ORIGINAL ARTICLE

Inhibition of DNA-PK potentiates the synergistic effect of NK314 and etoposide combination on human glioblastoma cells

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Abstract

Etoposide (VP-16) is the topoisomerase 2 (Top2) inhibitor used for treating of glioma patients however at high dose with serious side effects. It induces DNA double-strand breaks (DSBs). These DNA lesions are repaired by non-homologous DNA end joining (NHEJ) mediated by DNA-dependent protein kinase (DNA-PK). One possible approach to decrease the toxicity of etoposide is to reduce the dose while maintaining the anticancer potential. It could be achieved through combined therapy with other anticancer drugs. We have assumed that this objective can be obtained by (1) a parallel topo2 α inhibition and (2) sensitization of cancer cells to DSBs. In this work we investigated the effect of two Top2 inhibitors NK314 and VP-16 in glioma cell lines (MO59 K and MO59 J) sensitized by DNA-PK inhibitor, NU7441. Cytotoxic effect of VP-16, NK314 alone and in combination on human glioblastoma cell lines, was assessed by a colorimetric assay. Genotoxic effect of anticancer drugs in combination with NU7441 was assessed by comet assay. Cell cycle distribution and apoptosis were analysed by flow cytometry. Compared with VP-16 or NK314 alone, the combined treatment significantly inhibited cell proliferation. Combination treatment was associated with a strong accumulation of DSBs, modulated cell cycle phases distribution and apoptotic cell death. NU7441 potentiated these effects and additionally postponed DNA repair. Our findings suggest that NK314 could overcome resistance of MO59 cells to VP-16 and NU7441 could serve as sensitizer to VP-16/NK314 combined treatment. The combined tripartite approach of chemotherapy could reduce the overall toxicity associated with each individual therapy, while concomitantly enhancing the anticancer effect to treat human glioma cells. Thus, the use of a tripartite combinatorial approach could be promising and more efficacious than mono therapy or dual therapy to treat and increase the survival of the glioblastoma patients.

Keywords NU7441 · NK314 · Etoposide · Glioma · Topoisomerase 2 inhibitors

Introduction

DNA double-strand breaks (DSBs) are the most serious form of DNA damage [1]. Many anticancer drugs used in anticancer therapy cause DSB including topoisomerase 2 (Top2, EC:5.6.2.2) inhibitors. Top2 is an ubiquitous ribozyme which plays a crucial role in DNA stress and supercoiling reduction by DSBs formation [2]. There are two different Top2 isoforms: α and β with about 70% sequence similarities in humans [3]. Both isoforms are necessary for DNA replication, chromosome stability and sister chromatid segregation mechanisms, however Top2α high expression is observed during various cancer development [4]. Agents that target Top2 have been successfully in clinical use in cancer therapy for over 30 years. The typical example of this class of anticancer drugs is etoposide (VP-16). It actively acts mainly on isoform β than α. Unfortunate, this is associated with serious side effects such as high toxicity and secondary malignancies [5]. One possible approach to decrease the toxicity of etoposide could be to reduce the dose while maintaining the anticancer potential. It could be achieved through combined therapy with other anticancer drugs. We have assumed

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that this objective can be obtained by (1) a parallel topo2 α inhibition and (2) sensitization of cancer cells to DSBs. The first one could be obtained with a novel, potent and specific Top2 α inhibitor, NK314 [6, 7]. Inhibitors of catalytic subunit of DNA-dependent protein kinase (DNA-PKcs, EC:2.7.11.1), protein involved in non-homologous DNA end joining (NHEJ) pathway, could have potential application as sensitizing agents to increase the effectiveness of Top2 inhibitors, since NHEJ is the major pathway for the repair of DSBs in humans cells [8, 9]. The aims of the study were to analyse the therapeutic effect of Top2 α and β inhibitors in combined chemotherapy and to identify mechanism of sensitization of cancer cells to Top2α and β inhibitors combined chemotherapy via NHEJ repair inhibitor—NU7441. We used glioma cell lines MO59 K and MO59 J as in vitro model in our study for two reasons. Firstly, glioblastoma still remains palliative, with a very poor prognosis. In addition, there is a limited number of drugs available for glioblastoma treatment, due to the blood–brain barrier (BBB) [10, 11]. BBB is characterized by selective mechanisms regulating the transcellular traffic and the expression of tight junctions between adjacent endothelial cells. Thus, the paracellular transport as well as transcellular is very limited. Therefore only small (molecular mass not higher than 400–500 Da) and highly hydrophobic molecules with a can diffuse through this barrier [12]. Secondly, these cell lines provide useful model systems in which to study the role of DNA-PKcs in cellular and molecular processes involving DNA damage recognition and repair as MO59 J is a mutant form of MO59 K without expression of DNA-PKcs [13, 14].

Materials and methods

Cell lines

Human glioblastoma cell lines, MO59 K and MO59 J (LGC Standards, Teddington, UK) were cultivated in the DMEM/F12 1:1 Medium (Sigma Aldrich, Saint Louis, MO, USA) supplemented with 10% v/v FBS (Lonza, Basel, Switzerland,), 1% (v/v) MEM Non-essential amino acids solution (Sigma Aldrich, Saint Louis, MO, USA) and 1% (v/v) penicillin/streptomycin solution (Sigma Aldrich, Saint Louis, MO, USA), at humidity atmosphere (90%), 5% CO_2 and 37 °C. The difference between these cells is the presence of DNA-PK protein: MO59 K are DNA-PK-proficient cells but MO59 J are DNA-PK-deficient cells.

Anticancer drugs

VP-16 (Sigma Aldrich, Poznan, Poland) and NK314 (Adooq Bioscience, Irvine, USA) were dissolved in the dimethyl sulfoxide (DMSO) (Sigma Aldrich, Poznan, Poland)

to the stock concentration 20 mM and stored at − 20 °C. NU7441 (Selleck Chem, Munich, Germany) was dissolved in the DMSO to the stock concentration 5 mM and stored at − 20 °C.

Cytotoxicity assay

MO59 K and MO59 J cell lines were seeded into 96-wells plates at 5×10^3 density (per well) and allowed to attach for at least 12 h. Next, anticancer drugs alone or in combination were added. In experiments with NHEJ inhibition NU7441 was added 1 h prior to anticancer drugs. The optimal dose of NU7441 was previously determined at 10 μM. Control samples received DMSO. After 24 h of incubation cells were stained with CCK-8 kit (Sigma Aldrich, Saint Louis, MO, USA) and OD_{450} nm was measured according to manufacture suggestion on BioRad 550 microplate reader (Hercules, CA, USA) after 1 h.

Combination index analysis

Cells were treated similarly to the cytotoxicity assay with one exception for drug concentrations. VP-16 and NK314 were added to the cells alone and in combination at constant ratio concentration $1/4IC_{50}$, $1/2IC_{50}$, IC_{50}, $2IC_{50}$ and $4IC_{50}$. Combination index was calculated with CompuSyn 1.0 software (ComboSyn, Inc., Paramus, USA).

DNA damage and repair analysis (comet assay)

DNA damage level and efficiency of DNA repair was analysed by alkaline version of comet assay as described previously [15–17]. Cell lines were incubated for 4 h with the combination of etoposide and NK314 at total dose of 1/4 IC_{50} alone or after 1 h pre-incubation with 10 μM NU7441. Negative control was medium only with DMSO. 20 μM hydrogen peroxide solution served for positive control. Next, cells were mixed with 40 μL of 0.75% low-melting point agarose and placed into microscopic slides coted previously with 0.5% normal-melting point agarose. For DNA repair reaction, after 6 h incubation with drugs medium was replaced by fresh medium and incubated up to 6 h. All samples were lysed in the buffer containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris and 1% v/v Triton X-100 (at least 1 h, 4 °C) and unwinded for 20 min (buffer containing 300 mM NaOH and 1 mM EDTA, 4 °C). Electrophoresis was performed in the buffer composed of 30 mM NaOH and 1 mM EDTA under conditions: 20 min, RT, 17 V, 32 mA. The final results of DNA damage analysis are the mean of two and for DNA repair efficiency analysis of three independent measurements. Dry slides were stained by DAPI (4′,6-diamidino-2-phenylindole) under dimmed light and analyzed under Eclipse fluorescence microscope

(Nikon, Tokyo, Japan) with the COHU 4910 video camera (San Diego, USA) and UV filter block. The percentage of the DNA in the tail which correspond to the DNA damage was analyzed by Lucia-Comet v. 4.51 system. From each slide was taken 100 random samples (50*2 repeats for each sample).

Flow cytometry analysis

Distribution of cells among cell cycle phases was analysed by flow cytometry and propidium iodide (PI) staining. Cell lines were seeded into 10 cm dishes at density 5×10^5 cells/dish and after at least 12 h incubation (5% CO_2, 37 °C) combination of drugs alone or with 10 μM NU7441 were added into 24 h. We used only fresh medium with DMSO as a negative control and nocodazole at 10 μM as positive control. Cells were mixed with iced ethanol (96%) at 1:1 volume with PBS after samples preparation ($2 \times$ PBS washing and pellets resuspension in PBS and 15 min on ice incubation). Prepared in this way cell pellets was resuspended in the mixture of 300 μL PBS + 1.5 μL RNAse A + 1.5 μL PI and incubated in the dark at least 30 min in 37 °C. The final results are the mean of three independent measurements.

For apoptosis analysis, cells were seeded into 10 cm dishes at density 10^6 cells/dish and incubated at least 12 h. After 4 h incubation with the combination of drugs alone or with 10 μM NU7441, cells were mixed with 1 mL $1 \times$ Binding buffer and 100 μL of them (10^5 cells) mixed with 5 μL PI and 5 μL FITC-Annexin V. After 15 min incubation in the dark to each sample 400 μL of $1 \times$ Binding buffer was added and samples measured by flow cytometry. The final results are the mean of three independent measurements.

Statistical analysis

The data was analyzed by STATISTICA 12.5 software (Stat-Soft Inc.,Tulsa, OK, USA). For cytotoxicity, CI and flow cytometry data was presented as mean ± SD and for results from comet assay as median ± SE. t-Student test was used for the analysis of sample differences for normal distribution and U-Mann–Whitney test for these without normal distribution.

Results

NU7441 in a combinatorial treatment with VP-16 or NK314 induces MO59 K and MO59 J cells death

To determine the cytotoxic effects of the drugs on cell viability, CCK-8 assays were done. We tested whether NU7441/Top2 α and β inhibitors combination could affect the viability of studied cell lines. The cells were exposed to increasing concentrations of VP-16, NK314 at fixed concentration of NU7441 that was not harmful for used in this study cancer cells (10 μM). We found that both cell lines showed significant reduction of viability when treated with Top2 α and β inhibitors as well as the combination NU7441/Top2 α and β inhibitors (Figs. 1, 2 a, b and Table 1). The combined treatment reduced the IC_{50} values of NK314 in MO59 K up to 2.23 times (from $IC_{50} = 9.6$ μM to 4.3 μM). The similar reduction factor we observed for VP-16 (from $IC_{50} = 323.8$ μM to 142.6 μM). On the other hand the NU7441/NK314 combination did not show a significant effect on viability of MO59 J cells ($IC_{50} = 4.4$ μM and 4.6 μM) but we observed a significant loss of viability after treatment with NU7441/VP-16. Generally in response to Top2 α and β inhibitors, M059 J cells were more sensitive than M059 K cells.
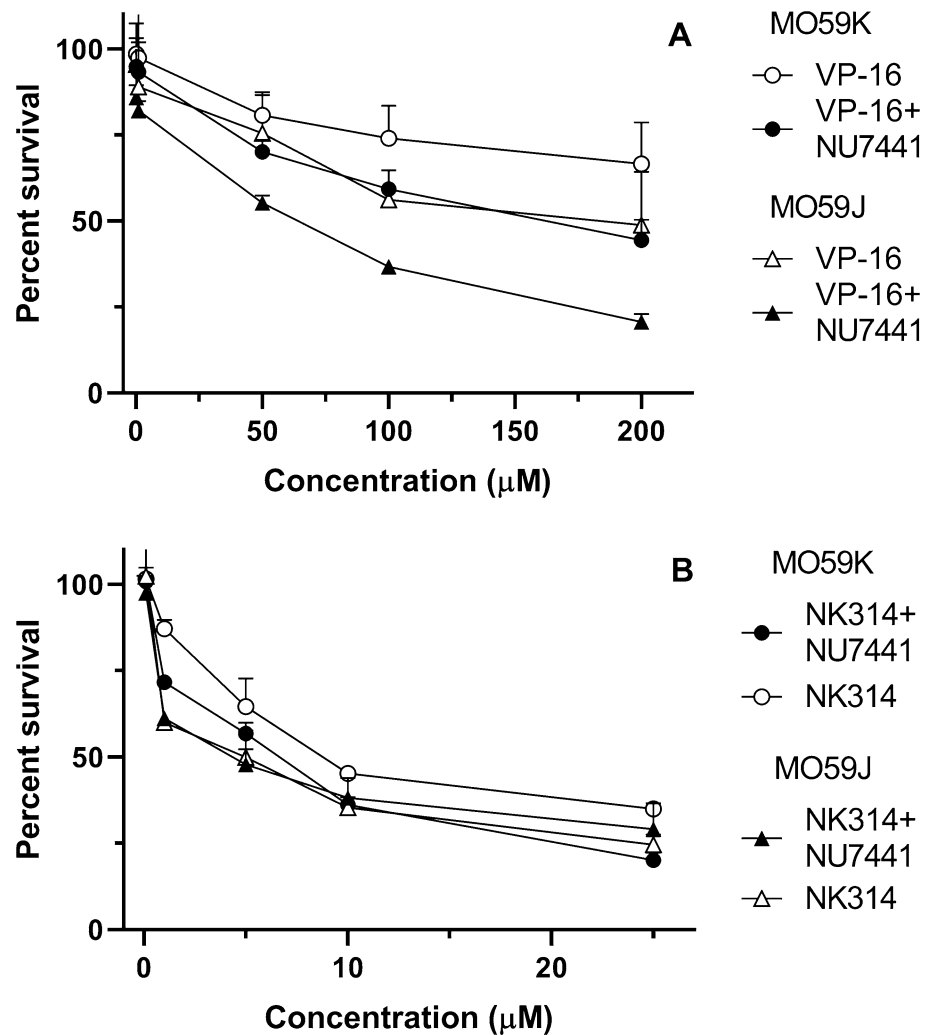
The combination of Top2 α and β inhibitors (NK314 and VP-16) exerts a synergistic anticancer effect in glioma-derived cell lines M059-K and M059-J

The combination index (CI) may be used to determine if a drug combination may have synergistic effect [18]. To assess whether the combined effect of Top2 α and β inhibitors represented additive or synergistic effects we calculated the CI values based on the median-effect equation. The cells were exposed to increasing concentration of VP-16 and NK314 at fixed ratios (0.25 IC_{50}, 0.5 IC_{50}, 1 IC_{50}, 2 IC_{50}, 4 IC_{50}) as seen in Fig. 2a. For example, it means that M059 K cells were incubated with NK314 at 9.6 μM combined with VP-16 at 323.8 μM (1 IC_{50} fixed ratio). We found that the CI of combined treatment with NK314/VP-16 on all cells showed a strong CI below 0.1. It suggest very strong synergistic anticancer effect of NK314 and VP-16. Moreover, preincubation with NU7441 enhanced this effect for 0.25 IC_{50}, 0.5 IC_{50} total doses (Fig. 2b, Table 2). We selected 0.25 IC_{50} combination doses for further studies. We used 0.25 IC_{50} because we observed high synergistic effect at this total dose as well as strong CI reduction with NU7441 (see Table 2). Please note that 0.5 IC_{50} dose has also desired characteristics but VP-16 concentrations were far beyond range of physiologically relevant concentrations.

The combination of NK314 and VP-16 induces DNA damage in glioma-derived cell lines M059-K and M059-J

The alkaline version of comet assay was performed to determine level of DNA damage and repair efficiency after 6 h exposure to etoposide and NK314 combination at total dose ¼ IC_{50} alone or in combination with 10 μM NU7441. Figure 3 shows combined drugs increase in DNA lesions in both cell lines and these levels were increased as all doses

in the presence of NU7441. The level of DNA lesions was increased about 15% when NU7441 was added. Interestingly, this effect was shown for both cell lines indicating that it is not DNA-PKcs status depended. It suggests that NU7441 sensitization to NK314 and VP-16 combined treatment is correlated with DNA lesions induction.

NU7441 postponement repair of DNA lesions induced by NK314 and VP-16

Figure 4 shows the extent of DNA damage in MO59 K and J cells, respectively, exposed to Top2 inhibitors in the presence and absence of NU7441 at the beginning and during 6 h of repair incubations conducted in drug-free medium. DNA damage induced by NK314 and VP-16 in combinations was repaired after 6 h, however please note that repair in MO59 J cell was not changed after 2 h and DNA damage remained at the same level (~ 10%) to the end of incubation period. In the presence of NU7441 levels of unrepaired DNA lesions evoked by comet assay were significantly higher than for

drugs alone. However the observed effect was more spectacular for MO59 K than J cells suggesting that it is DNA-PKcs status dependent.

NU7441 modulates cell cycle phases distribution

Figure 5 shows the effect of NU7441 on cell cycle phases in MO59 K and J cells treated with the Top2 inhibitors in combination. As seen, in MO59 K cells, treatment with NK314 and VP-16 caused an accumulation of cells in G1 (60% vs. 71%, p < 0.05) and decreased in cell number in S (29% vs. 24%, p < 0.05) and G2/M (11% vs. 5%, p < 0.05) phases compared to control cells. Pre-treatment of cells with NU7441 intensify this effect (60% vs. 76%, p < 0.05; 29% vs. 21%, p < 0.05 and 11% vs. 3%, p < 0.05). Quite contrary effect we observed for MO59 J cells, where we observed accumulation of cells in S phase (27% vs. 36%, p < 0.05) as well as (G2/M 6% vs. 8%). Addition of NU7441 has no statistically significant effect.
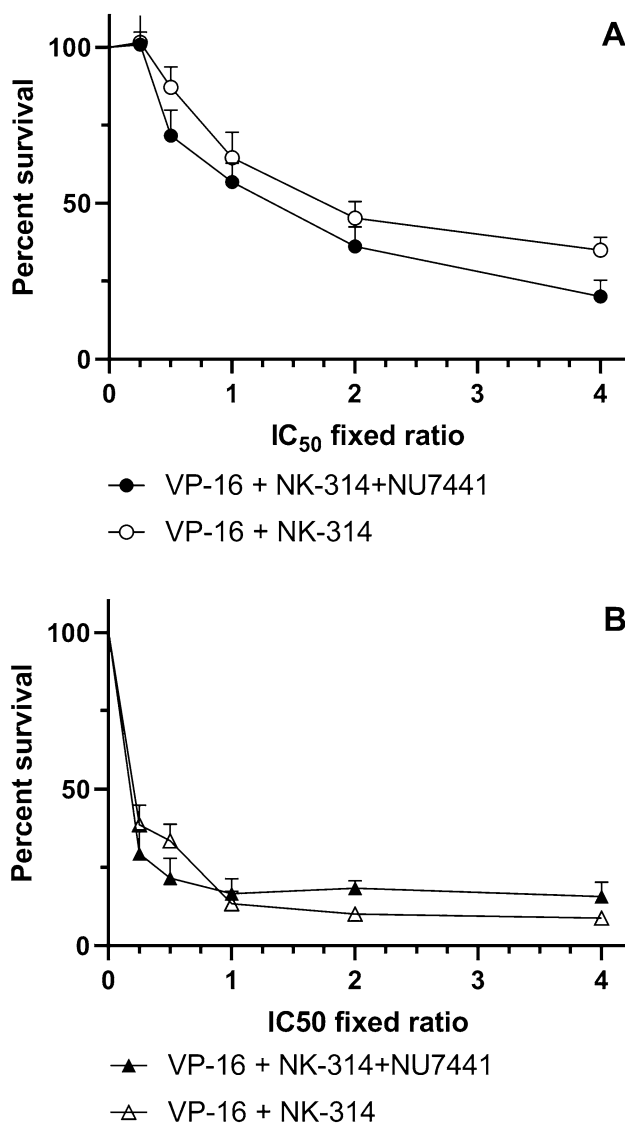
Fig. 2 NU7441 (black symbols) enhanced synergistic effect of NK314/VP-16 combination in glioblastoma cell lines MO59 K (circle) and MO59 J (triangle). Cells were grown in 96-well plate and treated with VP-16/NK314 combination (at constant ratio- $1/4IC_{50}$, $1/2IC_{50}$, IC_{50}, $2IC_{50}$ and $4IC_{50}$) for 1 day in the absence (white symbols) or presence (black symbols) of 10 μM NU7441. Next, viability test was performed using CCK-8 assay. All tested chemical stocks were prepared in absolute DMSO and further diluted in culture medium. The final concentration of DMSO was kept in all samples (including control) at < 1%. Data are expressed as mean ± SD of three separate replication experiments

NU7441 does not affect NK314 and VP-16-induced apoptosis

To address the effect of NU7441 on cell apoptosis, MO59 K and J cells were pre-incubated with NU7441 for 1 h prior to addition of NK314 and VP-16 combination. As shown on Fig. 6, the apoptosis rate of MO59 K in the combined treatment was 36% (total percentage of Annexin V and Annexin V-PI double positive cells), which was significantly higher than the 22% observed in the control and comparable with positive control (32%). Addition of NU7441 has no statistically significant effect. Similar trend we noticed for MO59 J cells, however apoptosis rate was much lower than in MO59 K cells.

Discussion

Resistance of astrocytic gliomas to common used chemotherapeutic drugs contribute to a poor clinical response (reviewed at [19]). The combined therapeutic approach is one important strategy to overcome cancer resistance. This strategy involves the simultaneous use of two drugs with different molecular targets [20]. The most important challenge with this approach is to find two synergistic drugs. In the present study we demonstrate for the first time that the Top2 α inhibitor—NK314 acts synergistically with Top2 β inhibitor VP-16 in two human glioma cell lines M059 J and M059 K. Both cell lines were isolated from a 33-year-old male patient with untreated glioblastoma and differ in their sensitivity to radiation [13]. M059 J cells are much more sensitive than M059 K cells to radiation, however these two cell lines are generally classified as resistant as compared with other cancer cell lines [14]. There are two reasons for increased sensitivity of MO59 J cells to radiation: the frameshift mutation in *PRKDC* gene and elevated expression of miR-100 [14, 21, 22]. These resulted in the absence of DNA-PKcs and the low-expression of ATM (EC:2.7.11.1), which in consequence leads to reduction of overall DSBs repair efficiency. Thus, this difference should be seen for all agents that induce DSBs. Indeed, we observed the difference within sensitivity of MO59 K and MO59 J cells to single and combined treatment with studied Top2 inhibitors. We also observed potentiation of cytotoxicity of Top2

Table 1 Growth inhibition by VP-16 or NK314 and NU7441 in MO59 K and MO59 J cells

Cell line	IC50 of VP-16 (μM)		Reduction factor (R)	IC50 of NK314 (μM)		Reduction factor (R)
	Alone	Combination		Alone	Combination	
MO59 K	323.8 ± 2.63	142.6 ± 1.47	2.27	9.6 ± 0.48	4.3 ± 0.42	2.23
MO59 J	155.6 ± 4.48	56.9 ± 1.02	2.73	4.4 ± 0.37	4.6 ± 0.32	0.95

Table 2 Synergistic effect of NK314 and VP-16 in MO59 K and MO59 J cells

	MO59 K				MO59 J			
	Total dose (µM)	VNK CI value	CI value with NU7441	Reduction factor (R)	Total dose (µM)	VNK CI value	CI value with NU7441	Reduction factor (R)
0.25 IC50	82.4	0.048	0.031	1.52	39.9	0.058	0.020	2.87
0.5 IC50	164.8	0.037	0.018	2.04	79.7	0.074	0.014	4.99
IC50	333.4	0.039	0.052	0.76	160.0	0.009	0.013	0.63
2 IC50	659.2	0.037	0.050	0.73	318.8	0.008	0.036	0.22
4 IC50	1318.4	0.032	0.035	0.93	637.6	0.113	0.046	2.45

inhibitors by DNA-PKcs inhibitor NU7441, however this effect was clearly smaller in MO59 J cells lacking DNA-PKcs expression. These data gentle suggest that NU7441 sensitization to Top2 inhibitors in studied cells is mediated not only by DNA-PKcs and ATM but probably by other PIKK kinases. Similarly, Tavecchio et al. demonstrated that NU7441 is weak inhibitor of other than DNA-PKcs PIKK kinases, however this effect is only seen when DNA-PKcs is absent [23]. We also showed that cells lacking DNA-PKcs with low ATM expression were more sensitive to Top2 inhibitors. This is in agreement with observation by Johnson and Jones. They found that hamster cells deficient in DNA-PKcs were threefold more sensitive to etoposide than control cells [24]. These data strongly suggest that DNA-PKcs and ATM is absolutely essential for Top2 inhibitors-induced DNA lesions. Some authors has postulated that VP-16-induced DNA lesions are repaired via two different DNA repair pathways DNA-PKcs dependent and DNA-PKcs independent one [17, 25, 26]. It should be mentioned here that there are two NHEJ pathways in eucariotic cells, canonical (cNHEJ) and alternative, known as backup (bNHEJ), micro homology-mediated end joining (MMEJ) or non-canonical (ncNHEJ). The first one needs DNA-PK and ligase IV (EC:6.5.1.1) whereas the second one relies on PARP1 (EC:.4.2.30), 53BP1, MRE11 (EC 3.1.-.-) and ligase III (EC:6.5.1.12). There are also differences in the kinetics and fidelity of repair process to the detriment of the ncNHEJ. NcNHEJ is slower and more error prone than cNHEJ. Nc NHEJ is active mainly cNHEJ deficient cells, however also exist in normal cells (review at [27]. Therefore, we cannot exclude possibility that small fraction of VP-16-induced DNA lesions are repaired by ncNHEJ and they are independent of DNA-PKcs and its inhibitors. Similar effect we observed after combined treatment of VP-16 and NK314 where inhibition of NHEJ via NU7441 resulted in enhancing of very strong synergistic effect. Interestingly, effect of NU7441 was clearly seen for 0.25 and 0.5 total dose and above 0.5 total dose this effect was abolished. The possible explanation of this difference would be that remaining doses of

Top2 inhibitors combinations were very toxic for MO59 glioma cells.

We demonstrated that combination of VP-16/NK314 induced DNA strand breaks visualized by comet assay. The level of DNA strand breaks was increased when NU7441 was added in both cells, however this difference was not statistically significant. Statistically significant difference we observed when we compared repair of DNA lesions induced by combinations of VP-16/NK314 in presence and absence of NU7441 in MO59 K cells. DNA breaks induced by drugs were not efficiently repaired in the presence of NU7441 and DNA repair curve was similar to those obtained for MO59 J cells. It suggest the role of NU7441 as inhibitor of DNA repair, which was previously shown [23, 28]. Detailed analysis of DNA repair kinetics curves was very interesting. In DNA-PKcs proficient cells most DNA lesions evoked by studied Top2 inhibitors where repaired by 2 h and ends within 6 h, wherever in DNA-PKcs deficient cells 10% of DNA lesions where detectable in this time point. Similar data we obtained when NU7441 was added to the DNA-PKcs proficient cells. It strongly suggest that in MO59 K cells cNHEJ is responsible for repair of VP-16/NK314 induced DNA lesions but they could be also partially repaired via slower ncNHEJ.

We presumed that accumulation of unrepaired DNA lesions following inhibition of DNA-PKcs by NU7441 would evoke cell cycle alternation. Indeed, we observed the alternation in the cell cycle after Top2 combo treatment in both cell lines. However, only in MO59 K we observed further alternation in the cell cycle after NU7441 treatment. This is consistent with others and with generally accepted model of cellular DNA damage response encompasses the DNA repair pathways as well as cell cycle checkpoints [29]. These checkpoints, stop cell cycle progression when DNA is damaged, allowing time for repairs. They also are involved in death signalling leading to cell death (mainly apoptosis) due to unrepaired DNA lesions which block DNA replications or transcription. These signaling needs also efficient DNA repair pathways as DNA repair proteins often initiate the signal. Any alternations in DNA repair pathway
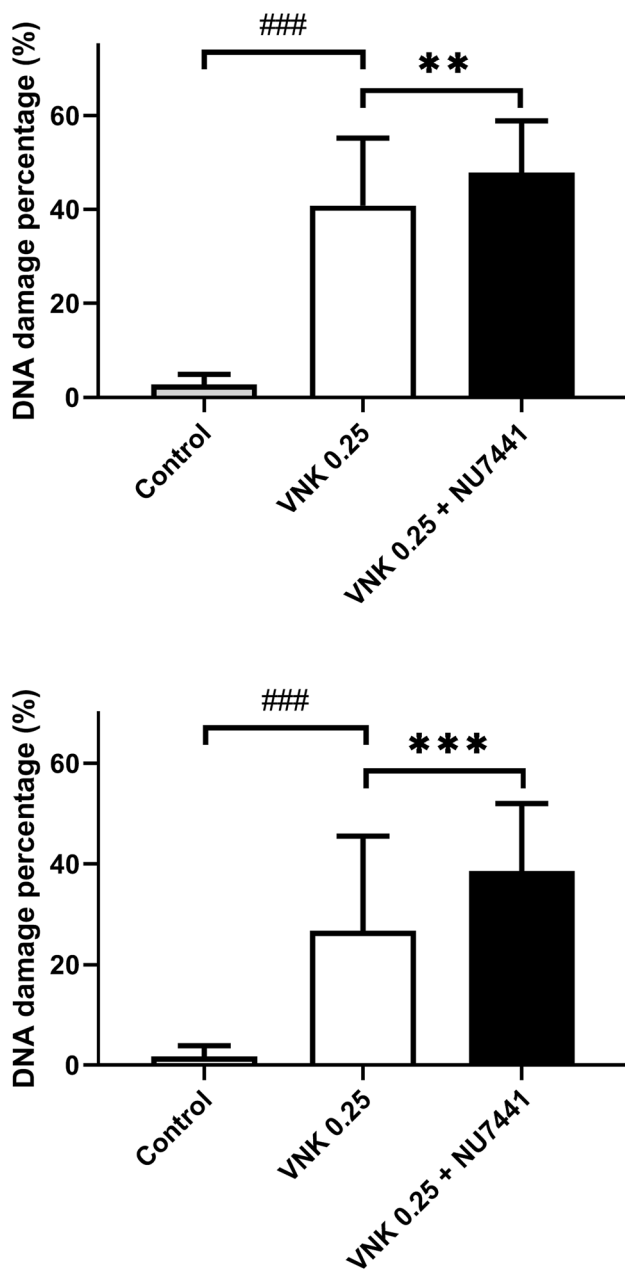
Fig. 3 NU7441 increased DNA damage induced by VP16/NK314 combination at constant ratio ¼ IC_{50} in glioblastoma cell lines MO59 K (**a**) and MO59 J (**b**). Cells were grown in 24-well plate and treated with VP16/NK314 combination at constant ratio ¼ IC_{50} for 6 h in the absence (white bars) or presence (black bars) of 10 µM NU7441. Next, DNA damage was analyzed as percentage in the tail DNA in comet assay. All tested chemical stocks were prepared in absolute DMSO and further diluted in culture medium. The final concentration of DMSO was kept in all samples (including negative control—empty bars) at < 1%. One hundred cells were analyzed in each sample. Data are expressed as mean ± SD of three independent experiments. Statistically significant difference: **P < 0.01, ***P < 0.001 compared to control
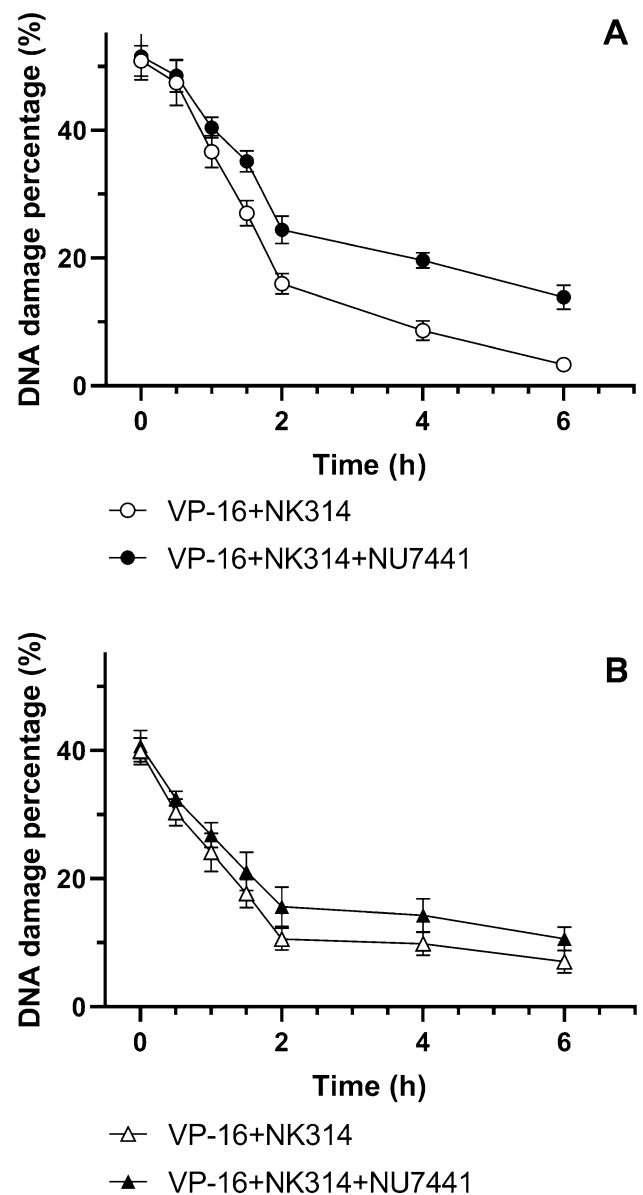
Fig. 4 NU7441 delayed repair of DNA damage induced by VP-16/NK314 combination at constant ratio ¼ IC_{50} in glioblastoma cell lines MO59 K (**a**) and MO59 J (**b**). Cells were grown in 24-well plate and treated with VP-16/NK314 combination at constant ratio ¼ IC_{50} for 6 h. Next, medium was replaced with fresh one (without VP-16/NK314) and repair incubation was conducted for 6 h in the absence (white symbols) or presence (black symbols) of 10 µM NU7441. DNA damage was analyzed as percentage in the tail DNA in comet assay immediately at the beginning of repair incubation and after 0.5, 1, 1.5, 2, 4 and 6 h. All tested chemicals were prepared in absolute DMSO and further diluted in culture medium. The final concentration of DMSO was kept in all samples at < 1%. One hundred cells were analyzed in each sample. Data are expressed as mean ± SD of three independent experiments
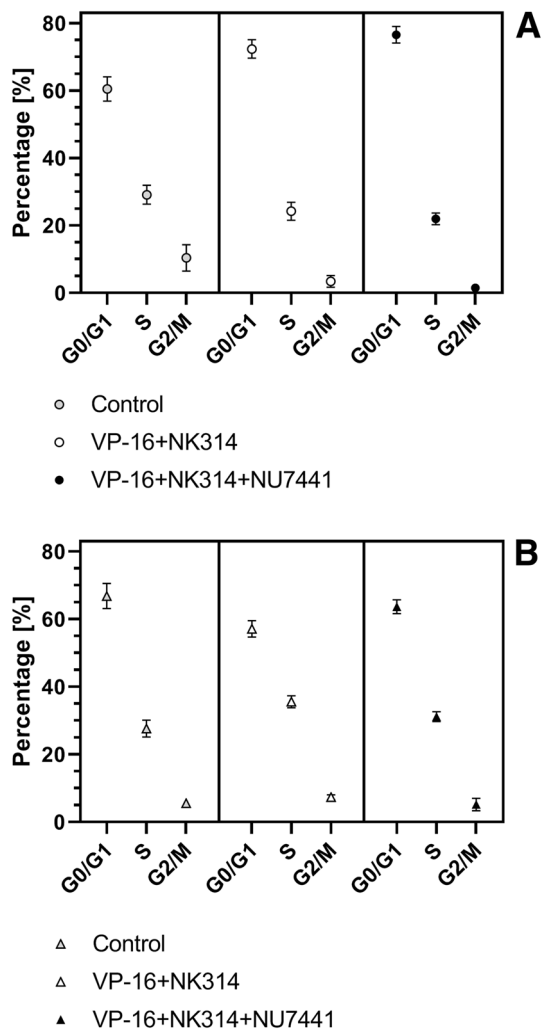
Fig. 5 NU7441 increased modulation of cell cycle phases distribution evoked by VP-16/NK314 combination at constant ratio ¼ IC$_{50}$ in glioblastoma cell lines MO59 K. Percentage cells distribution between cell cycle phases: G0/G1, S and G2/M after 24 h exposure to VP-16/NK314 combination with (black symbols) or without NU7441 (white symbols) in glioblastoma cell lines MO59 K (**a**, circle) and MO59 J (**b**, triangle). Control cells (grey symbols) received only medium with DMSO. The final concentration of DMSO was kept in all samples at < 1%. Data are expressed as mean ± SD of three independent experiments

Fig. 6 NU7441 did not affect apoptosis induced by VP-16/NK314 combination at constant ratio ¼ IC$_{50}$ in glioblastoma cell lines MO59 K (**a**) and MO59 J (**b**). Percentage cells after 6 h of exposure to VP-16/NK314 combination with (black bars) or without NU7441 (white bars) in glioblastoma cell lines MO59 K (**a**) and MO59 J (**b**). Control cells (light grey bars) received only medium with DMSO. The final concentration of DMSO was kept in all samples at < 1%. Apoptosis in positive control cells (dark grey bars) was induced by 100 μM camptothecin. Data are expressed as mean ± SD of three independent experiments. ***P < 0.001 compared to control

decreased sensitivity of such signaling and resulted in lower apoptosis ratio in DNA repair deficient cells as compare with cells with untouched DNA repair pathway. Our results are in agreement with this model as we observed higher apoptosis ratio in MO59 K cells than MO59 J. Some authors reported that topoisomerase inhibitors increased apoptosis following addition of NU7441 [30, 31] but we did not observed this in both MO59 cells. However, impact of NU7441 on induced by topoisomerase inhibitor apoptosis seems to be controversial and strongly depends not only on type of cancer but also on NU7441 doses [31, 32].
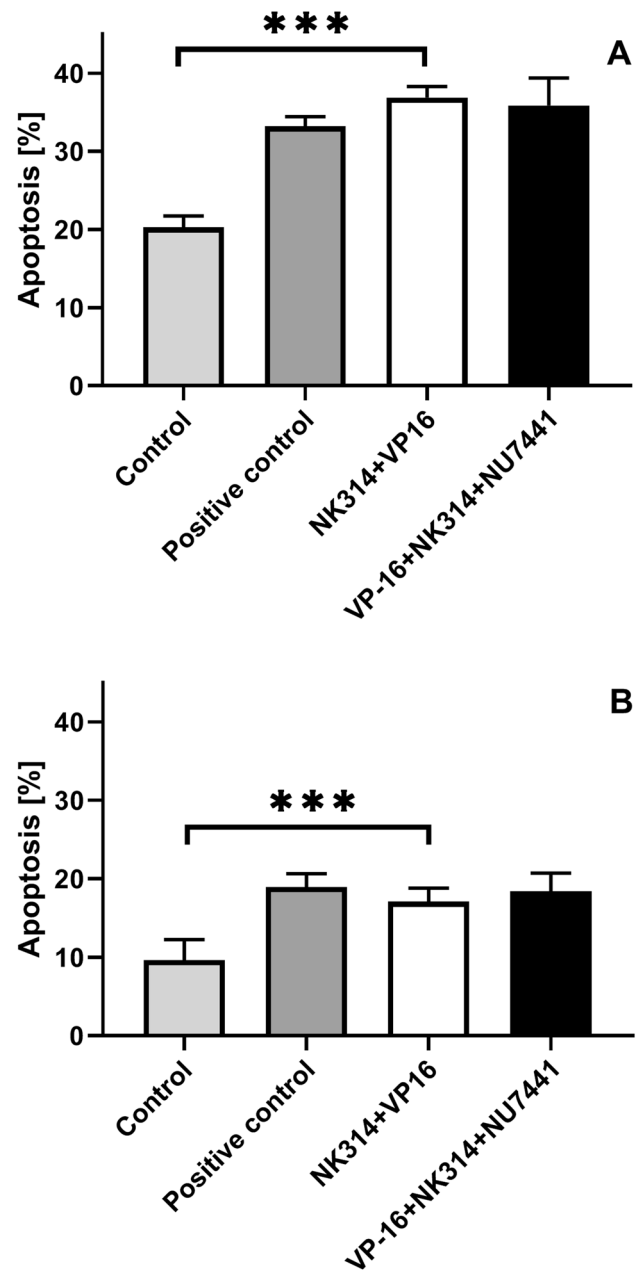
In conclusion, the present findings for the first time demonstrated that NK314 could overcome resistance of MO59 cells to VP-16 and NU7441 could serve as sensitizer to VP-16/NK314 combined treatment. The combined tripartite

approach of chemotherapy could reduce the overall toxicity associated with each individual therapy, while concomitantly enhancing the anticancer effect to treat human glioma cells. Thus, the use of a tripartite combinatorial approach could be promising and more efficacious than mono therapy or dual therapy to treat and increase the survival of the glioblastoma patients. Successful drug delivery across the blood–brain tumor barrier remains a separate issue. VP-16 penetrates the blood–brain tumor barrier [5]. There is no data about penetration blood–brain barrier (BBB) of NK314 and NU7441, however the first one belongs to benzo[c]phenanthridines. This class of compounds traverse BBB, and have relative high concentration in brain [33]. It is possible to increase the BBB permeability with melanotransferrin antibody or and tamoxifen conjugated on NU7441 [34]. Using this approach Kuo and Wang enhanced delivery of etoposide across BBB. In the worst case scenario, NU7441 could be replaced by another DNA-PKcs inhibitor. Wortmannin seems to be good candidate for NU7441 replacement as it penetrates BBB, however it is less potent than NU7441.

Compliance with ethical standards

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