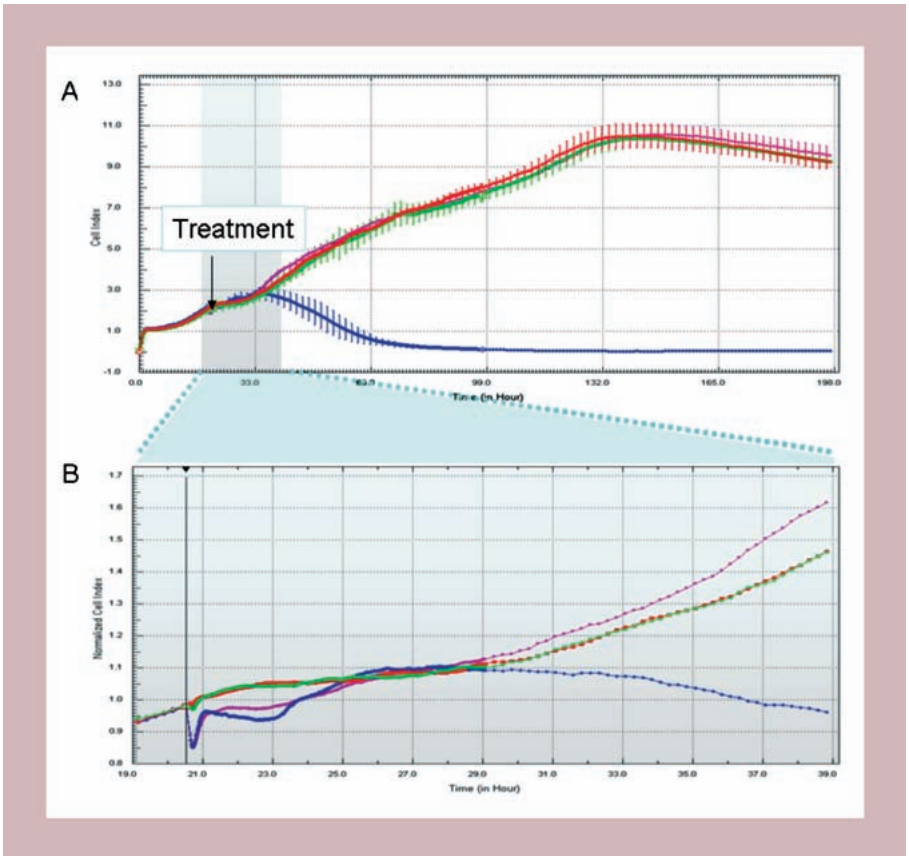

Figure 2: Continuous monitoring of cellular response using the xCELLigence System.
A) CI profiles were recorded over 200 hours.
B) A magnified view depicting only the early phase after treatment, indicates the early cellular response.
CI values were normalized to the time point of compound addition, indicated by the vertical black line. The effect of the different experiments in triplicate is shown for nutlin 3a (blue line), nutlin 3b (pink line), DMSO control (green line), medium control (red line).

2. Cell Viability

Cell viability was analyzed using the Cell Proliferation Reagent WST-1 (Roche), measuring metabolic activity. In cells with normal metabolism, a tetrazolium salt is converted to formazan, measurable by change in absorbance.

In SJSA1 cells treated with nutlin 3a, metabolic activity decreased between 8 hours and 24 hours after treatment. There was a significant decrease after 24 hours, with formazan becoming undetectable 72 hours after treatment (see Figure 3). Both the DMSO and nutlin 3b experiments showed no effect on SJSA1 cell viability and metabolism.

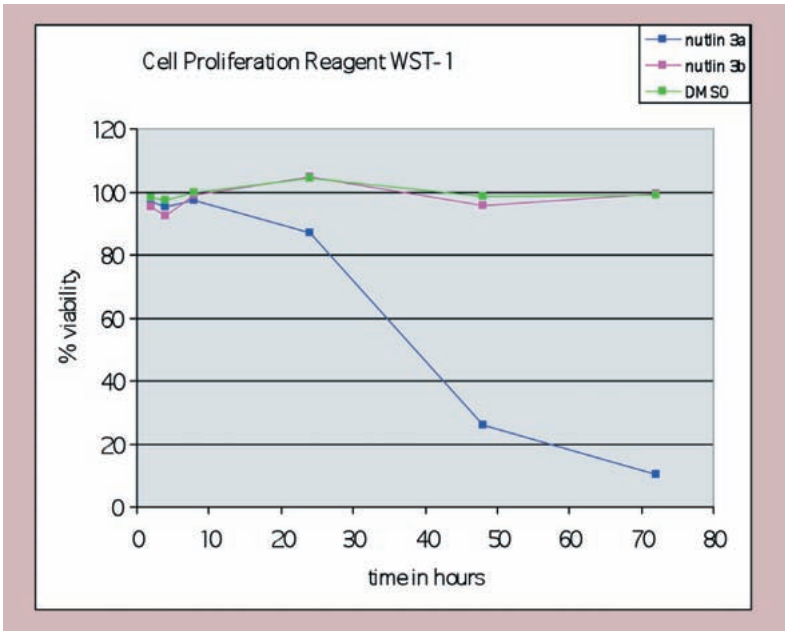

Figure 3: Nutlin 3a treated SJSA1 cells show a dramatic decrease in cell viability. Minimal metabolic activity was detectable 72 hours after treatment. In contrast, no significant change was found in cells exposed to nutlin 3b or DMSO. The effect of different experiments in triplicates is shown for nutlin 3a (blue line), nutlin 3b (pink line), DMSO control (green line).

3. Cell Proliferation ELISA, BrdU (chemiluminescent)

Cell proliferation was analyzed by measuring DNA synthesis using the Cell Proliferation ELISA, BrdU (chemiluminescent) Kit (Roche). The incorporation of the nucleotide analog BrdU is detected by ELISA using an anti-BrdU-POD antibody and the chemiluminescent substrate luminol.

After SJSA1 cells were treated with nutlin 3a, no significant changes were detected in DNA synthesis during the first 8 to 24 hours. After that, cell proliferation was sharply reduced in comparison to control cells. In contrast, all control cells proliferated to nearly the same extend (see Figure 4).

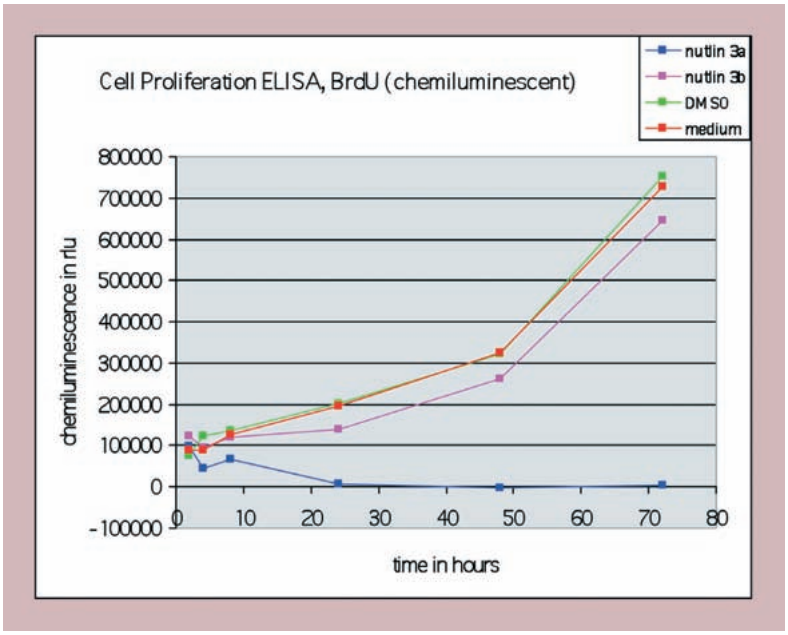

Figure 4: Cell proliferation was decreased after nutlin 3a treatment. In contrast, control cells proliferated to nearly the same extent after treatment. The effect of different experiments in triplicates is shown for nutlin 3a (blue line), nutlin 3b (pink line), DMSO control (green line), medium control (red line).

4. Cytotoxicity Assay

The cytotoxicity of nutlin 3a and nutlin 3b was analyzed using the Cytotoxicity Detection Kit^{PLUS} (LDH) (Roche). This assay detects dead or plasma membrane-damaged cells, using LDH enzyme activity in the culture supernatant. A significant increase in LDH was measured after 48 hours exposure to nutlin 3a, indicating cells are dying and cell lysis is ongoing. This process continues after 72 hours of nutlin 3a treatment (see Figure 5).
No significant changes were detected in the presence of nutlin 3b and the control cells, indicating cells were functional and viable.

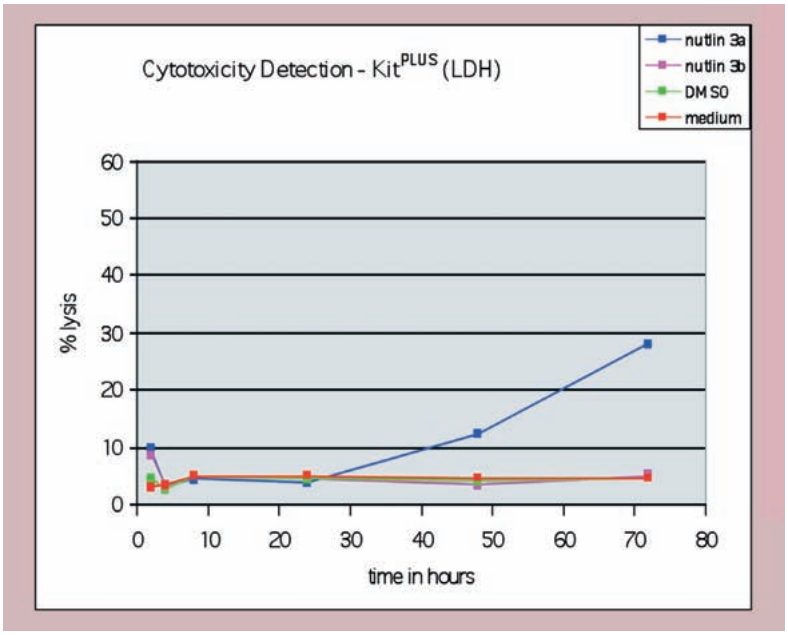

Figure 5: SJSA1 cells treated with nutlin 3a show an increase in LDH in the cell supernatant, indicating ongoing cell death. There was no indication of cell lysis in nutlin 3b treated, DMSO and medium-only control cells. The effect of different experiments in triplicates is shown for nutlin 3a (blue line), nutlin 3b (pink line), DMSO control (green line), medium control (red line).

5. Analysis of Apoptotic and Necrotic Cells

To discriminate apoptotic and necrotic cell death, the Cell Death Detection ELISA^{PLUS} (Roche) was used. Necrosis is accompanied by increased ion permeability of the plasma membrane, and is detected in the supernatant. Apoptosis is characterized by membrane blebbing (zeiosis), condensation of cytoplasm, and the activation of an endogenous endo-nuclease creating cytoplasmic histone-associated DNA fragments, detectable in the cell pellet. Nutlin 3a, but not nutlin 3b, was found to significantly increase the fraction of apoptotic cells in SJSA1 cells (see Figure 6).

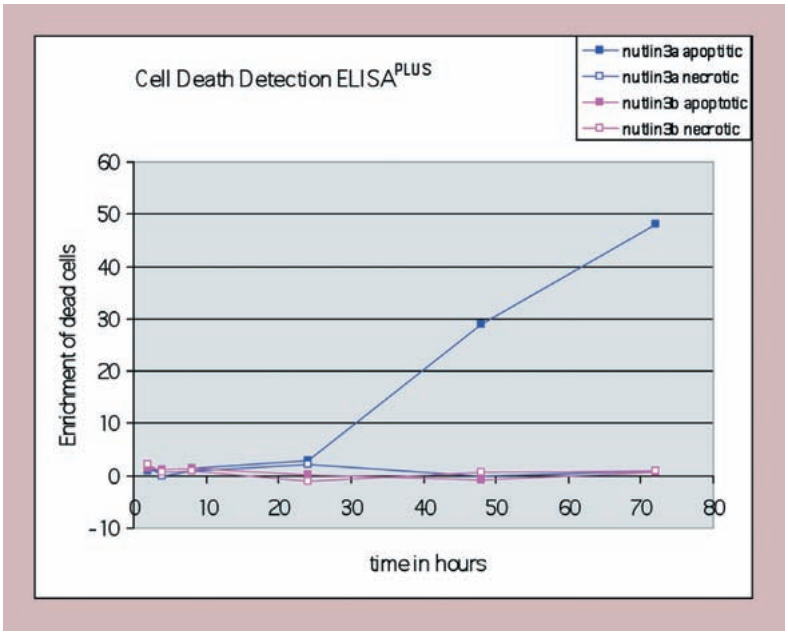

Figure 6: Nutlin 3a treated SJAS1 cells show a sharp increase in the number of apoptotic cells. The enrichment factor represents the fold increase of the number of dead cells relative to DMSO control cells. No necrotic cells were detected in either control or nutlin 3a treated cells. The effect of different experiments in triplicates is shown for nutlin 3a (blue line) and nutlin 3b (pink line).

6. Western Blot Analysis

Total protein extracts were collected 24 hours after treatment. The level of p21, p53 and MDM2 proteins was determined using western blotting. Compared to the controls, nutlin 3a led to a significant increase of p21, p53 and MDM2 protein expression (see Figure 7). Actin was used as a control for equal loading.

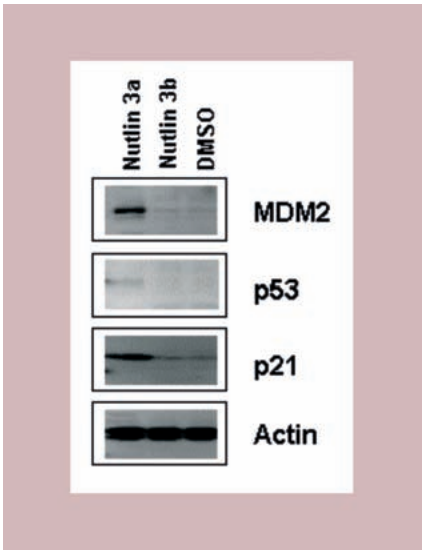

Figure 7: Nutlin 3a treatment elevates cellular levels of p21, p53 and MDM2 protein compared to the control cells; 5 µg total protein extracts were loaded per lane and analyzed with specific antibodies. Anti-actin antibody was used as control for equal loading.

To further investigate the increase in cellular levels of p21, p53 and MDM2 protein, western blot analysis was done 1, 2, 4, 8 and 24 hours after treatment (see Figure 8).

Figure 8: Kinetics of p21, p53 and MDM2 protein level changes in the presence of 10 µM nutlin 3a; 5 µg total protein extracts were loaded per lane and analyzed with specific antibodies. Anti-actin antibody was used as a control for equal loading.

7. qRT-PCR Analysis

RNA quality is crucial for reliable qRT-PCR analysis. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche). RNA integrity was confirmed using the Agilent Bioanalyzer. Samples showed high RNA Integrity Number (RIN) values between 7 and 9, indicating high RNA quality for qPCR. Total RNA was isolated 6 and 24 hours after treatment.

Quality Control of the cDNA Synthesis

cDNAs were synthesized using the LightCycler® RNA Pre-Amplification Kit (Roche). cDNA quality was analyzed using qPCR using the ß2M housekeeping gene as reference gene. All cDNAs showed high quality and were used for qPCR assays and with the RealTime ready Focus Panels (see Figure 9). Data created were also used to standardize the cDNA concentration for use with the panels to enable comparison.

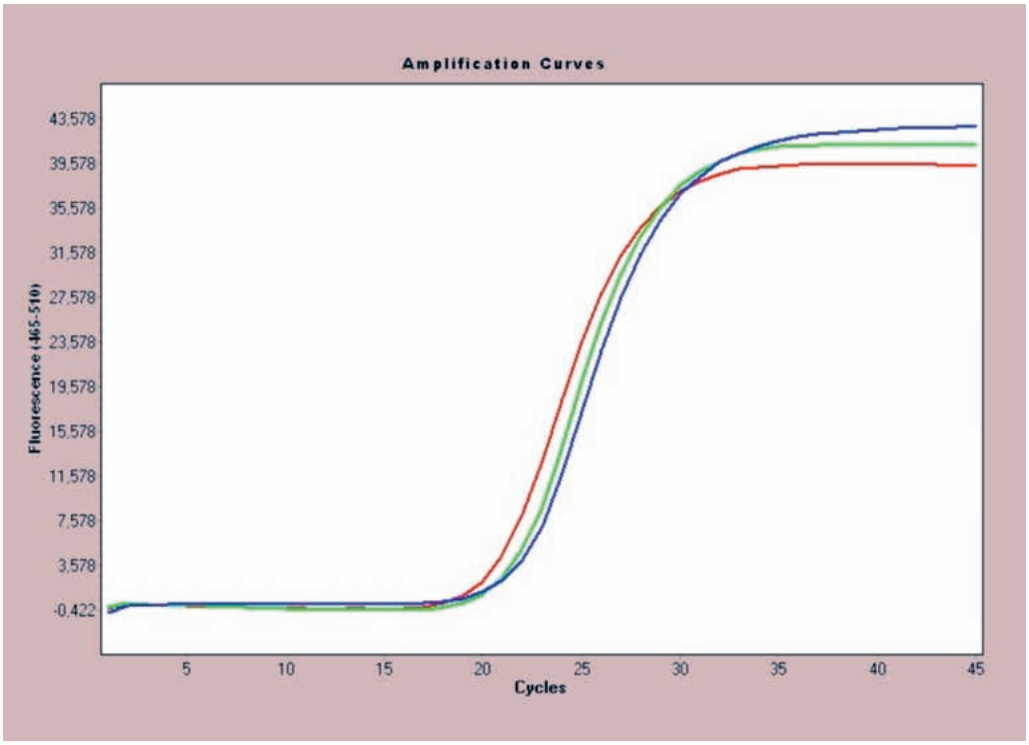

Figure 9: Analysis of cDNA quality. The ß2M gene, which is also one of the reference genes on the RealTime ready Focus Panels, was used for the control PCR using the Universal ProbeLibrary format. Corresponding cDNAs for cells treated at the 24 hours time point with DMSO (green line), nutlin 3a (blue line) and nutlin 3b (red line) were assessed with respect to quality for the individual PCRs and the focus panels.

Individual PCR Assays for MDM2, p53 and p21

Three key genes in the p53 pathway of nutlin-treated SJSA1 cells, p53, MDM2 and p21, were analyzed at 6 hours and 24 hours.

Table 1: Real-Time qPCR derived △△Cp values produced for p21, MDM2 and p53 using the LightCycler® 480 Instrument. For relative quantification analysis, GAPDH and ß2M were the reference genes. Relative quantification results, for nutlin 3a and nutlin 3b treated samples, were normalized to the DMSO controls (△△Cp).

target	treatment	△△Cp (6 hours)	△△Cp (24 hours)
p21	DMSO	1.0	1.0
	nutlin 3a	22.9	55.2
	nutlin 3b	1.5	1.0
MDM2	DMSO	1.0	1.0
	nutlin 3a	9.9	33.7
	nutlin 3b	1.2	1.0
p53	DMSO	1.0	1.0
	nutlin 3a	0.7	1.0
	nutlin 3b	1.0	0.9

RealTime ready Focus Panel Data

We analyzed 372 different apoptosis related genes using the RealTime ready Human Apoptosis Panel, 384. Relative quantification showed a more than threefold change in gene expression levels for a large number of genes (see Figure 10). Out of the 372 genes, 22 genes were found to be up regulated, and 24 genes were found to be down regulated, after 6 hours of nutlin 3a treatment. After 24 hours treatment, this number was even higher with 63 up regulated and 43 down regulated genes.

The activated p53 pathway may lead to both, the induction of apoptosis and/or a cessation in cell proliferation. We analyzed 84 different cell cycle related genes using the RealTime ready Human Cell Cycle Regulation Panel, 96.

Six and 24 hours after nutlin 3a treatment, 15 and 26 genes were found to be down regulated at least threefold, and only a fraction of these genes was up regulated.

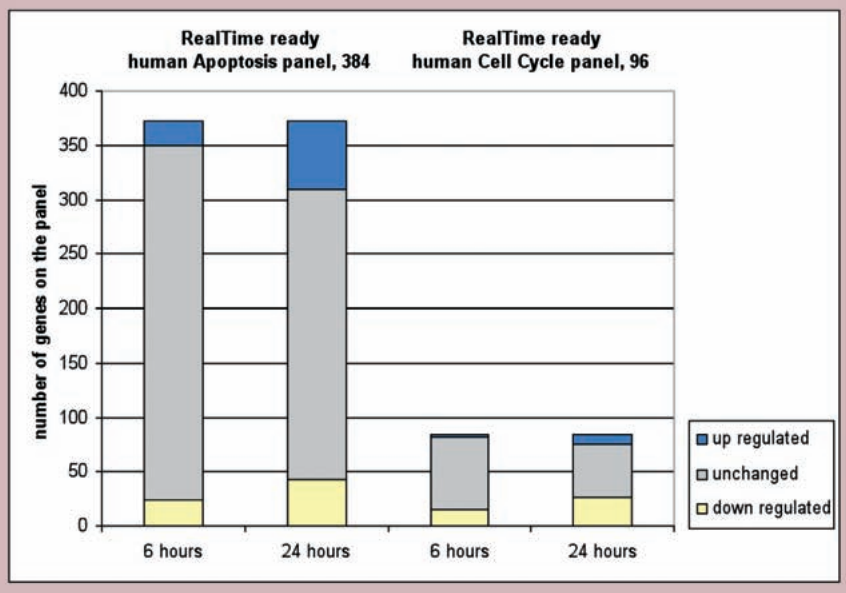

Figure 10: Fractions of at least threefold changed genes in response to nutlin 3a treatment based on the RealTime ready Human Apoptosis Panels, 384 and the RealTime ready Human Cell Cycle Regulation Panels, 96.
All genes with threefold and higher changes in gene expression were regarded as significant.

Table 2 identifies a subset of eight genes identified using the RealTime ready Focus Panels, already known to belong to the most relevant genes in the p53 pathway.

	6 hours			24 hours		
HGNC symbol	Apoptosis 384	Apoptosis 96	Cell Cycle 96	Apoptosis 384	Apoptosis 96	Cell Cycle 96
BAK1	1.09	1.04	/	4.43	3.59	/
BCL2	0.19	0.21	0.19	0.03	0.04	0.08
CASP3	0.89	0.84	/	3.20	2.68	/
CASP8	0.34	0.39	/	0.46	0.57	/
CDKN1A (p21)	22.69	/	22.05	41.43	/	48.31
TNFRSF10B	3.15	2.99	/	6.20	5.68	/
TP53 (p53)	0.80	0.80	0.77	0.80	0.72	1.24
TP53I3	5.60	5.28	/	20.16	20.61	/

Table 2: Summary of results from the RealTime ready Focus Panels. Significant changes in gene expression in the p53 pathway were found. The qPCR data, created using the three different panels (RealTime ready Human Cell Cycle Regulation Panel, 96, RealTime ready Human Apoptosis Panel, 96 and 384) were highly reproducible.

4 Conclusion

The xCELLigence System proved to be the ideal way to constantly monitor cellular events. The xCELLigence System continuously records cellular responses in real-time without using exogenous labels. Impedance measurements provide quantitative information about biological cell status, including cell number, cell viability, and cell morphology.

In our experiments using the xCELLigence System, changes in the CI profiles of SJSA1 cells were visible already within the first 6 hours after nutlin 3a treatment. Looking at this cell morphology "body language" allowed precise determination of the time points for the endpoint assays and the western blot analysis at 1, 2, 4, 8, 24, 48 and 72 hours.

Nutlin 3a treated SJSA1 cells were analyzed using four different endpoint assays for cell viability, cell proliferation, cell lysis or apoptosis/necrosis. Changes in the CI profiles could thus be attributed to a cessation in DNA synthesis, 24 hours after nutlin 3a treatment. This finding is consistent with a reduction in cell viability, followed by an increase in the number of apoptotic cells, and the onset of cell lysis by 48 hours after nutlin 3a treatment.

Western blot analysis of p53, MDM2 and p21 confirmed the accumulation of these proteins as a consequence of nutlin 3a treatment and induction of the p53 pathway during the first 24 hours of treatment. P53 increased already two hours after treatment, reaching a peak 24 hours later. p21, which was activated by p53, was already present 4 hours after treatment.

For a more detailed investigation of transcriptional activation over time, qPCR analysis was also performed in the very early phase. As previously shown (Vassilev et al.), most transcriptional targets of activated p53 reach their maximum level 24 hours after nutlin 3a treatment.

The individual analysis of gene expression for transcriptional p53 targets MDM2 and p21 by qPCR, confirmed the expected increase in the respective mRNAs. No significant change was detected in p53 mRNA expression in concordance with previous publications showing that the turnover of p53 is regulated in response to nutlin 3a treatment at the protein level.

p53 is a transcription factor that activates or inhibits hundreds of genes either directly or indirectly leading to diverse cellular effects. Our findings using two RealTime ready Focus Panels confirm this for the selective p53 activator nutlin 3a. More than 50 genes were up or down regulated by 6 hours after nutlin 3a treatment, with more than 140 genes in the apoptosis pathway, 24 hours after nutlin 3a treatment. Both RealTime ready Focus Panels provided a comprehensive set of apoptosis and cell cycle related genes corresponding specifically to nutlin 3a transcriptional activation. Further analyses of the role of these genes should provide more insight into the function of p53 and its complex regulation.

We are grateful to Dr. Lyuobomir Vassilev for providing us with nutlin 3a and nutlin 3b, and for very helpful discussions throughout these experiments and critical reading of the manuscript.

References

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Ordering Information

Product	Cat. No.	Pack Size
High Pure RNA Isolation Kit	11 828 665 001	up to 50 reactions
RTCA MP Station	05 331 625 001	1 instrument
RTCA Analyzer	05 228 972 001	1 instrument
RTCA Control Unit	05 454 417 001	1 instrument
E-Plate 96	05 232 368 001	1 x 6 plates
Cell Proliferation Reagent WST-1	05 015 944 001	8 ml (800 tests)
Cytotoxicity Detection Kit^{PLUS} (LDH)	04 744 926 001	1 kit (400 tests in 96 wells)
Cell Death Detection ELISA^{PLUS}	11 774 425 001	1 kit (96 tests)
Cell Proliferation ELISA, BrdU (chemiluminescent)	11 669 915 001	1 kit (1,000 tests)
Lumi-Light^{PLUS} Western Blotting Kit (Mouse/Rabbit)	12 015 218 001	1 kit
cOmplete Lysis M	04 719 956 001	1 kit
LightCycler® RNA Pre-Amplification Kit	05 190 894 001	1 kit (32 reactions)
LightCycler® 480 Instrument II	05 015 278 001	1 instrument (96-well version)
LightCycler® 480 Probes Master	04 887 301 001	10 x 500 reactions, 20 µl volume
RealTime ready Human Apoptosis Panel, 96	05 392 063 001	2 plates (each containing 96 assays)
RealTime ready Human Cell Cycle Regulation Panel, 96	05 339 359 001	2 plates (each containing 96 assays)
RealTime ready Human Apoptosis Panel, 384	05 339 316 001	2 plates (each containing 384 assays)

For supplementing information about the panels,
please visit our web pages at

https://www.roche-applied-science.com/pack-insert/5392063a.pdf
for RealTime ready Human Apoptosis Panel, 96 and

https://www.roche-applied-science.com/pack-insert/5339359a.pdf
for RealTime ready Human Cell Cycle Panel, 96.

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