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FAST TRACK

A New Multiplex Assay Allowing Simultaneous Detection of the Inhibition of Cell Proliferation and Induction of Cell Death

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Abstract The efficacy of distinct anti-cancer drugs used in the chemotherapy of human malignancies varies between tumor tissues and depends largely on the ability of the therapeutic agents to simultaneously inhibit cell proliferation and to eliminate malignant cells by apoptosis. Especially, detection of early apoptotic changes seems to be important because early stages of apoptosis differ from those of necrosis. Therefore, the development of a novel test allowing fast and concomitant screening of the anti-proliferative and pro-apoptotic action of a number of anti-cancer drugs is of great interest. For this purpose, we choose as an experimental model a well characterized anti-proliferative and pro-apoptotic effect of cisplatin (CP) on human cervical carcinoma $HeLaS_3$ cells. As previously reported, exposure of $HeLaS_3$ to CP resulted in a concomitant inhibition of cell proliferation and induction of apoptosis in a dose- and time-dependent manner. In the present study we performed two independent approaches. In the first approach, we examined the cell proliferation and activity of caspases-3/7 in two separate microtiter plates using the CellTiter-Glo™ Luminescent Cell Viability Assay and the Caspase-Glo™ 3/7 Assay, respectively. In the second approach, we determined the same parameters sequentially in one microtiter plate by a mutiplexing assay using CellTiter-Blue™ Cell Viability Assay and Caspase-Glo™ 3/7 Assay. The both approaches gave very similar results indicating that this new multiplexing assay offers an important advantage for simultaneous detection of cell number and activation of caspases-3/7. The new multiplexing assay offers a range of benefits over standard assays. J. Cell. Biochem. 96: 1–7, 2005.

Key words: apoptosis; cell cycle arrest; activation of caspases-3/7; degradation of PARP-1

Human cervical carcinoma $HeLaS_3$ cells represent a cell line widely used in cancer research. Although $HeLaS_3$ cells are characterized by wild-type p53, they lack normal control of the cell cycle [Scheffner et al., 1990, 1993]. The proper regulation of cell cycle is abrogated by the presence of small proteins encoded by high-risk human papilloma virus HPV16/18 [Heck et al., 1992; Slebos et al., 1994]. Cells infected with high-risk HPVs express the two virally encoded E6 and E7 proteins that inactivate the function of tumor suppressor proteins wt p53 and retinoblastoma, respectively, thereby disturbing the regulation of the checkpoints in the cell cycle [Heck et al., 1992; Kessis et al., 1993; Slebos et al., 1994]. Recently, we reported that during therapy of HeLa cells by cisplatin (CP) cell proliferation and cell cycle progression were efficaciously inhibited [Wesierska-Gadek et al., 2002; Schloffer et al., 2003]. This was accompanied by induction of apoptosis [Horky et al., 2001; Wesierska-Gadek et al., 2002; Schloffer et al., 2003]. We characterized distinct steps of CP mediated programmed cell death (PCD) and found out that apoptosis was executed in two consecutive steps [Horky et al., 2001; Wesierska-Gadek et al., 2002]. In the first step, caspase-3 mediated cleavage of cytokeratin 18 and the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) was observed. In the later stage, dissipation

Abbreviations used: CP, cisplatin; DAPI, 4,6-diamino-2-phenylidine; DOX, doxorubicine; PARP-1, poly(ADP-ribose) polymerase-1; PVDF, polyvinylidene difluoride membrane; WCL, whole cell lysate.

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of mitochondrial membrane resulting in the release of mitochondrial proteins into the cytosol, DNA degradation and chromatin fragmentation was observed. Considering the fact that caspase-3 was activated in the early stages of CP-induced apoptosis, this experimental model was designed to develop and to test a novel assay allowing fast and concomitant evaluation of the anti-proliferative and pro-apoptotic action of a number of anti-cancer drugs.

In the present study we exposed exponentially growing HeLa S_3 to CP and evaluated the drug action in two independent approaches performed in multiwell plates. In the first approach, we examined the cell proliferation using CellTiter-Glo^{TM} Luminescent Cell Viability Assay and the activity of caspases-3/7 in two separate 96-well plates. In the second approach, we combined the two assays and determined the same parameters sequentially in the same microtiter plate by a mutiplexing assay using the CellTiter-Blue^{TM} Cell Viability Assay and the Caspase-Glo^{TM} 3/7 Assay. Moreover, we used a culture supernatant for determination of lactate dehydrogenase (LDH) and caspases-3/7 activity released into medium. Both approaches gave very similar results indicating that this new multiplexing assay offers an important advantage for simultaneous detection of cell number and activation of caspases-3/7, thereby, allowing rapid evaluation of the effects of tested drugs on cell proliferation, cell viability, and induction of apoptosis. The most important benefit of the new multiplexing assay over standard assays is the possibility to normalize the measured activity of caspase-3/7 to the cell number in which it was determined.

MATERIALS AND METHODS

Cell Culture

The human cervical carcinoma cell line $HeLaS_3$ was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum. Cells were grown up to 60%–70% confluence and then treated with CP (Lachema, Czech Republic) at a final concentration of 1–40 μM for indicated periods of time.

Antibodies

We used the following antibodies: monoclonal anti-PARP-1 antibodies (C-2-10) from Dr. G. Poirier (Laval University, Quebec), polyclonal anti-PARP-1 (N-20) antibodies recognizing the epitope within aa 1-20 in the amino-terminus (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal antibodies p85 recognizing selectively caspase-3 cleaved PARP-1 (Promega Corporation, Madison, WI). Monoclonal anti-actin (clone C4) antibodies were from ICN Biochemicals (Aurora, OH). Appropriate secondary antibodies linked to horseradish peroxidase (HRP) were from Amersham International (Little Chalfont, Buckinghamshire, UK).

Determination of Number and Viability of Cells

Sensitivity of human $HeLaS_3$ to increasing concentrations of CP was determined by two independent multiwell plate viability assays: the CellTiter-Glo^{TM} Luminescent Cell Viability Assay and the CellTiter-Blue^{TM} Cell Viability Assay (Promega Corporation). As described recently in more detail [Wesierska-Gadek et al., 2004a] the CellTiter-Glo^{TM} Luminescent Cell Viability Assay generating luminescent signals is based on quantification of the ATP levels. The CellTiter-Blue^{TM} Cell Viability Assay provides a fluorometric method for estimating the number of viable cells. Living cells posses the ability to reduce resazurin into resosurfin, which is highly fluorescent. Nonviable cells, due to loss of metabolic capacity, do not reduce the CellTiter-Blue reagent and thus do not generate a fluorescent signal. Tests were performed in quadruplicates. Luminescence and fluorescence was measured in the Wallac 1420 Victor, a multilabel, multitask plate counter and GENiosPro^{TM}— a multi-functional reader (Tecan Austria, GmbH, Grödeg, Austria).

Each point represents the mean ± SD (bars) of four values from one representative experiment.

Analysis of DNA Content in Cells by Flow Cytometry

The measurement of DNA content in propidium iodide-stained nuclei was performed by flow cytometry as described previously [Vindelov et al., 1983; Wesierska-Gadek and Schmid, 2000]. The stained cells were analyzed using a FACScan cytometer (Becton Dickinson). Distribution of cells in distinct cell cycle phases was determined using ModFIT cell cycle analysis software.

Detection of Apoptotic Cells by CytoDEATH Staining

Apoptotic cells were identified by M30 CytoDEATH monoclonal antibodies (Roche Molecular

Biochemicals) recognizing caspase-3 cleaved cytokeratin 18. Apoptotic cells were detected in situ by indirect immunofluorescence microscopy and were additionally quantified by flow cytometric analysis. For microscopic investigations cells were plated on slides in chambers and appropriately cultivated. After treatment for indicated time, cells were washed three times in PBS and immediately fixed in ice-cold methanol and stained. The fixed cells were incubated according to the manufacturer's protocol with the fluorescein-coupled monoclonal antibodies M30. For visualization of nuclei, cells were sequentially stained with 4, 6-diamidino-2-phenylindole (DAPI) dissolved in PBS at a final concentration of 1 μg/ml [Wesierska-Gadek et al., 2002; Schloffer et al., 2003].

Cells were inspected under a fluorescence microscope (Inverted microscope Eclipse TE300, Nikon Corporation, Tokyo).

Determination of Caspase-3/7 Activity

The activity of both caspases was determined using the APO-ONE Homogenous Caspase-3/7 Assay (Promega), which uses the caspase-3/7 substrate rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R100) [Wesierska-Gadek et al., 2004b]. This compound exists as a pro-fluorescent substrate prior to its turnover. Upon cleavage and removal of the DEVD peptide by caspase-3/7 activity and excitation at 499 nm, the removed rhodamine 110 groups becomes intensely fluorescent.

Alternatively, the Caspase-Glo™ 3/7 Assay was used. This test provides a proluminescent caspase-3/7 substrate, which contains the caspase-3 specific tetrapeptide sequence DEVD in a reagent optimized for cell lysis, and determination of caspases and luciferase activity. The addition of a Caspase-3/7 reagent results in cell lysis, followed by caspase-mediated cleavage of the Z-DEVD, release of aminoluciferin, which is a substrate for luciferase reaction and finally the generation of luminescence.

$HeLaS_3$ cells were plated in 96-well microtiter plates. Different multiwell plates were used. Besides conventional black and white plates also those with transparent bottom were tested. Twenty-four hours after plating cells were exposed for 24 h to CP at concentrations ranging from 1 to 40 μM. Thereafter, culture supernatant was transferred into another microtiter plate and the caspase activity was determined separately in cells and in culture medium. Then an equal volume of caspase substrate was added and samples were incubated at 37°C for different periods of time to assess the best signal-to-background ratio. The fluorescence was measured at 485 nm. Culture medium was used as a blank. "No-cell background" values were determined. Luminescence and fluorescence was measured in the Wallac 1420 Victor, a multilabel, multitask plate counter and GENiosPro™— a multi-functional reader (Tecan Austria).

Each point represents the mean ± SD (bars) of four values from one representative experiment.

Electrophoretic Separation of Proteins and Immunblotting

Total cellular proteins dissolved in SDS sample buffer were separated on 10% SDS slab gels, transferred electrophoretically onto polyvinylidene difluoride membrane (PVDF) (Amersham International, Little Chalfont, Buckinghamshire, England) and immunoblotted as previously described [Wesierska-Gadek et al., 2002]. Equal protein loading was confirmed by Ponceau S staining. In some cases, blots were used for sequential incubations.

RESULTS AND DISCUSSION

Exposure of $HeLaS_3$ cells to increasing CP concentrations resulted in an inhibition of cell proliferation accompanied by induction of apoptosis (Fig. 1). These data are in strict concordance with our previous reports [Horky et al., 2001; Wesierska-Gadek et al., 2002; Schloffer et al., 2003]. The CP-mediated activation of caspase-3 in $HeLaS_3$ cells was evidenced by three independent tests. First, the caspase-3 mediated cleavage of cytokeratine 18 detected in situ by CytoDEATH antibody was observed solely in CP treated cells and only in cells exhibiting chromatin changes characteristic for apoptosis such as chromatin condensation and fragmentation of nuclei (Fig. 1). The quantification of CytoDEATH positive cells by flow cytometry revealed that after exposure of HeLa cells to 40 μM CP for 15 h about 65% cells were apoptotic. Moreover, the increase of caspase-3 activity upon CP treatment of HeLa cells became evident after analysis of PARP-1, an excellent nuclear substrate of caspase-3 [Kaufmann et al., 1993; Lazebnik et al., 1994]. Caspase-3 recognizes the DEVD motif within the amino-terminal domain of PARP-1 and

cleaves the nuclear enzyme between aa 214 and 215 [Kaufmann et al., 1993; Lazebnik et al., 1994; Wesierska-Gadek et al., 2004b]. Monitoring of PARP-1 by immunoblotting revealed its specific proteolytic degradation appearing in CP, DOX, and VP-16 treated HeLa cells. A p85 protein band representing the caspase-3 cleaved PARP-1 carboxy-terminal fragment was generated in HeLa cells after exposure to anti-cancer drugs (Fig. 1). Finally, the activation of caspase-3/7 measured by the APO-One test was evidenced. A strong increase of caspase-3/7 activity was detected in HeLa cells exposed to 40 μM CP for 15 h (Fig. 1). In the light of these results and considering the fact that CP also inhibited proliferation of HeLa cells [Wesierska-Gadek et al., 2002; Schloffer et al., 2003] and induced G_1 cell cycle arrest (Fig. 2),

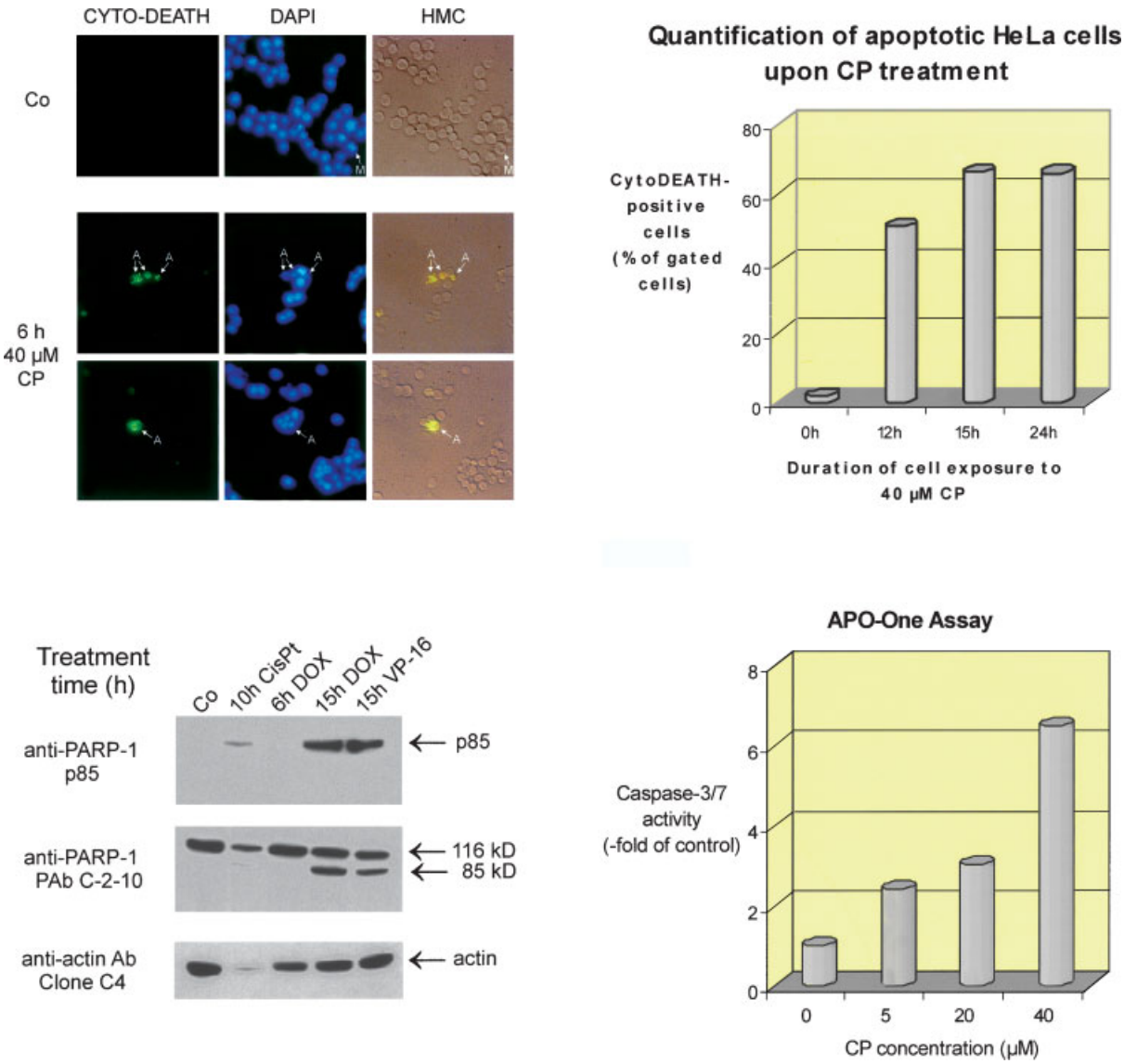

Fig. 1. Activation of caspase-3 in apoptotic HeLa cells after treatment with cisplatin (CP). Exponentially growing $HeLaS_3$ were treated with 40 μM CP for indicated periods of time. The cells were fixed according to the manufacturer's recommendations and stained using antibodies recognizing specifically caspase-3 cleaved cytokeratine 18 (CytoDEATH) (**upper panel**) or with antibodies recognizing specifically caspase-3 cleaved poly(ADP-ribose) polymerase-1 (PARP-1) (p85) (**lower left panel**). In cells fixed on slides the chromatin was visualized by sequential staining with DAPI. Cells present in the field were visualized by Hoffman modulation contrast (HMC). The right image was prepared by simultaneous detection of the CytoDEATH staining and HMC. Caspase-3/7 activity was determined in multiwell plate using APO-One assay.

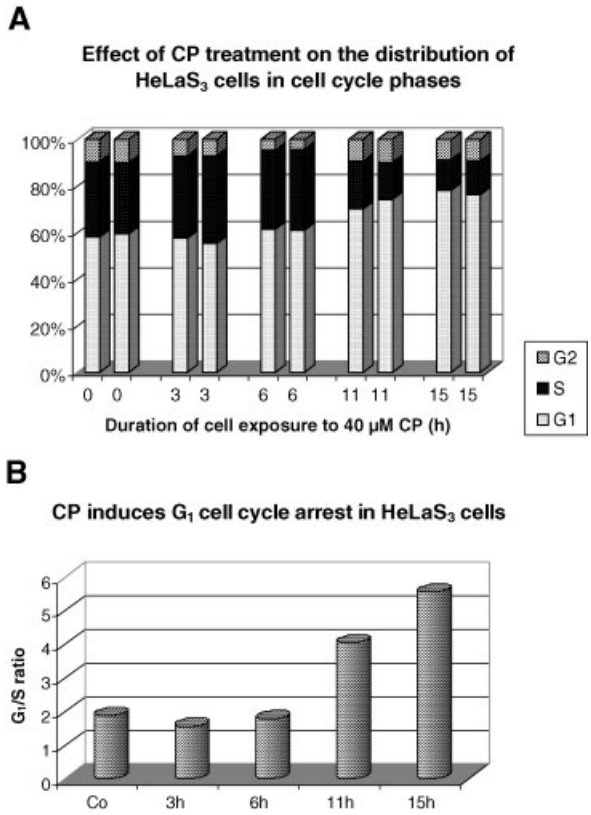

Fig. 2. CP arrests HeLa cells in G_1 phase of the cell cycle. HeLa cells were treated with 40 µM CP for indicated periods of time. Control and CP treated cells in duplicates were harvested and used for propidium iodide staining as described previously [Wesierska-Gadek and Schmid, 2000]. DNA content in single cells was measured using the Becton Dickinson FACsan. The distribution of HeLa cells in distinct cell cycle phases (**A**) and G_1/S ratio (**B**) are shown. The values of the duplicates were very similar.

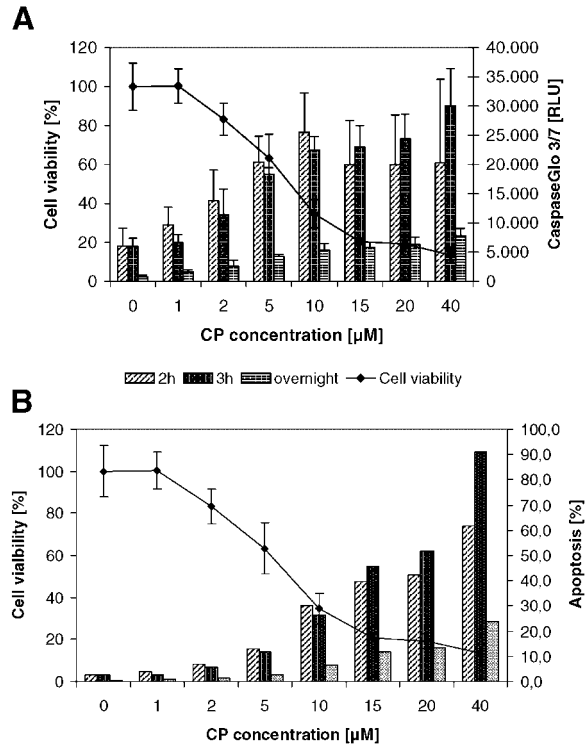

Fig. 3. CP mediated reduction of the number of viable cells associates with the activation of caspase 3/7. CellTiter-Glo™ Luminescent Cell Viability Assay and Caspase-Glo™ 3/7 were performed in separate plates. Luminescence generated by activated caspase-3/7 was measured after incubation for 2, 3 h, and overnight. **A**: Cell viability (% of control) was compared with the absolute caspase-3/7 activity. The increase of caspase-3/7 activity after exposure of cells to 5, 10, 15, 20 and 40 µM CP was statistically significant ($^{**}P < 0.01$). Statistical significance was valuated with the ANOVA test. **B**: Cell viability (% of control) was compared with the caspase-3/7 activity normalized to the cell number.

CP treated HeLa cells offered a very attractive model to establish and to test a novel assay allowing simultaneous detection of the effects of tested drugs on cell proliferation and induction of apoptosis.

In the first approach, we determined the cell proliferation using the CellTiter-Glo™ Luminescent Cell Viability Assay and the activity of caspases-3/7 independently in two seperate 96-well plates. To assess the optimal signal-to-background ratio for caspase-3/7 activity, luminescence was measured after incubation for 2, 3 h, and overnight. As shown in Figure 3A the reduction of the number of viable cells was accompanied by the activation of caspase-3/7. The best signal-to-background ratio for caspase-3/7 was observed after incubation for 3 h. The reduction of the cell number by 50% occurred at a final CP concentration of 6 µM. Measurements of serial three and ten-fold dilutions of HeLa cells from 100 to 250,000 revealed a linear responsiveness of the CellTiter-Glo™ Luminescent Cell Viability Assay within the tested cell number range. Therefore, we tried to correlate the reduction of the number of viable cells with the relative caspase-3/7 activity normalized to the number of cells per well. As shown in Figure 3B the frequency of apoptosis estimated in such a way correlated very well with the number of apoptotic cells determined by flow cytometry (Fig. 1). The results evidenced that the caspase-Glo™ 3/7 Assay was very sensitive and the activation of caspases-3/7 even in a low number of cells was detectable. The comparison of the results obtained by the APO-One test and by the Caspase-Glo™ 3/7 Assay (not shown) indicates that the latter is more sensitive. Moreover, the

luminescent assay avoids interference from inherent fluorescence and for these reasons seems to be suitable to combine it with the cell viability assay based on generation of fluorescent signals.

Therefore, in the second approach, we combined two assays and determined the same parameters sequentially in the same microtiter plate by mutiplexing the two tests: the CellTiter-Blue™ Cell Viability Assay and the Caspase-Glo™ 3/7 Assay. Moreover, we used a culture supernatant for determination of LDH and caspases-3/7 activity released into medium that reflect the integrity of the plasma membrane. The CellTiter-Blue™ Cell Viability Assay is predestined to be used in combination with other assays for several reasons. First, resazurin used as an indicator of the metabolic activity of cells can penetrate living cells and it becomes reduced inside living cells to the highly fluorescent resorufin, which is further reduced to nonfluorescent hydroresorufin [O'Brien et al., 2000; Gloeckner et al., 2001]. Resazurin can be reduced by action of several different redox enzymes in different subcellular compartments [Gonzalez and Tarloff, 2001]. It has been shown that reductases in cytosolic, mitochondrial, and microsomal fractions are able to convert resazurin to resorufin. The fluorescent dye resorufin generated upon reduction can diffuse out of cells into the medium. Therefore, the CellTiter-Blue reagent used in the assay is diluted in an isotonic buffer, which does not impair the integrity of the plasma membrane during the recommended incubation time. Moreover, this assay can be combined with the other tests, for example, sequential determination of caspase activity by luminescent assay. As shown in Figure 4 the reduction of the cell number after exposure to higher CP concentrations coincided with the activation of caspase-3/7. At about 5 μM CP the cell number was reduced by 50%. Quadruplicate fluorescent values measured in black or white multiwell plates using conventional fluorometer slightly varied. Therefore, we decided to perform the multiplexing test in black or white plates with transparent bottom. In these plates the direct control of the cell distribution by microscopy prior to measurement was possible. Inspection of the plates revealed the non-uniform distribution of cells within the wells. It indicated that the variability of the fluorescent signals may be attributable to the low efficiency of the detection of fluorescent signals if cells grow on the well's periphery. The serial tests using the GENiosPro™ multifunctional reader revealed that signal fluctuations can be eliminated when fluorescence measurements at a few sites per well were performed. Moreover, it became evident that the mode of reading of the fluorescent signals (top vs. bottom) may be of importance for adherent cells. Surprisingly, the well-to well fluorescence crosstalk in plates with transparent bottom was very low and ranged from 0.1% to 0.3%.

Both approaches gave very similar results indicating that this new multiplex assay offers an important advantage for simultaneous detection of cell number and activation of caspases-3/7 thereby allowing rapid evaluation of the effects of tested drugs on cell proliferation, cell viability, and induction of apoptosis.

The possibility to determine exactly the cell number in which caspase activity was measured is an important benefit of the new multiplexing assay. On the other hand, the additional analysis of LDH activity and caspase activity in the culture supernatant allows monitoring of the integrity of the plasma membrane. It is known that in the early stages of apoptosis the plasma membrane remains intact. Therefore, appearance of caspase-3/7 activity in culture medium would indicate the loss of the integrity of the plasma membrane, which usually occurs in the late apoptotic phase. As shown in Figure 4 no activated caspase-3/7 was released into the culture medium after treatment with CP at a final concentration of 5 and 10 μM. These treatments significantly activated cellular caspases. The above results clearly evidence that during the execution of apoptosis after exposure of HeLa cells to lower CP doses the integrity of the plasma membrane was maintained.

However, the multiplexing of the two assays has some important limitations. First, the caspase-Glo™ 3/7 Assay generates very good luminescent signals in cells expressing both caspases. However, in cells devoid of caspase-3, for example, human breast cancer MCF-7 cells the fluorescent signals are weaker. Moreover, some drugs at higher concentrations, for example, doxorubicin after intracellular accumulation produce a fluorescence that can interfere with the CellTiter-Blue™ Cell Viability Assay. The doxorubicin emitted fluorescence can result in the enhancement of fluorescent signals generated by resorufin and lead to overestimation of

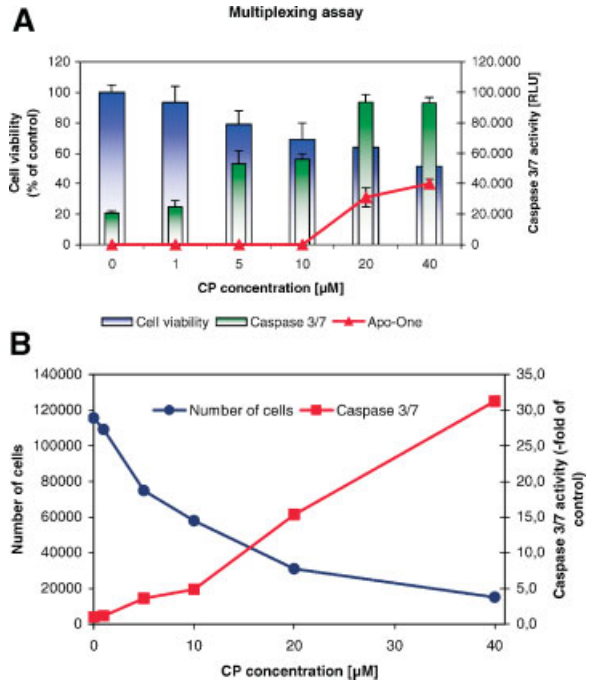

Fig. 4. Multiplexing assay combining CellTiter-Blue™ Cell Viability Assay and Caspase-Glo™ 3/7 Assay. Both tests were performed sequentially in the same multiwell plate. **A**: Cell viability (% of control) was compared with the absolute caspase-3/7 activity. The increase of cellular caspase-3/7 activity after exposure of cells to 5, 10, 15, 20 and 40 µM CP was statistically significant (**$P < 0.01$). Statistical significance was valuated with the ANOVA test. The activity of caspases-3/7 released into the culture medium determined by the APO-One Assay was measured separately in the culture supernatant taken from each well before addition of CellTiter-Blue reagent. Cell exposure to CellTiter-Blue reagent for 4 h did not impair the integrity of plasma membrane. **B**: Cell viability (% of control) was compared with the caspase-3/7 activity normalized to the cell number.

the results of the CellTiter-Blue™ Cell Viability Assay. However, in such a case, the determination of the spontaneous fluorescence prior to the addition of CellTiter-Blue reagent can help to resolve this problem.

Taken together, multiplexing of several assays allows to determine simultaneously cell number, activity status of caspases-3/7 and integrity of plasma membrane, thereby, allowing rapid evaluation of the effects of tested drugs on cell proliferation, cell viability, and induction of apoptosis.

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