Biochemical Pharmacology 79 (2010) 1387–1397

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Increased cytotoxicity of an unusual DNA topoisomerase II inhibitor compound C-1305 toward HeLa cells with downregulated PARP-1 activity results from re-activation of the p53 pathway and modulation of mitotic checkpoints

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ARTICLE INFO

Article history:
Received 24 October 2009
Accepted 31 December 2009

Keywords:
PARP-1
Topoisomerase II
Combination therapy
Mitotic catastrophe
p53

ABSTRACT

Our previous studies have shown that murine fibroblast cells, in which *PARP-1* gene was inactivated by gene disruption, are extremely sensitive to triazoloacridone compound C-1305, an inhibitor of DNA topoisomerase II with unusual properties. Here, we show that pharmacological inhibition of PARP-1 activity by its inhibitor compound NU1025, sensitizes human cervical carcinoma HeLa cells to compound C-1305 compared to treatment with drug alone. Cytotoxic effect of drug/NU1025 of other topoisomerase II inhibitors varied depending on the dose of PARP-1 inhibitor. Increased cytotoxicity of topoisomerase II inhibitor/NU1025 combinations was attributable to the re-activation of the p53 pathway in drug-treated HeLa cells. This lead to a more stringent cell cycle checkpoint control during G2 and M and enhanced cell death by mitotic catastrophe induced by drug/NU1025 combinations. Interestingly, treatment of HeLa cells with NU1025 alone also increased p53 expression. This effect is, at least in part, related to the inhibition of proteasome activity by drug treatments. Together, our results show that concomitant inhibition of topoisomerase II and PARP-1 leads to the synergistic cytotoxic effect toward tumor cells that may be important for combination therapies with NU1025 and topoisomerase II inhibitors. We also confirmed our earlier work and show the important role of PARP-1 activity in the maintenance of the G2 arrest induced by DNA damaging drugs. Finally, based on our studies we propose that NU1025 and possibly other inhibitors of PARP-1 may be used as non-genotoxic agents to activate p53 in tumor cells with non-functional p53 pathways.

1. Introduction

Poly(ADP-ribose) polymerase-1 (PARP-1) is a sensitive detector of DNA damage. This enzyme is strongly activated by DNA strand breaks and has important functions in DNA repair and in the maintenance of genome stability as well as plays a key role in the initiation of cell death induced by different stimuli [1–3]. The important role of PARP-1 in base excision repair and cell death led to the development of new highly specific inhibitors of PARP-1 [4,5]. Recently, inhibition of PARP-1 activity by chemical inhibitors has been shown to potentiate the cytotoxic effect of ionizing radiation, DNA methylating agents such as temozolomide, oxidative damage, and topoisomerase I inhibitors [6–10]. In line with that, several different PARP-1 inhibitors are being tested in clinical trials in combination with DNA damaging agents as a new strategy to sensitize tumor cells to conventional antitumor drugs.

Triazoloacridone C-1305 is an inhibitor of DNA topoisomerase II with unusual properties, which exhibits potent antitumor activity toward solid tumors [11,12]. In our earlier studies, antiproliferative action of C-1305 was investigated in nontransformed mouse fibroblasts and two mutant cell lines in which the *PARP-1* gene was disrupted by homologous recombination in exon 2. Unexpectedly, C-1305 very strongly affected proliferation of cells lacking functional PARP-1, whereas the action of another topoisomerase II inhibitor acridine *m*-AMSA as well as biologically inactive triazoloacridone compound C-1533 toward normal and PARP-1-null cells was comparable [13]. We also showed that oversensitivity of cells with non-functional PARP-1 to compound C-1305 is associated with the important role of the PARP-1 status in the maintenance of the G2 arrest induced by compound C-1305 and possibly other topoisomerase II inhibitors.

The objective of the studies described in this report was to determine whether unusual oversensitivity of cells with genetic

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4′,6-diamidino-2-phenyindole diacetate; NU1025, 8-hydroxy-2-methylquinazoline-4-one; m-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.

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doi:10.1016/j.bcp.2009.12.023

defect in the *PARP-1* gene to triazoloacridone C-1305 may be observed in tumor cells in which PARP-1 activity is downregulated by its chemical inhibitor, compound NU1025. For comparison, in our studies we also included classic inhibitors of topoisomerase II, antitumor drugs amsacrine (*m*-AMSA) and etoposide. We show that pharmacological inhibition of PARP-1 activity by NU1025, sensitizes human cervical carcinoma HeLa cells to compound C-1305 compared to treatment with either drugs alone. Unexpectedly, we show that inhibition of PARP-1 activity leads to the activation of p53 in HeLa cells that is further potentiated in the presence of studied drugs. The molecular mechanism of this effect involves stabilization of the p53 protein, i.e. at least in part, associated with the inhibition of proteasome activity by C-1305 and NU1025. This leads to re-establishment of the DNA damage checkpoint control in G2 and M and enhanced cell death of drug-treated cells by mitotic catastrophe.

2. Materials and methods

2.1. Drugs, antibodies and chemicals

Compounds *m*-AMSA and C-1305 were synthesized in our department by Dr. Marek Konieczny and Dr. Barbara Horowska. MTT, propidium iodide, RNase A and DAPI were from Sigma, NU1025 was from Merck. Cell culture media, antibiotics and serum were from GIBCO (Paisley, UK). Monoclonal mouse anti-Cdk1/Cdc2 (clone 17), polyclonal rabbit anti-cyclin B1 (clone GNS1), polyclonal goat anti-actin (clone I-19) and monoclonal mouse anti-p53 (clone DO-1) antibodies were from Santa Cruz (Santa Cruz, CA), monoclonal anti-PARP-1 antibodies were from Cell Signaling (Beverly, MA). Monoclonal anti-topoisomerase IIalpha and anti-topoisomerase IIbeta antibodies were from BioTrend Chemikalien GmbH (Koln, Germany). Horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat IgG antibodies were obtained from Jackson ImmunoResearch Labs (West Grove, PA). FITC-conjugated anti-mouse antibodies were from Sigma.

2.2. Cell lines

Human cervical carcinoma HeLa S3 cells were obtained from ATCC. Cells were grown in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were maintained at 37 °C in 10% CO_2/air atmosphere and screened routinely for Mycoplasma by the PCR method with Mycoplasma *Plus* PCR Primer Set (Stratagene, La Jolla, CA).

2.3. Cytotoxicity assay

Exponentially growing HeLa S3 cells, cells were seeded at 4000 cells/well, left overnight and exposed to different doses of NU1025 and then exposed to drugs for 24 h, washed and post-incubated in a drug-free medium for 5 days. The IC_{50} values are defined as the drug concentration resulting in 50% reduction of viable cells, compared to untreated control cells.

2.4. Immunofluorescence microscopy and image analysis

For determination of intracellular localization of p53, cells were attached to microscopic slides, treated with studied drugs or drug combination and fixed in 1% paraformaldehyde at room temperature for 30 min. After two washes in PBS, cells were permeabilized with 0.1% Triton X-100 for 5 min on ice. The cells were first treated with 1% BSA/PBS to block non-specific staining and were then incubated with anti-p53 antibodies at 1:200 dilution for 1 h at 37 °C. Following two washes in PBS containing 0.5% BSA and 0.2% Tween-20, cells were incubated with anti-mouse FITC-conjugated antibodies diluted at 1:50 for 30 min at room temperature. After two additional washings with PBS with 0.5% BSA and 0.2% Tween-20, samples were stained in PBS containing 0.1 μg/ml DAPI for 10 min at room temperature and mounted in 90% glycerol/10% 10 mM Tris–HCl, pH 8, containing 25 mg/ml diazabicyclo[2,2,2]octane (DABCO).

2.5. Detection of mitotic and apoptotic cells

Mitotic fractions were determined in drug-treated cell populations after Carnoy's fixation, staining with 0.1 μg/ml DAPI and counting under fluorescent microscope. Apoptotic/dead cells were identified after 20 μg/ml fluorescein diacetate staining for 15 min at 37 °C followed by the addition of 5 μg/ml propidium iodide and analysis by fluorescence microscopy.

2.6. DNA fragmentation

Following drug treatment, cells were harvested by centrifugation and washed twice with ice-cold PBS. Internucleosomal DNA fragmentation in cells undergoing apoptosis was assayed by inverted pulse field gel electrophoresis as described earlier [14]. Briefly, cells were washed twice in PBS and embedded in 0.75% low melting InCert agarose (Cambrex Bioscience, Rockland, ME, USA). Plugs were then incubated in lysis buffer (10 mM Tris–HCl, pH 8, 10 mM NaCl, 25 mM EDTA, 0.9% sarcosyl, 0.1% SDS, 1 mg/ml proteinase K) for 24 h at 50 °C, washed 3 times in washing buffer (10 mM Tris–HCl, pH 8, 50 mM EDTA) and stored in 50 mM EDTA, pH 8 at 4 °C until analysis. Samples, including 50–1000 kbp lambda DNA marker (BioRad, Hercules, CA, USA), were analysed in a 1% SeaKem Gold agarose gel (Cambrex Bioscience) in 0.5× TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8.3) with temperature maintained at 12 °C using FIGE Mapper system (BioRad). The pulsewave switcher was programmed to provide initial 9 s forward and 3 s reverse pulses, with linear ramp 3:1 and a constant voltage of 180 V throughout 12 h. Gels were stained and photographed under UV illumination.

2.7. Flow cytometry

The cell cycle distribution was measured by flow cytometry using an FACScan flow cytometer (Becton Dickinson) equipped with an argon laser to give a 488 nm light. The cells were fixed in 70% ethanol at −20 °C, rehydrated in PBS and stained with propidium iodide (20 μg/ml) and RNAse A (100 μg/ml) at room temperature for 30 min. The percentage of cells in each phase of the cell cycle was calculated by MultiPlus software (Phoenix Flow Systems, San Diego, CA).

2.8. Western blot analysis

Cells were lysed in RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl, pH 8) containing a protease inhibitor cocktail (Roche Diagnostic, Meylan, France) and phosphatase inhibitors (50 mM sodium fluoride, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate) for 15 min on ice. Lysates were centrifuged at 20,000 × *g* for 10 min at 4 °C and supernatants collected. Protein concentrations in cellular lysates were determined by the BCA assay (Pierce). Equal amounts (50 μg protein per lane) were loaded in Laemmli buffer and separated by the SDS-PAGE electrophoresis in polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech). After transfer, membranes were blocked in 5% non-fat milk in TBS buffer (10 mM Tris–HCl, 150 mM NaCl, pH 8) and washed in TBST buffer (TBS buffer containing 0.05% Tween-20). Membranes were

incubated with primary antibodies diluted in TBST containing 0.5% bovine serum albumin at 1:100 (anti-cyclin B1, anti-Cdk1) or 1:1000 (anti-actin) for 1–6 h at room temperature. Alternatively, antibodies were diluted in TBST containing 5% fat-free milk at 1:3000 (anti-PARP-1) and membranes were incubated overnight at 4 °C. After three washes in TBST, membranes were incubated with secondary antibodies diluted at 1:40,000 in TBST for 1 h at room temperature. Results were revealed by the ECL kit (Pierce).

2.9. Determination of proteasome activity

Cytosolic fractions were prepared by cell lysis in 50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl, 2 mM ATP and 1% Triton X-100 for 30 min. on ice followed by centrifugation at 20,000 × *g*/+4 °C/30 min. The peptidase activity of cellular lysates toward fluorogenic peptides Z-Leu-Leu-Leu-AMC and suc-Leu-Leu-Val-Tyr (Alexis Biochemicals) was determined by measuring the fluorescence of the generated aminomethylcoumarin, as described [15]. The reaction mixture contained 25 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.05% NP-40, and 0.001% SDS, 25 μM substrate and an aliquot of cytosol fractions in the presence or absence of proteasome inhibitor, MG132 (5 μM). Fluorescence was measured in a PerkinElmer Victor ^{3}V 1420 Multilabel Counter with excitation and emission filters set at 380 and 440/460 nm, respectively. The proteasome activity was normalized for the protein content of cytosolic fractions and expressed in arbitrary units.

Table 1
Cytotoxicity of both studied topoisomerase II inhibitors either alone or in combination with two doses of NU1025. Cells were pre-incubated with NU1025 and co-incubated with NU1025 in combination with topoisomerase II inhibitors for additional 24 h. Drug-containing medium was subsequently removed, cells washed and post-incubated for additional 72 h in a drug-free medium. The cytotoxic effect was determined by the MTT assay. Data are means ± standard deviation from three independent experiments, each done in duplicates.

	IC_{50} [μM]		
	No NU1025	+100 μM NU1025	+200 μM NU1025
C-1305	14.74 ± 0.07 (1)	7.85 ± 0.08 (1.9)	3.57 ± 0.06 (4.1)
m-AMSA	0.63 ± 0.05 (1)	0.32 ± 0.01 (2.0)	1.0 ± 0.04 (0.6)
Etoposide	0.81 ± 0.05 (1)	1.48 ± 0.04 (0.55)	1.6 ± 0.03 (0.5)
Cisplatin	3.20 ± 0.05 (1)	4.92 ± 0.04 (0.65)	5.00 ± 0.03 (0.64)

Potentiation factor, ratio of IC_{50} determined without NU1025 pre-treatment and IC_{50} for drug/NU1025 combination are within brackets.

3. Results

3.1. Cytotoxicity of NU1025 and topoisomerase II inhibitors toward HeLa cells

We first determined the cytotoxic activity of different topoisomerase II inhibitors in the absence or presence of NU1025. For combination studies, HeLa cells were pre-treated with either 100 or 200 μM NU1025 and then co-incubated with NU1025 and studied drugs for additional 24 h. These two doses of NU1025 doses have been shown to inhibit PARP activity in tumor

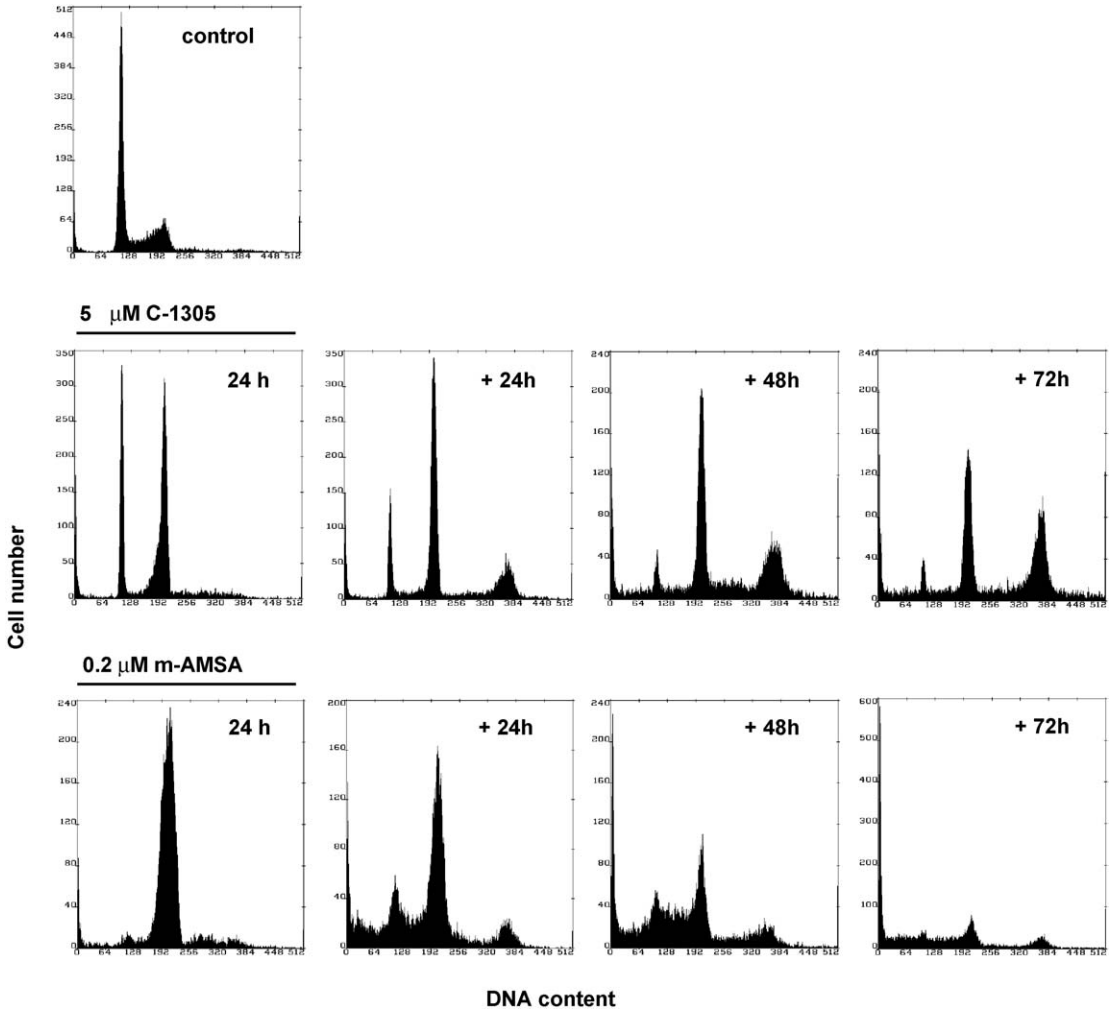

Fig. 1. Changes in the cell cycle distribution of HeLa cells treated with C-1305 or *m*-AMSA for 24 h and post-incubated in drug-free medium for the time indicated.

cells by >90% [7,16]. Compound NU1025 at both studied concentrations, i.e. 100 and 200 μM had a very low toxicity, with less than 5% non-viable cells after a 48 h treatment.

Combination of C-1305 and NU1025 led to enhanced cytotoxicity toward HeLa cells by about 2- and 4-fold for 100 and 200 μM NU1025, respectively, compared to the effect of C-1305 alone (Table 1). Interestingly, combination of *m*-AMSA with PARP inhibitor led to about 2-fold increased cytotoxicity only at the lower dose of NU1025 (100 μM) whereas at higher concentration of NU1025 we observed reduced cytotoxic effect by about 2-fold, compared to the drug alone. The protective effect of PARP inhibition by NU1025 was also observed for two other antitumor drugs, another topoisomerase II inhibitor etoposide and DNA crosslinking agent cisplatin, for which combination of both these drugs with NU1025 at both studied doses led to about 2-fold decreased cytotoxicity (Table 1).

3.2. Effect of topoisomerase II inhibitors and drug/NU1025 combinations on cell cycle progression of HeLa cells

To establish the effect of C-1305, *m*-AMSA, NU1025 on cell cycle progression, we exposed HeLa cells to both topoisomerase II

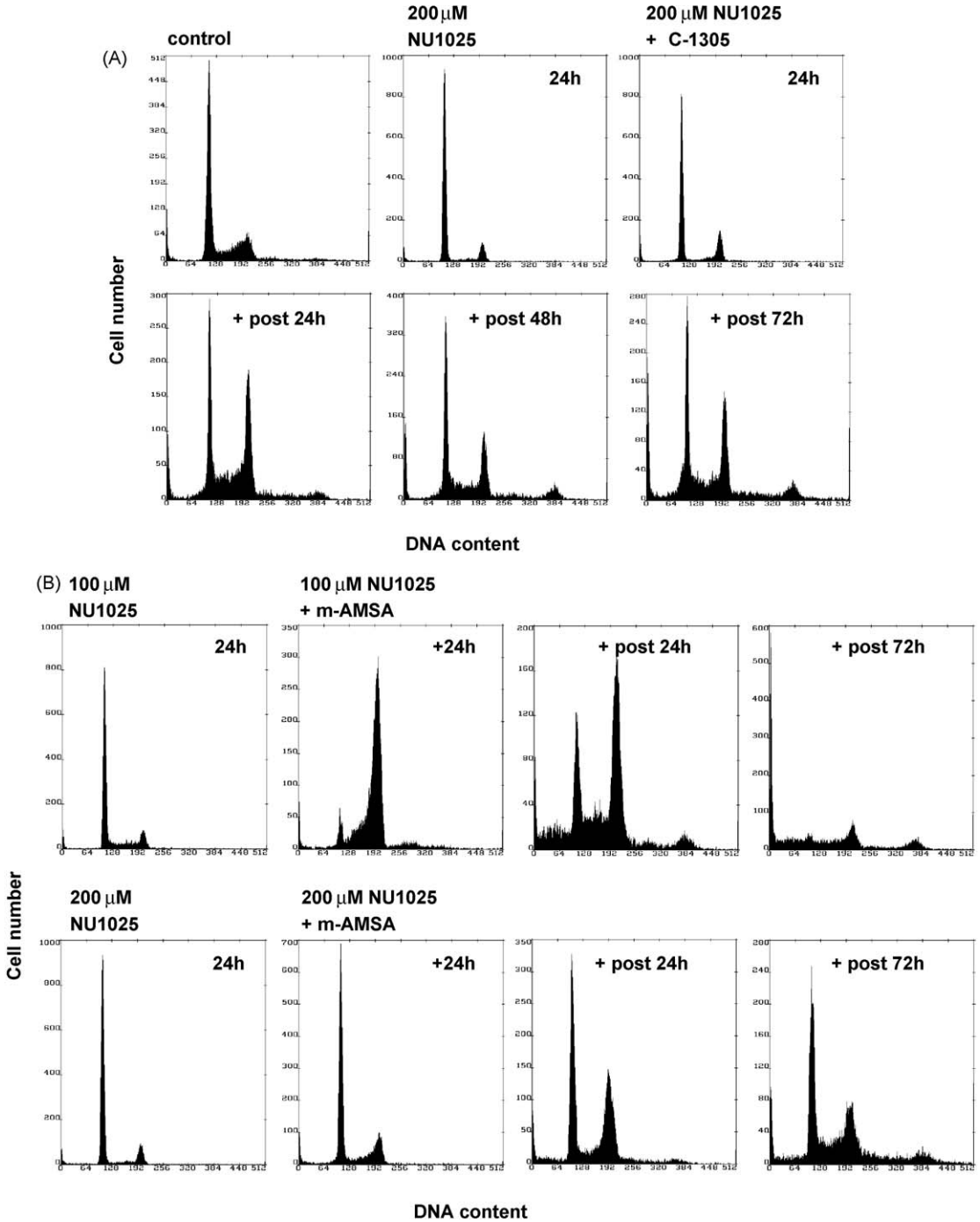

Fig. 2. (Panel A) Changes in cell cycle distribution of HeLa cells treated with C-1305 in combination with NU1025. Cells were pre-incubated with 200 μM NU1025 for 24 h, co-incubated with NU1025/5 μM C-1305 for additional 24 h and post-incubated in drug-free medium for the time indicated. (Panel B) Changes in cell cycle distribution of HeLa cells treated with *m*-AMSA in combination with NU1025. Cells were pre-incubated with either 100 or 200 μM NU1025 for 24 h, co-incubated with NU1025/0.2 μM *m*-AMSA for additional 24 h and post-incubated in drug-free medium for the time indicated.

inhibitors for 24 h and post-incubated in drug-free medium. Alternatively, cells were pre-incubated with NU1025 for 24 h, co-incubated with either C-1305 or *m*-AMSA and post-incubated in drug-free medium. In these studies, we used drug doses which induce total growth inhibition after a 24 h exposure to studied topoisomerase II inhibitors and post-incubated in drug-free medium.

Cells exposed to C-1305 for 24 h arrested in G1 and G2 (Fig. 1) and after post-incubation without the drug a substantial fraction of cells entered polyploidy (about 25% at 72 h post-incubation). Effect induced by the equitoxic dose of *m*-AMSA was quite different and most of drug-treated HeLa cells were in late S and G2/M after a 24 h of drug treatment. No major changes in cell cycle distribution was observed when cells were post-incubated in drug-free medium, except an appearance of a small fraction of polyploid cells (Fig. 1).

PARP-1 inhibitor alone produced a very modest effect on cellular growth and cell cycle distribution of HeLa cells. Lower dose of NU1025 (100 μM) did not induce apparent changes in the distribution in the cell cycle of HeLa cells, higher dose led to a partial emptying of S phase fraction and accumulation of cells in G1 and G2/M (Supplementary Fig. 1). When HeLa cells were pre-incubated with 200 μM NU1025 and subsequently co-treated with 5 μM C-1305 most cells arrested in G1 and G2/M, with no detectable S fraction (Fig. 2, panel A). After removal of both drugs, cells exited G1 and G2/M and DNA histograms resembled normal cell cycle distribution, however, a higher fraction of G2/M cells was still apparent. Combined treatment with 100 μM NU1025 and 5 μM C-1305 produced similar changes in the cell cycle distribution (data not shown).

Effect of combined treatment of HeLa cells with *m*-AMSA and NU1025 was dependent on the dose of PARP inhibitor used. When cells were exposed to 100 μM NU1025/0.2 μM *m*-AMSA combination, changes in cell cycle distribution induced by this treatment were very similar to that observed for *m*-AMSA alone, with higher fraction of hypodiploid cells (sub-G1) (compare Figs. 1 and 2, panel B). Pre-treatment with 200 μM NU1025 followed by co-incubation with 0.2 μM *m*-AMSA and post-incubation in drug-free medium led to a transient G2/M arrest

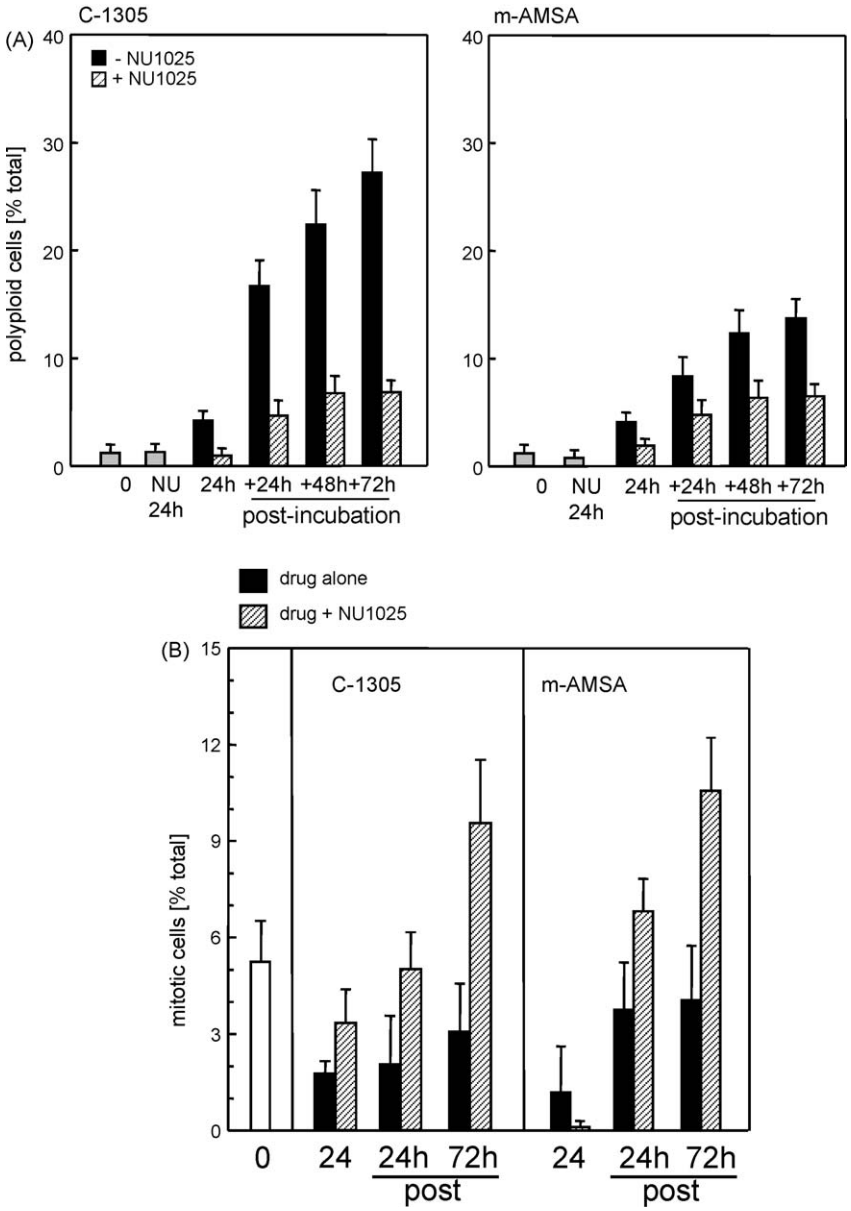

Fig. 3. Fraction of polyploid (with >4N DNA content) HeLa cells (panel A) and mitotic cells (panel B) after treatment with 5 μM C-1305 or 0.2 μM *m*-AMSA or combination of 5 μM C-1305/200 μM NU1025 or 0.2 μM *m*-AMSA/100 μM NU1025 for 24 h and post-incubated in drug-free medium for the time indicated.

(Fig. 2, panel B). At later post-incubation times, a small fraction of drug-treated cells entered polyploidy and cell cycle distribution progressively changed to that observed for non-treated cells. This result is compatible with lower cytotoxicity of *m*-AMSA in combination with 200 μM NU1025 (Table 1).

3.3. Induction of polyploidy and cell death in drug-treated cells

The most striking difference between the effect induced by two studied drugs alone and drug/NU1025 combinations was in the fraction of polyploid cells (with >4N DNA content). These fractions were reduced by 2–3-fold when cells were exposed to studied topoisomerase II inhibitors in combination with NU1025 (Fig. 3, panel A) and effect of PARP-1 inhibition on the number of polyploid cells was much more pronounced for cells treated with compound C-1305.

Polyploidization of HeLa cells after drug treatment may be indicative of an abnormal mitotic transition. Therefore, we also followed the number of mitotic cells in drug-treated cell populations. As shown in Fig. 3 (panel B), combined treatment of HeLa cells with drug/NU1025 led to greatly increased mitotic fraction, compared to the effect of both drugs alone.

We also compared fractions of dying cells in drug-treated cell populations. As presented in Fig. 4 (panel A), combined treatment of HeLa cells with C-1305/NU1025 increased the fraction of dying cells at both doses of PARP-1 inhibitor, compared to C-1305 alone. In contrast, the fraction of non-viable cells increased only for NU1025/*m*-AMSA combination at the lower dose (100 μM) of PARP inhibitor whereas at higher dose the number of dead cells was reduced. These results are in agreement with the cytotoxic activity of both drugs and drug/NU1025 combinations (see Table 1). Microscopic examinations of cell and nuclear morphology revealed that HeLa cells treated with drug/NU1025 combinations died mostly during mitosis as we observed accumulation of abnormal mitotic figures with morphological features of mitotic death, as shown for *m*-AMSA/NU1025 treatment (Fig. 4, panel B).

3.4. Expression of cell cycle regulators in cells treated with topoisomerase II inhibitors in the presence or absence of PARP inhibitor

We next followed changes in the expression of regulators of G2/M transition as well as apoptosis-related PARP-1 cleavage. Exposure of HeLa cells to both topoisomerase II inhibitors led to increased expression of cyclin B1 and the appearance of phosphorylated, inactive forms of Cdk1 kinase. HeLa cells express the E6 oncoprotein, which facilitates p53 degradation [17]. After treatment with *m*-AMSA, p53 levels were induced at 24 h and progressively decreased to undetectable levels during post-incubation in drug-free medium (Fig. 5, panel A). However, after treatment with C-1305, p53 levels increased only after 48 and 72 h post-incubation without drug. Interestingly, p21 was only induced in cells treated with C-1305. We also observed cell death-related PARP-1 cleavage in cells exposed to *m*-AMSA whereas progressive downregulation of PARP-1 expression to non-detectable levels in cells treated with C-1305 (Fig. 5, panel A).

Quite different pattern of protein expression was observed after treatment of HeLa cells with drug/NU1025 combinations. Pre-treatment with NU1025 induced p53 levels which was further potentiated during co-incubation with *m*-AMSA or C-1305 (Fig. 5, panel B). Greatly elevated levels of p21 levels were observed after a 24 h treatment with 200 μM NU1025 which further increased after addition of both drugs. Effect of drug/NU1025 combination on the expression of mitotic regulators cyclin B1 and cdk1 was similar to that observed for either drug alone. Only for

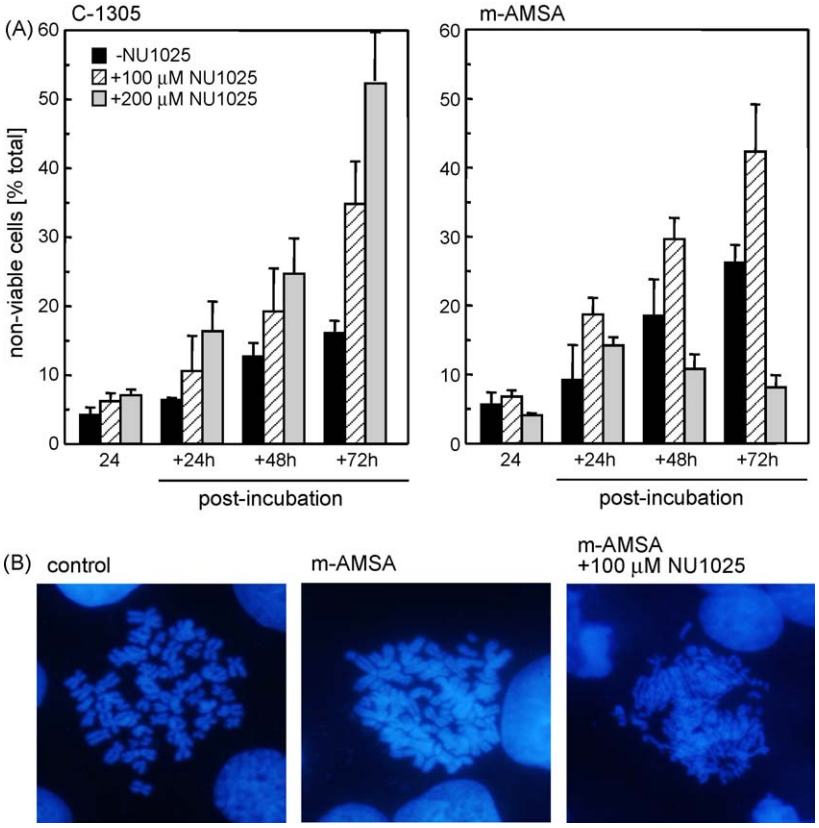

Fig. 4. (Panel A) Changes in apoptotic fraction after treatment with 5 μM C-1305 or 0.2 μM *m*-AMSA or combination of 5 μM C-1305 or 0.2 μM *m*-AMSA and different doses of NU1025 for 24 h and post-incubated in drug-free medium for the time indicated. (Panel B) Abnormal mitoses observed after treatment of HeLa cells with *m*-AMSA and *m*-AMSA/NU1025 combination for 24 h.

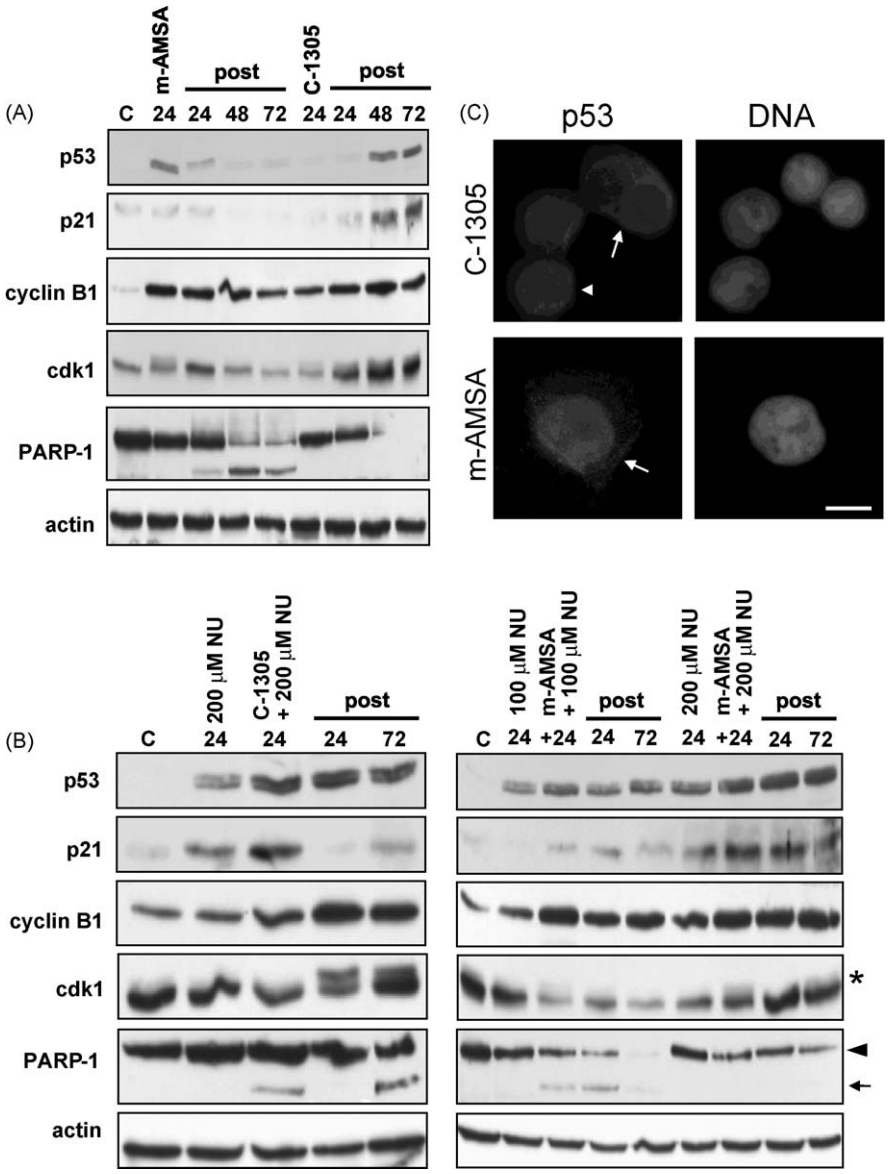

Fig. 5. Expression of different cell cycle regulators following treatment of HeLa cells with C-1305 or *m*-AMSA alone (panel A) or in combination with different doses of NU1205 (panel B). Cells were treated as described in the legend to Fig. 2. Hyperphosphorylated form of Cdk1 is marked with an asterisks, full length and cleaved forms of PARP-1 are marked with arrowheads and arrows, respectively. (Panel C) Intracellular localization of p53 in HeLa cells treated C-1305 and *m*-AMSA in combination with 200 and 100 μM NU1205, respectively. Cells with cytoplasmic (arrow) or nuclear (arrowhead) localization of p53 are shown. Original magnification 600×, bar 5 μM.

combination C-1305 and NU1025, high levels of both cyclin B1 and hyperphosphorylated forms of Cdk1 were observed during post-incubation without drugs (Fig. 5, panel B). Cell death-associated cleavage of PARP-1 was noted for *m*-AMSA/100 μM NU1025 and C-1305/200 μM NU1025 but not with combination of *m*-AMSA and 200 μM NU1025.

We wanted to clarify if increased p53 levels observed in drug-treated cells are accompanied with nuclear translocation of the protein. Immunofluorescence analysis showed that exposure to *m*-AMSA and *m*-AMSA/NU1025 led to nuclear accumulation of p53 whereas after treatment with compound C-1305 and C-1305/NU1025 increased p53 levels in the cytoplasm as well as in the nucleus were observed (Fig. 5, panel C and not shown).

3.5. p53 stability, effect of E6 expression and proteasome activity

We next wanted to assess whether elevated p53 levels after treatment with NU1025 and drug/NU1025 combinations result from the increased stability of p53 protein in drug-treated cells. To this end, we analysed p53 levels by Western blotting in cells treated with drug/NU1025 combinations in the absence or presence of cycloheximide, an inhibitor of protein synthesis. As shown in Fig. 6 (panel A), p53 protein levels were upregulated after a 24-h treatment with NU1025 and drug/NU1025 combinations. However, intracellular p53 rapidly disappeared (after about 1–3 h) when protein synthesis was inhibited by cycloheximide. In contrast, p53 levels were still measurable after a 6-h post-incubation in drug-free medium and cycloheximide. It follows that elevated levels of p53 in drug-treated HeLa cells result from the increased stability of the protein.

HeLa cells express viral E6 oncoprotein that leads to increased degradation of p53 and disruption of the p53 mediated cellular response to DNA damaging agents [17,18]. Earlier studies have shown that cisplatin as well as roscovitine downregulated the expression of E6 protein in HeLa cells and induced p53 accumulation [19,20]. Therefore, we assessed whether E6 levels in HeLa cells are downregulated by drug/NU1025 combinations. As shown in Supplementary Fig. 2, treatment of HeLa cells with C-

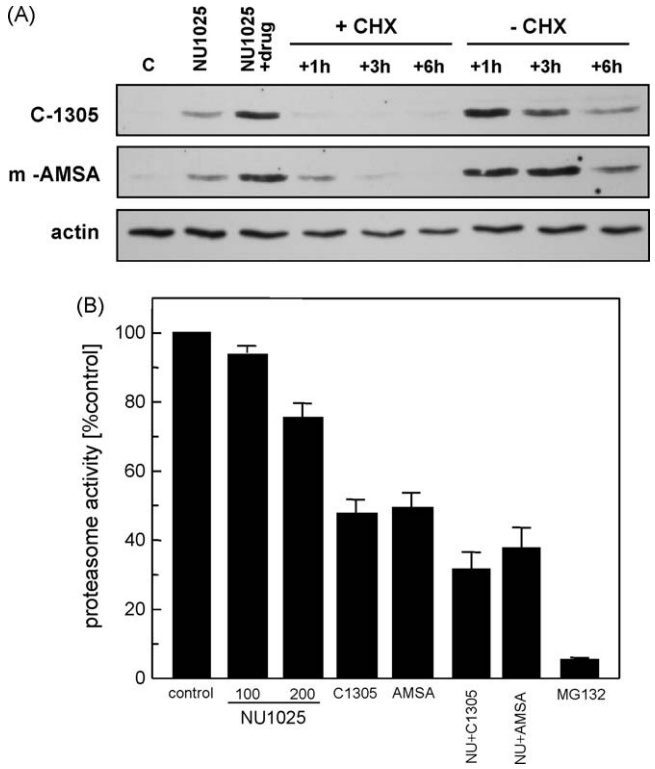

Fig. 6. Stability of p53 protein (panel A) and effect of drug treatment on proteasome activity (panel B) in HeLa cells treated with NU1025 alone or in combination with topoisomerase II inhibitors. Cells were treated with both topoisomerase II inhibitors alone or pre-incubated with NU1025 for 24 h, co-incubated with NU1025/drug combinations for additional 24 h post-incubated in drug-free medium with or without 20 μg/ml cycloheximide for the time indicated. Bar MGI132 in panel B corresponds to the effect of proteasome inhibitor at 5 μM for 24 h.

1305 and *m*-AMSA alone or in combination with NU1025 did not influence the expression of E6. In contrast, when tumor cells were treated with *m*-AMSA alone, greatly increased levels of E6 protein were observed. Similarly, no effect on E6 expression was observed in MCF7/E6 cells that are transfected with E6 encoding gene vector (not shown).

Finally, we measured proteasome activity in cellular extracts from HeLa cells treated with drugs or drug combinations. As shown in (Fig. 6, panel B), PARP-1 inhibition by NU1025 downregulated proteasome activity, as did both studied drugs and drug/NU1025 combinations produce an additive effect.

3.6. Changes in topoisomerase II expression in cells treated with studied inhibitors and drug/NU1025 combinations

Decreased proteasome activity in drug-treated HeLa cells may influence cellular levels of topoisomerase II isoenzymes [21]. For this reason, we determined whether drug/NU1025 combinations influence topoisomerase II expression by Western blotting analyses. Exposure of HeLa cells to *m*-AMSA had only marginal effect on the expression of topoisomerase IIalpha, however, cellular levels of the beta isoform increased by about 2-fold during post-incubation in drug-free medium (Supplementary Fig. 3, panel A). Similarly, treatment of HeLa cells with compound C-1305 did not significantly change expression of topoisomerase IIalpha but greatly increased the levels of topoisomerase IIbeta up to almost 6-fold at 72 h post-incubation without drug.

Similarly, combination of C-1305 and NU1025 had no effect on the expression of topoisomerase IIalpha but during post-incubation in drug-free medium increased levels of the beta isoform to up to 3-fold were observed (Supplementary Fig. 3, panel B). In contrast, treatment of cells with *m*-AMSA and 100 μM NU1025 had only minor effect on topoisomerase IIbeta expression but progressive downregulation of the alpha isoform was observed that can be attributed to cell death (Supplementary Fig. 3, panel B). Combination of 200 μM NU1025 and *m*-AMSA led to the initial

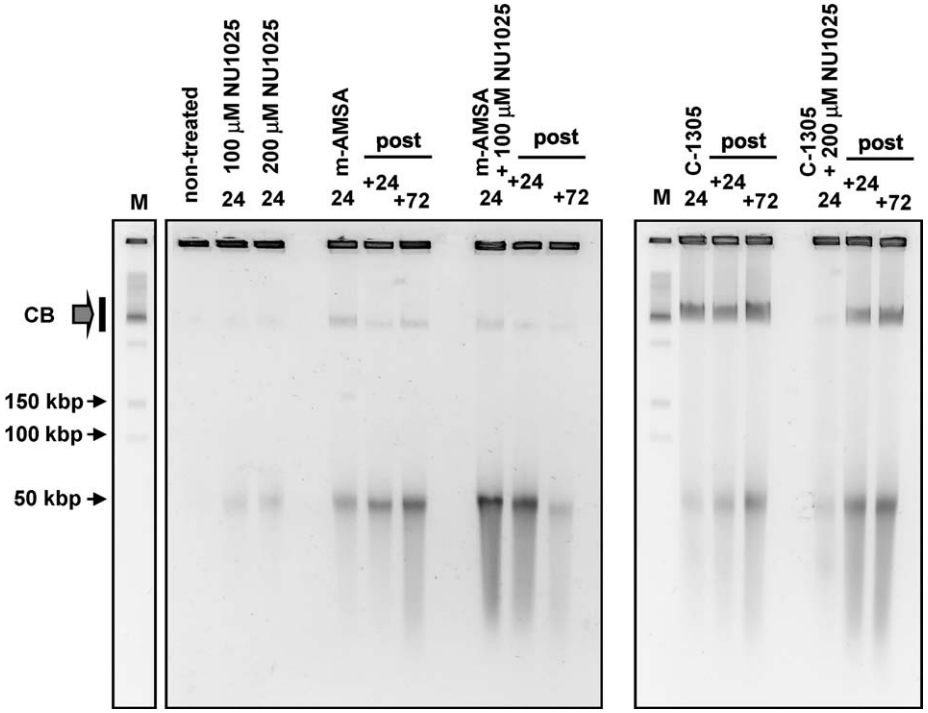

Fig. 7. DNA fragmentation induced in HeLa cells by topoisomerase II inhibitors alone or in combination with NU1205. Cells were treated with NU1025 and NU1025/drug combinations and post-incubated in drug-free medium for the time indicated.

suppression of both isoforms of topoisomerase II but cellular levels of topoisomerase IIalpha and beta returned to levels observed in non-treated cells. These results suggest that both drugs and drug/NU1025 combination influenced the expression of topoisomerase II but this was not directly associated with the cytotoxic effect toward HeLa cells exerted by both studied drugs.

3.7. DNA damage induced by studied topoisomerase II inhibitors and drug/NU1025 combinations

Decreased proteasome activity may also stabilize DNA-topoisomerase II cleavable complexes formed in the presence of topoisomerase II inhibitors, which leads to increased levels of double-stranded DNA breaks [22]. To clarify this point, we analysed DNA fragmentation induced by drug treatment by pulse field gel electrophoresis. As shown in Fig. 7, NU1025 by itself induced low but measurable DNA fragmentation in HeLa cells after a 24 h exposure to the inhibitor. Combination of *m*-AMSA/NU1025 led to increased DNA fragmentation, compared to the effect induced by the drug alone. Similar effect was observed for C-1305/NU1025 and this drug combination produced higher levels of DNA fragmentation, mainly to 50 kbp fragments compared to the effect induced by C-1305 alone (Fig. 7). Overall, these results suggest that increased cytotoxic activity of drug/NU1025 combinations may be related to increased DNA damage induced in drug-treated HeLa cells.

4. Discussion

Recent advent of more selective and less toxic inhibitors of PARP-1 opened new possibilities for combination therapies which could improve antitumor activity of DNA damaging agents [4,8–10]. However, enhanced sensitivity of tumor cells in the presence of PARP-1 inhibitors has been confirmed for some but not all DNA damaging drugs. For example, cytotoxic and antitumor activities of topoisomerase I inhibitors, alkylating agents and other DNA damaging agents have been shown to be potentiated by different inhibitors of PARP-1 activity [10,16,23–26]. In contrast, combination of PARP-1 inhibitors and anti-topoisomerase II-directed drug etoposide was less active toward tumor cells compared to the effect of this drug alone [7].

In our previous studies we have shown that mouse cells with genetically inactivated *PARP-1* gene are extremely sensitive to triazoloacridone compound C-1305 [13], an inhibitor of DNA topoisomerase II with unusual properties [12]. We attributed this oversensitivity of PARP-1 −/− cells to the induction of permanent arrest in G2 and M following treatment with C-1305. In this study, we wanted to clarify if one may reproduce unusual oversensitivity to our triazoloacridone of tumor cells with attenuated PARP-1 activity by chemical inhibitors of this enzyme. Our results show that pharmacological inhibition of PARP-1 by NU1025 sensitizes HeLa cells to compound C-1305 and another well-known topoisomerase II inhibitor, *m*-AMSA. We show that this increased cytotoxic effect of both studied drugs toward cells with low PARP-1 activity is associated with re-activation of the p53 pathway in drug-treated cells, at least partially due to a strongly reduced proteasome activity. Activation of p53 in drug-treated cells facilitated cell death during G2 and mitosis and, at the same time, prevented cells from entering polyploidy.

One of the unexpected findings of this study is that inhibition of PARP-1 in HeLa cells by NU1025 led to induction of p53 and this effect was further potentiated by both studied topoisomerase II inhibitors. Increased p53 intracellular levels were associated with increased stability of the protein in cells treated with drug/NU1025 combinations. Previous studies have shown that inhibition of PARP-1 leads to decreased 20S proteasome activity [27]. In agreement, our results show that greatly reduced proteasome activity is observed when cells were treated with both drugs and drug/NU1025 combinations. Intriguingly, NU1025 by itself at both studied doses had no or very little effect on the proteasome activity but still effectively induced p53 levels. However, it should be noted that after treatment of cells only with both studied drugs, p53 was induced for *m*-AMSA but not C-1305, whereas both drugs were equally efficient in inhibiting proteasome activity. It follows that it is not proteasome inhibition *per se* that is responsible for increased stability of p53 protein after drug treatment.

Proteasome has also been suggested to be involved in the repair/processing of topoisomerase II-DNA adducts induced by inhibitors of this enzyme [28] and inhibition of proteasome potentiates the cytotoxic activity of topoisomerase II inhibitors [29]. Decreased activity of 20S proteasome in tumor cells treated with PARP-1 inhibitor may explain higher DNA fragmentation observed in HeLa cells treated with drug/NU1025 combinations. This may result from a possible interference with the degradation of cleavable complexes between DNA and topoisomerase II, stabilized in the presence of its inhibitors. Differences in DNA damage induced by studied drugs do not result from changed topoisomerase II levels since no apparent correlation was observed between cellular levels of both isoforms of topoisomerase II and cytotoxic activity of both drugs or drug/NU1025 combinations. Yet another explanation of increased damage in cells treated with drug/NU1025 combination could be the effect of NU1025 on the repair of DNA strand breaks produced in the situation when topoisomerase II is inhibited. Recent studies have shown that NU1025 effectively inhibits rejoining of DNA breaks produced by hydrogen peroxide [30,31].

In HeLa cells, p53 protein is not detectable even after severe DNA damage induced by antitumor drugs, e.g. cisplatin due to E6-mediated degradation of p53 [19,20]. Another possibility was that both studied drugs repress the expression of E6 oncoprotein in HeLa cells that leads to increased stability of p53 protein. However, in our studies we observed unchanged or increased E6 levels in HeLa cells after treatment with both studied topoisomerase II inhibitors and NU1025. Thus, the cellular accumulation of p53 protein in HeLa cells exposed to studied drugs and drug combinations could not be attributed to the repression of E6 oncoprotein by drug treatment.

Re-activation of p53 functionality in HeLa cells by drug/NU1025 combinations greatly influenced the functionality of cell cycle checkpoints induced by DNA topoisomerase II inhibitors during G2 and mitosis. In consequence, we observed increased cytoxicity of drug/NU1025 combinations toward HeLa cells. We showed that the fraction of polyploid cells is greatly suppressed after treatment with C-1305 or *m*-AMSA in combination with NU1025, compared to the situation observed after treatment with either drug alone. This was associated with the accumulation of aberrant mitotic cells and suggests that HeLa cells treated simultaneously with PARP-1 inhibitor and topoisomerase II poisons die during mitosis, instead of undergoing endoreduplication. Interestingly, earlier studies showed increased but not decreased polyploidy in glioblastoma cells treated with topotecan/NU1025 combination [10]. Several reports showed that PARP-1 directly influences the DNA damage checkpoint regulators such as ATM, Aurora B and centromere proteins, which become inactivated by poly-ADP-ribosylation [32–34]. In addition, both spindle and M exit checkpoints are defective in PARP-1 −/− fibroblasts [35], although inhibition of PARP-1 activity by synthetic inhibitors does not lead to tetraploidy [36]. DNA damage during G2 and mitosis may activate spindle checkpoint [37] and our previous studies pointed to the possible crosstalk between DNA damage checkpoint in G2 and spindle checkpoint during mitosis [38].

In conclusion, we show here that inhibition of PARP-1 activity by NU1025 leads to increased sensitivity of HeLa cervical carcinoma cells to triazoloacridone C-1305 and *m*-AMSA, two different anti-topoisomerase II drugs. Our results show that this enhanced cytotoxic effect could be attributed to re-activation of p53 function by PARP-1 inhibitor alone and in combination with topoisomerase II inhibitors. Re-activation of p53 function leads to changed functionality of cell cycle checkpoints in cells with low PARP-1 activity and enhanced mitotic cell death induced by drug/NU1025 combinations. The molecular mechanism of p53 re-activation in HeLa cells treated with NU1025 and studied drugs involves, at least in part, inhibition of proteasome activity by drug treatment but not downregulation of E6 expression.

Re-activation of p53 in HeLa cells by drug/NU1025 combinations by inhibition of proteasome activity, is potentially important for combination therapies directed toward ovarian carcinoma cells. Inactivation of p53 protein due to expression of viral E6 oncoprotein is very frequent in cervical carcinomas. This leads to lower sensitivity of tumor cells to anticancer treatment due to defects in p53-dependent apoptotic pathway. Accordingly, clinical data show that inactivation of the p53 pathway is associated with poor outcome in patients with ovarian carcinomas [39]. Re-activation of p53 function is a promising approach to sensitize tumor cells to anticancer agents. This approach was recently successfully applied to E6-expressing tumors by use of small molecular weight compounds which were able to specifically suppress E6 protein and re-activated p53 function [40]. In addition to that, both isoforms of topoisomerase II are frequently overexpressed in cervical cancer patients [41]. In that way, another promising approach to sensitize E6-expressing cervical carcinomas is to use proteasome inhibitors in combination with topoisomerase II inhibitors or, as our results show, drugs or drug combinations that inhibit both proteasome and topoisomerase II activity.

Acknowledgement

The authors would like to acknowledge the financial support from the Polish Ministry of Science and Higher Education, grant no. N401 157 31/3526.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.12.023.

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