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ORIGINAL ARTICLE

Comparison of the effect of three different topoisomerase II inhibitors combined with cisplatin in human glioblastoma cells sensitized with double strand break repair inhibitors

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Abstract

Topoisomerase II (Topo2) inhibitors in combination with cisplatin represent a common treatment modality used for glioma patients. The main mechanism of their action involves induction of DNA double-strand breaks (DSBs). DSBs are repaired via the homology-dependent DNA repair (HRR) and non-homologous end-joining (NHEJ). Inhibition of the NHEJ or HRR pathway sensitizes cancer cells to the treatment. In this work, we investigated the effect of three Topo2 inhibitors—etoposide, NK314, or HU-331 in combination with cisplatin in the U-87 human glioblastoma cell line. Etoposide as well as NK314 inhibited Topo2 activity by stabilizing Topo2-DNA cleavable complexes whereas HU-331 inhibited the ATPase activity of Topo2 using a noncompetitive mechanism. To increase the effectiveness of the treatment, we combined cisplatin and Topo2 inhibitor treatment with DSB repair inhibitors (DRIs). The cells were sensitized with NHEJ inhibitor, NU7441, or the novel HRR inhibitor, YU238259, prior to drug treatment. All of the investigated Topo2 inhibitors in combination with cisplatin efficiently killed the U-87 cells. The most cytotoxic effect was observed for the cisplatin + HU331 treatment scheme and this effect was significantly increased when a DRI pretreatment was used; however, we did not observed DSBs. Therefore, the molecular mechanism of cytotoxicity caused by the cisplatin + HU331 treatment scheme is yet to be evaluated. We observed a concentration-dependent change in DSB levels and accumulation at the G2/M checkpoint and S-phase in glioma cells incubated with NK314/cisplatin and etoposide/cisplatin. In conclusion, in combination with cisplatin, HU331 is the most potent Topo2 inhibitor of human glioblastoma cells.

Keywords Human glioblastoma · Topo2 inhibitor · DNA double strand breaks · NHEJ · HRR

Introduction

Topoisomerases are present in both eukaryotic and prokaryotic organisms; the first member of this class of enzymes, the ω protein (EC 5.99.1.2) belongs to type I topoisomerases and was discovered in 1971 in *Escherichia coli* [1]. Human cells encode six different topoisomerases: Topo1, Topo1mt, Topo2α, Topo2β, Topo3α, and Topo3β [2]. They are responsible for controlling DNA topology and chromatin dynamics in various cellular processes including DNA repair. They can solve topological problems that appear during replication and transcription. This makes topoisomerases one of the essential components involved in the maintenance of genomic stability [3, 4]. Topoisomerases are very attractive targets in the development of new anticancer therapies [5–10]. The main molecular targets for topoisomerase inhibitors are monomeric topoisomerase 1 (Topo1) and multimeric topoisomerase 2 (Topo2). Mechanism of action of Topo1 involves formation of reversible single-strand breaks (SSBs) in DNA molecule, whereas one of crucial steps in Topo2 activity is breaking and rejoining double-strand breaks (DSBs). According to their structure and mechanism of

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action, two different subclasses (α and β) are present among Topo1 and Topo2 [4, 11].

Studies that concern topoisomerases as target for anticancer treatment are focused mainly on Topo2-specific inhibitors. Topo2 seems to be a more relevant target than Topo1 because of certain unique features: Topo2 can control and modify the topological state of chromosomes by introducing DSBs into DNA. Additionally, one of the Topo2 isoforms—Topo2α—is a cell cycle-dependent enzyme, overexpressed in fast-proliferating cells [12, 13]. According to their mechanism of action, Topo2 inhibitors can be divided into two main groups: topoisomerase poisons (e.g., etoposide, mitoxantrone, NK-314) or topoisomerase catalytic inhibitors (e.g., MST-16) [14, 15]. "Topoisomerase poisons" cause an increase of cleavable Topo2:DNA complexes. The level of complexes depends on the concentration of Topo2 in cells—it is higher in fast-proliferating cancer cells compared to normal cells [16]. In consequence, "topoisomerase poisons" that target Topo2 generate enzyme-mediated DSBs. In case of catalytic inhibitors of topoisomerases, a similar effect is observed, but following a longer exposure to these compounds [17]. Topoisomerase catalytic inhibitors are a group of compounds that exhibit structural diversity, and their mechanism of action is based on interactions with Topo2 in the different steps of the catalytic cycle [14].

In the recent years, Topo2 inhibitors are widely tested as potential anticancer drugs in the combined treatment against different types of human cancer. Promising results were obtained for combined treatment (doxorubicin with exportin 1 [XO1] inhibitor) of drug-resistant multiple myeloma [18], cervical cancer (Top2 inhibitor—Thiazolo[5,4-*b*]quinoline derivative, D3CLP—with cisplatin) [19], renal cell carcinoma (etoposide with 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, 15d-PGJ_2) [20], and brain tumors (etoposide with oncolytic herpes simplex virus) [21, 22].

In this study, we investigated the effect of combined treatment of cisplatin with three different Topo2 inhibitors on human glioblastoma cells. Although penetration of platinum-based compounds in to central nervous system is described as moderate, the effectiveness of its treatment against malignant brain tumors has been proven [23]. From among the known Topo2 inhibitors, we decided to choose three compounds. The first is etoposide. It is a widely used, model compound, which inhibits Topo2 activity and stabilizes the Topo2-DNA cleavable complex [24, 25]. Nevertheless, etoposide as well as other well-known Topo2 inhibitors exhibit some limitations in the form of severe side effects or as development of secondary tumors [16]. To overcome these limitations, novel topoisomerase inhibitors were tested. We decided to introduce two of them in our studies. The first one—NK314 is a synthetic benzo[*c*]phenanthridine alkaloid specific for Topo2α isoform [26]. The second one is HU331; it belongs to the group of quinones, which are described as compounds with promising anticancer properties. HU331 inhibits the ATPase activity of Topo2 using a noncompetitive mechanism [23]. To improve further the effect of the treatment of cisplatin combined with Topo2 inhibitors, which directly and indirectly introduce DSBs into glioblastoma cells, we decided to sensitize the U-87 glioblastoma cells by introducing DSB repair inhibitors (DRIs) of the two main DSB repair pathways: HRR and NHEJ. We found, that in combination with cisplatin, NK314 is the most potent Topo2 inhibitor of human glioblastoma cells.

Materials and methods

Cell culture

U-87 cell line was purchased from European Collection of Authenticated Cell Cultures from Sigma-Aldrich (St. Louis, MO). U-87 represent human glioblastoma astrocytoma and was derived by explant technique from a malignant glioma obtained from a female patient [24].

Cells were maintained in Eagle's Minimum Essential Medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate (NaP), 1% (v/v) of nonessential amino acids (NEAA), 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) penicillin/streptomycin. FBS and penicillin/streptomycin were obtained from Corning (Tewksbury, MA), the rest of the reagents for cell culture were purchased from Sigma-Aldrich (St. Louis, MO). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

Viability assay—CCK-8 Kit

Exponentially growing cells (5×10^3/well in 100 µL) were seeded into 96-well plates. After at least 24 h, drug(s) with and without pretreatment with DRI were added to the plate (at least three replicates). Cisplatin and etoposide were obtained from Sigma-Aldrich (St. Louis, MO). HU-331 and NU7441 were obtained from SelleckChem (Houston, TX), whereas NK314 was from Adooq Bioscience (Irvine, CA). YU238259 was synthesized according to previous reports [27] by TriMen Chemicals (Lodz, Poland). All of them were made up as stocks and stored at − 20 °C. Cisplatin was dissolved in water, whereas other drugs were dissolved in anhydrous dimethyl sulfoxide (DMSO).

After 48 h of incubation, growth inhibition was assessed by a Cell Counting 8 (CCK-8) kit (Sigma Aldrich, Poland). Reduction of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) to a water-soluble orange formazan allows detection of the activity of cellular dehydrogenases. The amount of formazan is proportional to the number of living cells. Plates were read on a plate reader at the wavelength of

450 nm. Results are presented as the percentage of control (untreated cells). All experiments were performed in triplicate. The concentration that induced 50% growth inhibition (IC_{50}) was estimated using Compusyn software [28].

Evaluation of the influence of DSB repair inhibitors on the interactions between drugs

As an indicator of chemosensitization, we used a reduction factor (Rf) value. Reduction factor was calculated from the ratios of the IC_{50} of the drug(s) without inhibitors to the IC_{50} obtained after pretreatment with inhibitors. Rf > 1 indicates chemosensitization. For combined treatment (cisplatin + Topo2 inhibitor), the ratio of drug I dose to drug II dose was kept constant (based on the IC_{50} value). To determine interactions between tested compounds, the combination index (CI) was calculated. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively. All of the calculations were performed using Compusyn software [28]. To determine the influence of DRI on cells treated with cisplatin and different Topo2 inhibitors, cells were exposed to tested compounds according to Table 1.

Comet assay

The level of DNA damage and the process of DSBs repair were investigated using alkaline version of comet assay. We decided to use alkaline version instead of neutral due to two main reasons: the neutral version is characterized by lower sensitivity compared to the alkaline one. Additionally, the neutral version still is not specific for DSBs [29]. DNA damage level was measured after treatment with the test compounds. To investigate the process of DNA repair, the test compounds were replaced with fresh, drug-free medium. Samples were collected after repair incubation in the timepoints between 0 and 6 h after removing tested compounds. The level of DNA damage was compared with the initial level obtained immediately after treatment with tested compounds.

The suspensions of U-87 cells in 0.75% LMP agarose were placed onto microscope slides precoated with 0.5% NMP agarose. The cells were then lysed overnight at 4 °C, pH 10 in buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% (v/v) Triton X-100. After lysis, the slides were equilibrated for the 20 min with buffer appropriate for the version of comet assay and electrophoresis was performed at pH > 13, under conditions: 17 V, 32 mA, 20 min. After electrophoresis, the slides were dried and stained with DAPI (5 µg/mL) (Sigma Aldrich, Poland).

DNA damage and repair analysis

Results were measured in an Eclipse fluorescence microscope (Nikon, Japan) attached to a COHU 4910 video camera (Cohu, USA) equipped with a UV filter block consisting of an excitation filter (359 nm) and a barrier filter (461 nm) and connected to a personal computer-based image analysis system, Lucia Comet 4.51 (Laboratory Imaging, Czech Republic). The level of DNA damage was expressed as percentage of DNA in the comet tail. Each experiment was performed in triplicate, for each experimental point the number of counted cells was 100.

Cell cycle analysis

Cells were treated with test compounds for 48 h, then were fixed with 96% ethanol and stained with PI (40 µg/mL), and DNase-free RNase (200 µg/mL) for 30 min at 37 °C. DNA content was analyzed with a LSRII flow cytometer (Becton Dickinson, USA). For each experiment, positive (cells exposed to 10 µM nocodazole), negative (untreated cells), and unstained control samples were prepared. All experiments were performed in triplicate. Cell cycle distribution was expressed as a percentage of cells in each phase of the cell cycle.

Detection of apoptosis

To detect apoptosis, we used the FITC Annexin V Apoptosis Detection Kit II (Becton Dickinson, USA). Annexin V conjugated with FITC has high affinity to phosphatidylserine, which is translocated to the outer part of cellular membrane in the early steps of apoptosis. To distinguish apoptosis and necrosis, propidium iodide (PI) staining was performed. After 6 h of treatment with the test inhibitors and drugs, cells were prepared as described in the manufacturer instructions and analyzed with a LSRII flow

Table 1 The scheme of sensitization human glioblastoma cells (treated with cisplatin and etoposide, NK314 and HU331) to DRI

I. Cisplatin + Topo2 inhibitor	II. Cisplatin + Topo2 inhibitor + HRR inhibitor	III. Cisplatin + Topo2 inhibitor + NHEJ inhibitor
Cisplatin + etoposide	Cisplatin + etoposide + YU238259	Cisplatin + etoposide + NU7441
Cisplatin + NK314	Cisplatin + NK314 + YU238259	Cisplatin + NK314 + NU7441
Cisplatin + HU331	Cisplatin + HU331 + YU238259	Cisplatin + HU331 + NU7441

cytometer (Becton Dickinson, USA). For each experiment, positive (cells exposed to 100 µM camptothecin), negative (untreated cells), and unstained control samples were prepared. It is known, that apoptosis can be measured after different times of exposure to compounds: from short (4–6 h), medium (24 h) to long (48 h). We decided to choose 6 h treatment with drugs due to high cytotoxic effect observed after treatment with drugs. The apoptosis ratio was defined as a percentage of apoptotic (FITC–Annexin V positive, PI negative) cells in the sample, while necrotic cells as the percentage of PI positive cells. All experiments were performed in triplicate.

Data analysis

All the values for viability tests and for DNA repair analysis were expressed as means ± SEM from three separate experiments. The analysis of interaction between drugs was derived from the mass-action law and based on the median-effect principle. Calculation of the CI value requires an IC_{50} value, which was calculated from median-effect plots for each of tested compounds and for their combination. After that, the corresponding dose for given level of effect (i.e. percentage of affected/non affected cells) was determined. The detailed equations needed for calculating CI values were described by Chou and Talalay [30]. Reduction factor (Rf) was calculated as a ratio of the CI obtained for the drug combination treatment in cells untreated with a DRI to the CI obtained after pretreatment with each of the DRI. For DNA damage analysis results were expressed as median, the lower and upper quartile represent observations outside the 9–91 percentile range. The differences were assessed with Mann–Whitney U test. Data analysis and figure drawing was performed using GraphPad Prism software v. 5 (GraphPad Software Inc., CA).

Results

Cisplatin and Topo2 inhibitors decrease the viability of U-87 cells

We compared the effect of three Topo2 inhibitors—etoposide, NK314, and HU-331 (Fig. 1) in combination with cisplatin on U-87 cells sensitized by the two DRIs (YU238259 and NU7441). The effect of each compound on the growth of U-87 cells was determined after 48 h of treatment using a colorimetric Cell Counting Kit-8. YU238259 and NU7441, from a low to high dose (0–200 µM), did not inhibit cellular growth. Cisplatin and Topo2 inhibitors (tested as single compounds) significantly decreased the viability of U-87 cells in a concentration-dependent way. The IC_{50} value was calculated for each compound using Compusyn software (Table 2).

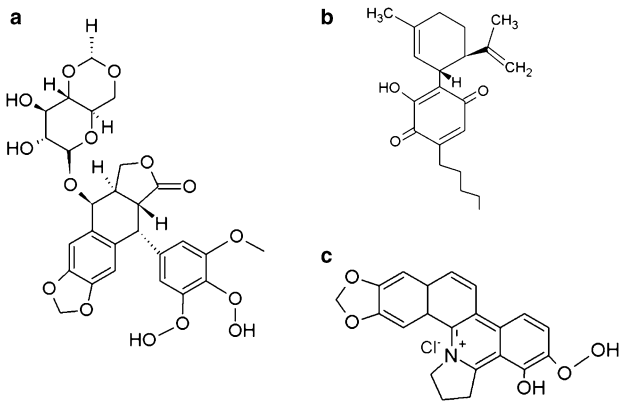

Fig. 1 Molecular structures of three topoisomerase II inhibitors used in the present study: etoposide (**a**), HU-331 (**b**) and NK-314 (**c**)

Table 2 IC_{50} values obtained for U-87 cells treated with cisplatin, Topo2 inhibitors (etoposide, NK314 and HU331) and double strand breaks inhibitors (DRI: YU238259 and NU7441)

Compound	IC_{50} value (µM)
Cisplatin	36.18 ± 1.55
Etoposide	48.43 ± 1.68
NK314	6.18 ± 0.78
HU331	9.51 ± 0.97
YU238259	> 1000 ± 2.47
NU7441	296.38 ± 3.27

IC_{50}—concentration that causes 50% growth inhibition ± SD

Among Topo2 inhibitors, the highest inhibitory effect on the U-87 cells growth was observed after treatment with NK314 (IC_{50} = 6.18 ± 0.78 µM).

Based on IC_{50} values obtained for each compound, the doses of the drugs for the combined treatment were calculated. To determine the effect of combined treatment, U-87 cells were exposed to increasing concentrations of cisplatin and Topo2 inhibitors. The ratio of drugs was constant (1:1) and concentrations were equal to ¼ IC_{50} for cisplatin + ¼ IC_{50} for each of Topo 2 inhibitors, ½ IC_{50} for cisplatin + ½ IC_{50} for each of Topo 2 inhibitors, IC_{50} for cisplatin + IC_{50} for each of Topo 2 inhibitors, 2 IC_{50} for cisplatin + 2 IC_{50} for each of Topo 2 inhibitors, and 4 IC_{50} for cisplatin + 4 IC_{50} for each of Topo 2 inhibitors (Tables 3, 4). Inhibitory effect on the U-87 cells growth was observed for each of the drug combination. The most significant decrease of the viability of U-87 cells was obtained after treatment with cisplatin and HU331, next NK314, and finally etoposide as seen on Fig. 2a.

Table 3 Combination index (CI) and reduction factor (Rf) values obtained for U-87 cells after treatment with cisplatin and Topo2 inhibitors: etoposide (a), NK314 (b) and HU331 (c)

(a)					
Total dose of cisplatin + etoposide (μM)	CI for cisplatin + etoposide	CI for cisplatin + etoposide + NU7441	Rf value	CI for cisplatin + etoposide + YU238259	Rf value
21.152	0.44	0.56	0.79	0.47	0.93
42.305	0.7	0.77	0.89	0.71	0.98
84.61	1.53	1.21	1.41	1.43	1.10
169.22	3.37	2.35	1.69	2.98	1.18
338.44	4.73	4.51	1.09	4.15	1.26
(b)					
Total dose of cisplatin + NK314 (μM)	CI for cisplatin + NK314	CI for cisplatin + NK314 + NU7441	Rf value	CI for cisplatin + NK314 + YU238259	Rf value
10.59	0.65	0.57	1.19	0.57	1.07
21.18	1.1	1.09	1.01	0.83	1.28
42.36	1.92	1.72	1.10	1.72	1.11
84.72	1.41	2.18	0.48	1.46	0.93
169.44	2.25	2.25	1.00	2.26	0.99
(c)					
Total dose of cisplatin + HU331 (μM)	CI for cisplatin + HU331	CI for cisplatin + HU331 + NU7441	Rf value	CI for cisplatin + HU331 + YU238259	Rf value
11.42	0.64	0.64	1.00	0.51	1.24
22.84	0.63	0.59	1.12	0.56	1.33
45.69	0.76	0.94	0.65	0.75	0.98
91.38	1.5	1.45	1.08	1.41	1.15
182.76	2.71	2.71	1.00	2.70	1.01

CI and Rf values were calculated using Compusyn software. CI < 1, CI = 1 and CI > 1 indicate synergism, additive effect and antagonism, respectively. Rf > 1 indicates chemosensitization

DSB repair inhibitor pretreatment enhances the cytotoxic effect of the combined treatment of cisplatin with Topo2 inhibitors

The effect of cisplatin and Topo2 inhibitors on U-87 cells sensitized by either of the two DRIs—YU238259 or NU7441—was measured after 48 h exposure to the test compounds. The cells were exposed to 10 μM DRI 60 min prior to the combined cisplatin/Topo2 inhibitor treatment (Fig. 2b–d).

Analysis of interactions between the drugs was based on two parameters: combination index (CI) and reduction factor (Rf) value. The CI was calculated with Compusyn software, based on the IC_{50} values determined for the combined drug treatment with and without pretreatment with DRI. CI < 1 indicated synergism between the test compounds, whereas CI > 1 indicated antagonism, and CI = 1 showed an additive effect. Rf value indicates the level of sensitization (Rf > 1) and was calculated as a ratio of the CI for the drug combination treatment in cells untreated with a DRI to the CI obtained after pretreatment with each of the DRI. We observed that introducing DRIs in the treatment scheme caused slight changes in interactions between cisplatin and Topo2 inhibitors. At lower concentration of drugs (¼ IC_{50}, ½ IC_{50}) interactions were synergistic, but introduction of the DRI did not affect this parameter, especially after treatment of cisplatin combined with etoposide and NK314. At higher concentrations, the interaction between drugs was more antagonistic; however, after exposure to DRIs, we observed the sensitization (Rf > 1) effect and a decrease in the CI value. This effect was more pronounced for NU7441 than for YU238259 (Table 3a, b). CI values obtained for cisplatin combined with HU331 revealed that interactions between these two drugs are synergistic at higher concentrations. Pretreatment with a DRI caused further decrease of this parameter. It suggested the ability of DRIs to sensitize U-87 cells to the combined treatment with cisplatin and HU331 (Table 3c).

DSB repair inhibitors cause an accumulation of DSBs in human glioblastoma cells treated with cisplatin and Topo2 inhibitors

The DSB level was measured by comet assay. Based on the results obtained in the viability test and on analysis of

Table 4 The ratio of cisplatin and three Topo2 inhibitors: etoposide (a), NK314 (b), and HU-331 (c) used in combination study

(a)	
Total dose of cisplatin + etoposide	Total dose of cisplatin + etoposide (μM)
¼ IC_{50} (cisplatin) +¼ IC_{50} (etoposide)	21.152 (9.045 + 12.107)
½ IC_{50} (cisplatin) + ½ IC_{50} (etoposide)	42.305 (18.09 + 24.215)
IC_{50} (cisplatin) + IC_{50} (etoposide)	84.61 (36.18 + 48.43)
2 IC_{50} (cisplatin) + 2 IC_{50} (etoposide)	169.22 (72.36 + 96.86)
4 IC_{50} (cisplatin) + 4 IC_{50} (etoposide)	338.44 (144.72 + 193.72)
(b)	
Total dose of cisplatin + NK 314	Total dose of cisplatin + NK 314 (μM)
¼ IC_{50} (cisplatin) +¼ IC_{50} (NK 314)	10.59 (9.045 + 1.545)
½ IC_{50} (cisplatin) + ½ IC_{50} (NK 314)	21.18 (18.09 + 3.09)
IC_{50} (cisplatin) + IC_{50} (NK 314)	42.36 (36.18 + 6.18)
2 IC_{50} (cisplatin) + 2 IC_{50} (NK 314)	84.72 (72.36 + 12.36)
4 IC_{50} (cisplatin) + 4 IC_{50} (NK 314)	169.44 (144.72 + 24.72)
(c)	
Total dose of cisplatin + HU 331	Total dose of cisplatin + HU 331 (μM)
¼ IC_{50} (cisplatin) +¼ IC_{50} (HU 331)	11.422 (9.045 + 2.377)
½ IC_{50} (cisplatin) + ½ IC_{50} (HU 331)	22.845 (18.09 + 4.755)
IC_{50} (cisplatin) + IC_{50} (HU 331)	45.69 (36.18 + 9.51)
2 IC_{50} (cisplatin) + 2 IC_{50} (HU 331)	91.38 (72.36 + 19.02)
4 IC_{50} (cisplatin) + 4 IC_{50} (HU 331)	182.76 (144.72 + 38.04)

Two DRIs (YU238259 and NU7441) were used for sensitization of cells before exposure to cisplatin and Topo2 inhibitors at a constant concentration equal to 10 μM for each of DRIs

synergy, for further research, we decided to choose concentrations equal to ½ IC_{50} for cisplatin and etoposide and ¼ IC_{50} for cisplatin combined with HU331 and NK314. U-87 cells were sensitized by preincubation with 10 μM of YU238259 or NU7441 for 60 min prior to treatment with drugs. Cells were then exposed to cisplatin and a test Topo2 inhibitor for 120 min. Exposure to cisplatin combined with any of the test Topo2 inhibitors caused an increase in DSB levels in U-87 cells, compared to untreated control. However, introduction of DRIs in the treatment scheme did not affect the DNA damage levels in U-87 cells treated with cisplatin and etoposide. Statistically significant increase in cisplatin/HU331-induced level of DNA damage was observed after pretreatment with one of DRIs—NU7441. The effect of both DRIs—NU7441 and YU238259—was more pronounced in the case of cells treated with cisplatin and NK314. The level of drug-induced DNA damage was significantly higher after sensitization of U-87 cells with both of the DRIs; however, this effect was more significant for cells sensitized with NU7441 (Fig. 3a).

DSB repair inhibitors modulate the process of DNA repair in human glioblastoma cells treated with cisplatin and Topo2 inhibitors

Efficiency of DNA repair was evaluated after 60 min of pretreatment with 10 μM DRI, 120 min of exposure to cisplatin combined with a Topo2 inhibitor and further 6 h of repair incubation. We observed decrease in the level of DSBs during repair incubation in drugs-free medium (Fig. 3b–d). The results are presented as a difference between initial level of DNA damage and level after pretreatment with DRI. We transformed the results of the comet assay to show the initial level of DNA damage as 100%. The effect of DRI is the difference between results obtained for each of experimental points and the initial 100%. After 6 h of repair incubation, we observed an efficient repair in U-87 cells treated with cisplatin and Topo2 inhibitors. We also observed differences in the efficiency of DNA damage repair between cells sensitized with DRI and cells treated only with cisplatin and Topo2 inhibitors. In cells exposed to cisplatin and etoposide, we observed positive effect after introducing NU7441 to the scheme of treatment (Fig. 3b). In contrast, in cells treated with cisplatin and NK314 the effect of YU238259 was more pronounced (Fig. 3c). Also in cells exposed to cisplatin and

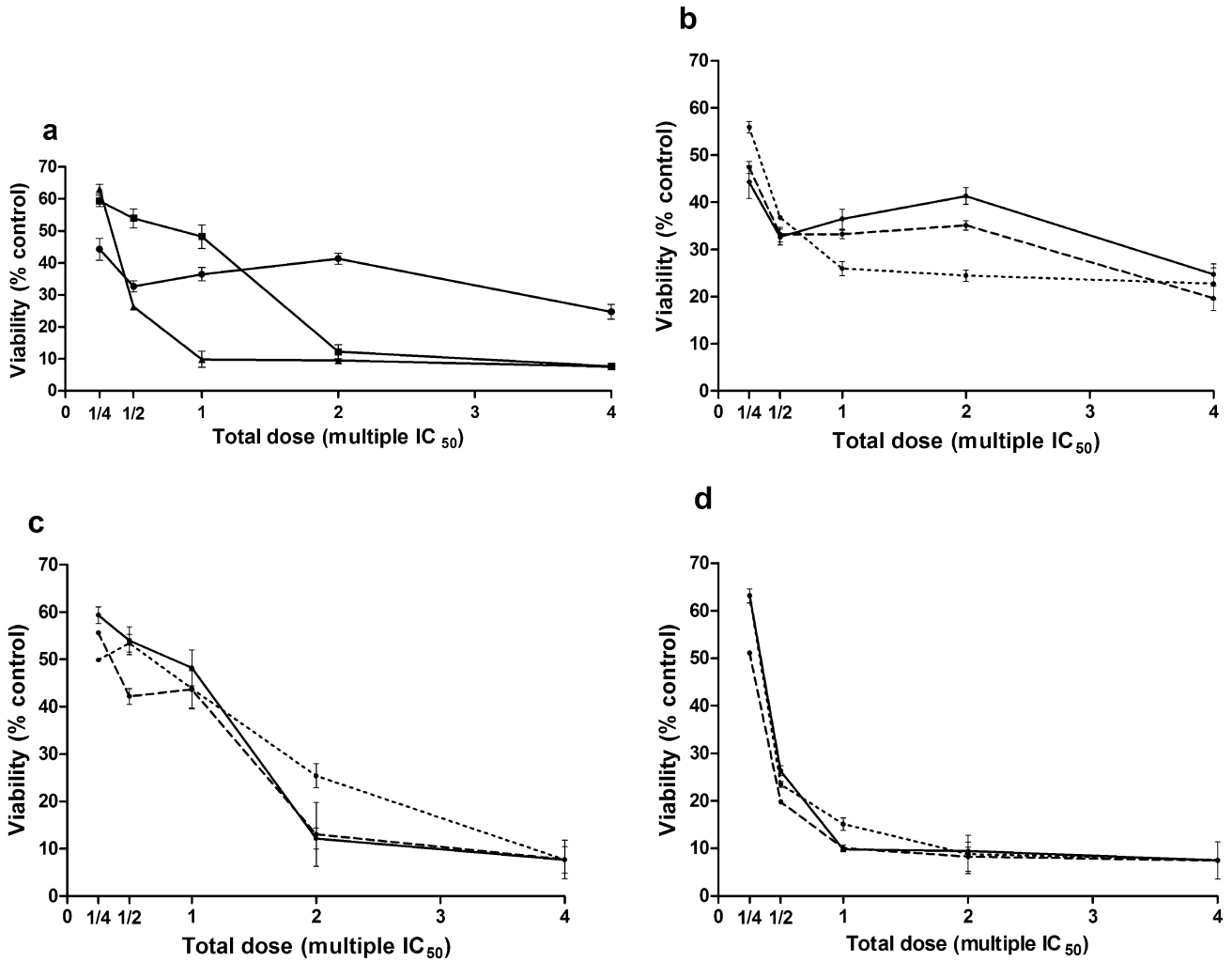

Fig. 2 Cytotoxic effect of cisplatin combined with three Topo2 inhibitors (**a**) and pretreated additionally with double-strand breaks inhibitors (DRI) (**c, d**) on U-87 cell line. Cisplatin combined with Topo2 inhibitors: etoposide (filled circle), NK314 (filled square) and HU-331 (filled triangle) caused concentration-dependent growth inhibition on U-87 cells. Cells were treated with increasing concentrations of drugs for 48 h (**a**). Growth inhibitory effect was observed for cells sensitized with DRI, treated with cisplatin (solid line) and Topo2 inhibitors: etoposide (**b**), NK314 (**c**) and HU-331 (**d**) on U-87 cells. Prior to treatment with drugs, cells were sensitized with double strand breaks inhibitors (DRI): YU238259 (dashed lines) and NU7441 (dotted lines). Cells were sensitized with 10 μM DRI by 1 h and then treated with increasing concentrations of drugs for 48 h. Cells were then stained with Cell Counting Kit-8 and the OD450nm was determined. Results are means ± SEM of at least three experiments

HU331, we observed an increase in the accumulation of DSBs, especially after pretreatment with NU7441 (Fig. 3d).

DSB repair inhibitors cause an accumulation of cells at the G2/M checkpoint of cell cycle in human glioblastoma cells treated with cisplatin and Topo2 inhibitors

We determined the influence of sensitization with two DRIs (YU238259, NU7441) on the cell cycle distribution in U-87 cells treated with cisplatin combined with Topo2 inhibitors (etoposide, NK314 and HU-331). Staining with propidium iodide (PI) and analysis by flow cytometry allowed determination of the percentage of cells in each phase of cell cycle. After 48 h treatment with cisplatin and Topo2, cells accumulated at the G2/M checkpoint, compared to untreated control. DRIs alone did not affect cell cycle distribution (data not shown). Introduction of DRIs to the scheme of treatment did not change the cell cycle distribution in U-87 cells treated with cisplatin and NK314. Among the two remaining schemes (cisplatin + etoposide, cisplatin + HU-331) we observed an increase in the number of cells accumulated in S phase and at the G2/M checkpoint (Fig. 4a–c).

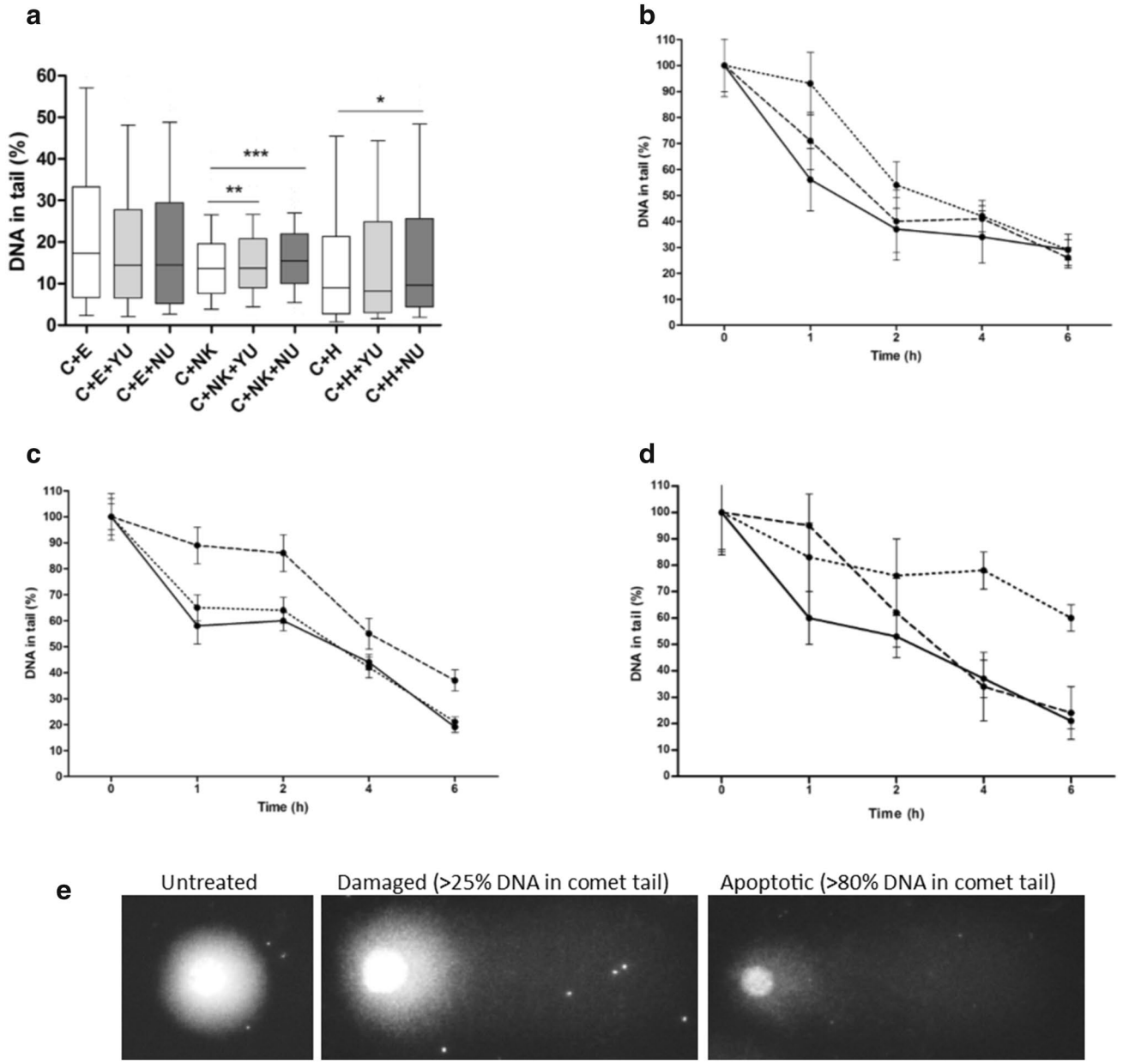

Fig. 3 Genotoxic effect of cisplatin combined with three Topo2 inhibitors and pretreated additionally with double-strand breaks inhibitors (DRI) on U-87 cell line. An effect of DRI: YU238259 and NU7441 on the cisplatin and Topo2—induced level of DNA damage on U-87 cells. Cells were treated with increasing concentrations of cisplatin combined with etoposide, NK314 and HU331 in the absence or presence 10 μM YU238259 and NU7441. After 60 min of pretreatment with DRI and 120 min exposure to the drugs, alkaline version of the comet assay was performed (**a**). The number of cells analyzed for each experimental point of comet assay was 100. The lower and upper quartile, represent observations outside the 9–91 percentile range. The diagram also shows the median results, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as compared with cells untreated with DRI. An effect of DRI: YU238259 and NU7441 on the repair of cisplatin and Topo2—induced level of DNA damage on U-87 cells. Cells were treated with increasing concentrations of cisplatin combined with etoposide (**b**), NK314 (**c**) and HU331 (**d**) in the absence (solid line) or presence 10 μM YU238259 (dashed lines) and NU7441 (dotted lines). Cells were exposed for 60 min to DRI and then for 120 min to the drugs. Efficiency of the DNA repair was evaluated after 6 h of repair incubation. Alkaline version of the comet assay was performed. Results are expressed as percentage of DNA in the comet tail, for each of time points the number of counted cells was 100. Representative microphotographs of results obtained in comet assay (**e**). C—cisplatin, E—etoposide, NK—NK314, H—HU331, YU—YU238259, NU—NU7441

Pretreatment with DSB repair inhibitors induce apoptosis in human glioblastoma cells treated with cisplatin and Topo2 inhibitors

To determine the influence of DRIs on induction of cellular death in U-87 cells treated with cisplatin and three Topo2 inhibitors, we decided to use Annexin V FITC apoptosis detection kit (Becton–Dickinson). Cells were exposed to 10 μM of a DRI prior to 6 h treatment with cisplatin and Top2 inhibitors. We observed that introduction of DRI to either the combined cisplatin/etoposide, or the cisplatin/NK314 and cisplatin/HU331 treatment scheme did not caused apoptosis or necrosis in U-87 cells (Fig. 4d–f).

Discussion

Standard treatment for gliomas involves surgery followed by radiotherapy and/or chemotherapy. Despite multimodal therapies, the median survival time of glioma patients is nearly 1 year and chemotherapy extends survival by about 2–2.5 months [31, 32]. Various combinations of chemotherapeutic drugs have been proposed for treatment against gliomas; however, the survival benefits for glioma patients remain unsatisfactory. Some of the current strategies are focused on the concurrent use of cisplatin and etoposide [33–35]. A combination of these drugs provides synergistic effects against glioma cells. Both compounds induce DSBs directly (etoposide) or indirectly. Cisplatin forms mono-, inter-, and intra-strand DNA adducts, which could be converted to DSBs during replication or transcription if unrepaired. Moreover, cisplatin adducts on DNA ends decreased the overall efficiency of NHEJ—a DSB repair pathway. Etoposide acts as a Topo2 inhibitor and forms Topo2-DNA cleavable complexes that are converted into DSBs. However, etoposide is not a perfect drug as it targets mainly Topo2β isoform. Inhibition of this isoform results in cardiotoxicity and etoposide therapy-related secondary tumors. Other Topo2 inhibitors such as NK314 and HU331 chosen for this study seem to lack these disadvantages. The first compound targets Topo2α isoform and the second one is a catalytic inhibitor that impedes the function of the Topo2 in an unknown, reversible or irreversible manner. In this study, we compared the anticancer effect of three Topo2 inhibitors: etoposide, NK314, and HU-331 in combination with cisplatin. We determined the nature of interaction calculating the combination index (CI) using median-effect analysis described by Chou and Talalay [28, 30]. The associations of cisplatin with HU331 were found to be the most effective among the tested combinations. In this combination, we observed the most synergic effect as compared to the cisplatin/etoposide and cisplatin/NK314 combinations. The synergism between cisplatin and HU331 was classified as moderate and was noted at low concentrations of the drugs. From a clinical point of view, it is significant that we observed synergism followed by strong cytotoxicity. This would allow decrease in drug doses during glioma treatment and lower any potential side effects. Similar associations were found for cisplatin/etoposide and these findings were consistent with those of other authors showing synergy

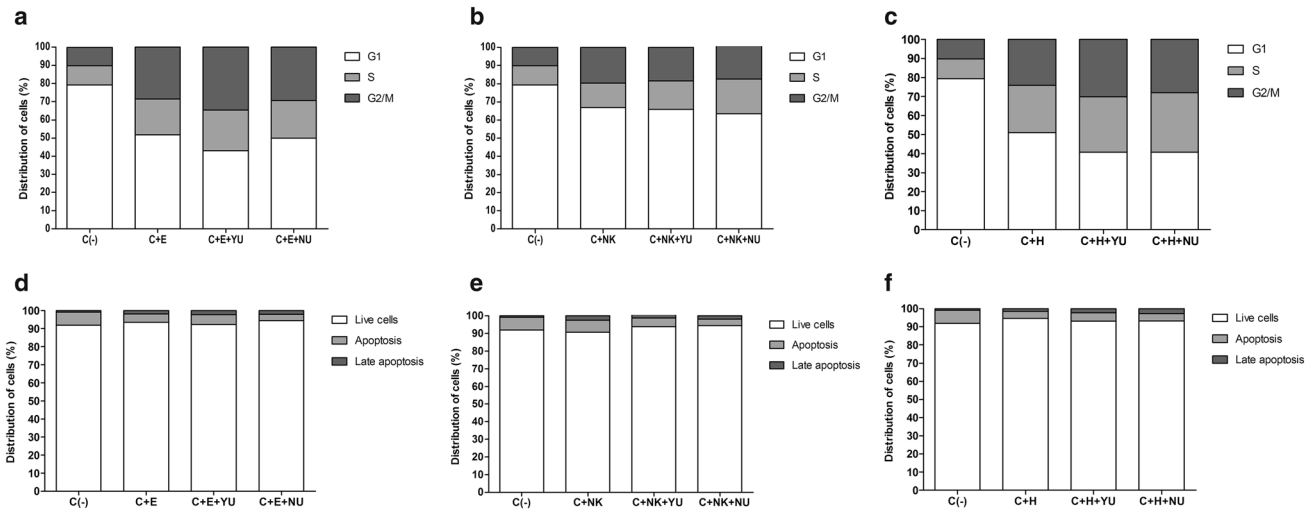

Fig. 4 An effect of DRI: YU238259 and NU7441 on cell cycle distribution and cellular death in U-87 cells. To analyze cell cycle distribution cells were treated with cisplatin combined with etoposide (**a**), NK314 (**b**) and HU331 (**c**) in the absence or presence 10 μM YU238259 and NU7441. Results are expressed as % of cells in the each phase of cell cycle. To detect apoptosis cells were treated with cisplatin combined with etoposide (**d**), NK314 (**e**) and HU331 (**f**) in the absence or presence 10 μM YU238259 and NU7441. Results are expressed as % of live, apoptotic and necrotic cells. C—cisplatin, E—etoposide, NK—NK314, H—HU331, YU—YU238259, NU—NU7441

between these two drugs in glioma treatment [36, 37]. However, synergism observed for cisplatin/etoposide combination has no impact on cytotoxic effect as we observed for cisplatin/HU331.

We have shown that cisplatin/NK314 is also a noneffective combination against glioma; although synergy was noted at lower concentrations, it was without appreciable cytotoxic effect. According to IC_{50} values the potency of NK314 is six fold higher than cisplatin and 1.5 fold higher than HU331 but NK314 and cisplatin combination did not work well. This effect seems to be typical for Topoisomerase 2 inhibitor/cisplatin combo treatment as we reported earlier. We have even observed an antagonistic effect between these drugs especially in DNA-PK-deficient cells. However, the detailed mechanism of this interaction remains unknown (please see [38] for details) but it can be reverse with DRI (as we shown here and at [38]). This suggest that the synergism between these two class of drugs requires at least DNA-PK and a functional DNA DSB pathway. Please note that DNA-PK is involved not only in direct DSB repair but also serve as a transcriptional modulator. It interacts with both the transcriptional machinery and transcription factors. These interaction that alter a variety of critical cellular processes associated with cancer including genomic instability, hypoxia, metabolism, and inflammatory response [39]. Thus, the observed effect could not be involved in direct DSA repair.

Synergistic effect observed for higher concentrations of Topo2 inhibitors combined with cisplatin can be a result of accumulation of different types of molecular interactions of these compounds with DNA. As it was mentioned above, cisplatin interacts with DNA thorough forming adducts. In contrast to cisplatin, etoposide was shown to be a poor DNA intercalator [40]. Etoposide alone has low affinity to DNA, but etoposide molecules are able to stabilize cleavable complex consisting of Topo2β and DNA by separation crucial catalytic residues of Top2β. It was determined, that etoposide prevents the religation of DNA ends by increasing the distance between the active-site tyrosine and the Mg^{2+}-chelating residues [41].

To better understand the potential mechanism of action of the studied combinations, additional analysis of synergy was performed using two DRI inhibitors. We presumed that introduction of DRIs to the treatment scheme would potentiate the cytotoxicity of the studied drugs as etoposide as well as cisplatin introduce DNA DSBs and such effect on glioma cells was reported by us earlier [38]. Surprisingly, the observed effect was smaller than expected from our other study [38]. Inhibition of NHEJ along with HRR has little effect on the cytotoxic action of the studied drug combinations but has a more pronounced effect on genotoxic action. There is a possible explanation. We used two DRIs, NU7441 and YU238259. The first of them is a DNA-PK—the phosphatidylinositol-3-kinase (PI3-K)-related protein kinase—inhibitor. DNA-PK is an important component in the NHEJ pathway. NU7441 targets the ATP-binding site of the kinase domain inhibiting all the canonical DNA-PK-dependent forms of NHEJ activity. However, cells could also use an alternative, slowly operating, error-prone backup pathway named B-NHEJ to repair DSBs. In contrast to D-NHEJ, it works without DNA-PK, and therefore NU7441 cannot inhibit this pathway [42]. An analogous situation exists for the second main DSB repair pathway—HRR—where there a single strand annealing (SSA) next to the HRR. In contrast with HRR, SSA is independent of the key HRR protein RAD51 but requires the activity of RAD52 [43]. Unfortunately, the molecular target for YU238259 in HRR pathway has not yet been elucidated at the cellular level, and therefore we cannot specify the homology-dependent DNA repair pathway inhibited by YU238259. In normal cells, the main DSBs pathways—NHEJ and HRR—suppress other alternative [44] pathways, but in cancer cells this imperative very often does not work. All glioma cells including the U-87 cell line, express multiple drug-resistant genes. This phenotype could affect DNA repair processes but NHEJ and HRR pathways seem to be untouched in U-87 as reported earlier [45]. However, the authors studied only the overall NHEJ and HRR efficiency without studying specific sub-pathways such as B-NHEJ or SSA. This could be a little confusing as cancer cells are characterized by genomic instability and imbalance of DNA damage signaling and repair. To survive, cancer cells must compensate defects in one DNA repair pathway by upregulation of a complementary one. Thus, we cannot exclude that alternative DSB repair pathways like B-NHEJ or SSA prevail over other canonical NHEJ and HRR pathways.

Can our study be of help in the clinic? Of course, these in vitro data using the simplest model of tumor are not predictive per se of the effectiveness of drug combinations in cancer patients. Moreover, gliomas displayed heterogeneity even within a single tumor [46]. We observed cisplatin/HU331 as the most effective against malignant glioma among all tested combinations, including the conventional (cisplatin/etoposide) chemotherapeutic agents. However, further studies are needed to determine the possibility of concerning the HU331 as new component of the combined treatment in glioma therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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