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Wortmannin potentiates the combined effect of etoposide and cisplatin in human glioma cells

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ABSTRACT

The combination of etoposide and cisplatin represents a common modality for treating of glioma patients. These drugs directly and indirectly produce the most lethal DNA double-stand breaks (DSB), which are mainly repaired by non-homologous DNA end joining (NHEJ). Drugs that can specifically inhibit the kinase activity of the catalytic subunit of DNA-dependent protein kinase (DNA-PK$_{cs}$), the major component of NHEJ, are of special interest in cancer research. These small molecule inhibitors can effectively enhance the efficacy of current cancer treatments that generate DNA damage. In this study, we investigated the effect of DNA-PK$_{cs}$ inhibitor, wortmannin, on the cytotoxic mechanism of etoposide and cisplatin in MO59K and MO59J human glioblastoma cell lines. These cell lines are proficient and deficient in DNA-PK$_{cs}$, respectively. Wortmannin synergistically increased the cytotoxicity of cisplatin and etoposide, when combined, in NHEJ-proficient MO59K cells. Surprisingly, wortmannin sensitizing effect was also observed in DNA-PK$_{cs}$-deficient MO59J cells. These data suggest that wortmannin sensitization to etoposide and cisplatin in human glioma cells is mediated by inhibition of not only DNA-PK$_{cs}$ activity but other enzymes from PI3-K family, e.g. ATM and ATR. A concentration-dependent increase in etoposide and cisplatin-induced DSB levels was potentiated by inhibitor in both cell lines. Moreover, drug-induced accumulation in the G2/M checkpoint and S-phase was increased by wortmannin. Wortmannin significantly inhibited drug-induced DSB repair in MO59 cells and this effect was more pronounced in MO59J cells. We conclude that the mechanism of wortmannin potentiation of etoposide and cisplatin cytotoxicity involves DSBs induction, DSBs repair inhibition, G2/M checkpoint arrest and inhibition of not only DNA-PK$_{cs}$ activity.

1. Introduction

The combined treatment of cisplatin and etoposide is one of the most active regimens for low-grade glioma and allows avoidance of radiotherapy in the vast majority of patients (Sardi et al., 2012). These two agents provide considerable synergy during treatment of glioma, although the mechanism of this synergy is not completely known (Nakagawa et al., 2007). Efforts have been made to understand the molecular aspects of cancer cell sensitivity or resistance to chemotherapeutic agents. This knowledge is an important determinant for the discovery of new agents to improve chemotherapy by enhancing chemosensitivity.

cis-Diamminedichloroplatinum (II) (cisplatin) is a widely used chemotherapeutic agent which acts by the formation of cisplatin-DNA covalent adducts, including DNA intra- and inter-strand cross-links (ICL). Such adducts causes cell cycle arrest, inhibition of DNA replication, transcription, and, if not repaired, apoptosis (Boeckman et al., 2005). Repair of cisplatin-induced ICL is mainly catalyzed by nucleotide excision repair (NER) pathway (Calsou and Salles, 1993; Köberle et al., 1997). However, cisplatin may induce DNA double-strand breaks (DSB) as an intermediate step during ICL repair.

Abbreviations: ATM, ataxia telangiectasia-mutated kinase; ATR, ataxia telangiectasia and Rad3-related kinase; CI, combination index; CLL, chronic lymphocytic leukemic cells; DNA-PK, DNA-dependent protein kinase; DNA-PK$_{cs}$, DNA-dependent protein kinase catalytic subunit; DSBs, DNA double-strand breaks; HR, homologous recombination; ICL, intra- and inter-strand cross-links; IR, ionizing radiation; NHEJ, non-homologous DNA end joining; PNK, polynucleotide kinase; PI3-K, phosphatidylinositol-3-kinase; XLF, XRCC4-like factor; XRCC4, X-ray cross complementation 4 protein.

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Etoposide, an epipodophyllotoxin, is a widely used antitumor agent which acts by the inhibition of topoisomerase II, a nuclear enzyme that controls DNA topology. Topoisomerase II cleaves the phosphodiester bond of DNA to generate a transient DSB through which unbroken DNA duplex can pass, and then the DSB is relegated. Topoisomerase II "poisons", such as etoposide, prevent the relegation step by covalently binding to enzyme and forming a stable drug-enzyme-DNA complex named "cleavable complex" (Chen et al., 1984). Cellular processing converts this complex into a DSB, which may be repaired by a DSB repair pathway.

DSB are repaired by non-homologous DNA end joining (NHEJ) and/or by homologous recombination (HR) repair pathways. The major contributor in DSB repair in mammals is NHEJ (Mahaney et al., 2009; Pastwa et al., 2009; Wang and Lees-Miller, 2013). Important component in this repair pathway is the phosphatidylinositol-3-kinase (PI3-K)-related protein kinase family of enzymes. These DNA damage-activated serine/threonine protein kinases include DNA-dependent protein kinase (DNA-PK) involved directly in NHEJ, ataxia telangiectasia-mutated kinase (ATM), and ataxia telangiectasia and Rad3-related kinase (ATR) involved in signal transduction (Durocher and Jackson, 2001; Pastwa and Blasiak, 2003). The DNA-PK holoenzyme consists of heterodimer Ku70/Ku80, which binds to DNA strand breaks, and catalytic subunit of DNA-PK termed DNA-PK_{cs} recruited by the heterodimer. Apart from DNA-PK, other components take part in NHEJ, including Artemis, X-ray cross complementation 4 protein (XRCC4), ligase IV complex and XRCC-4 like factor (XLF, also called Cernunnos) (Ahnesorg et al., 2006; Buck et al., 2006; Pastwa and Malinowski, 2007).

The MO59K and MO59J cells are human glioblastoma cell lines extracted from the same brain tumor, but are different in their ability to repair DSB (Allalunis-Turner et al., 1993; Kurimasa et al., 1999). MO59J is a null mutant for DNA-PK_{cs} whereas MO59K is a wild type with respect to DNA-PK_{cs} (Lees-Miller et al., 1995). The MO59J line is defective in the rejoining of ionizing radiation (IR)-induced DSB, and has been demonstrated to be hypersensitive to IR, bleomycin, and nitrogen mustard (Allalunis-Turner et al., 1993; Kurimasa et al., 1999).

In cancer cells, inhibition of NHEJ repair could increase the therapeutic effect, whereas efficient DSB repair could result in the development of resistance to both IR and drug-induced DNA damage. Consequently, drugs that can specifically inhibit the kinase activity of DNA-PK, the major component of NHEJ, are of special interest in cancer research (Davidson et al., 2013). The first inhibitor, which bounds covalently to the ATP-binding site of DNA-PK_{cs}, and other PI3-K (e.g. ATM, ATR) was the fungal metabolite wortmannin (Izzard et al., 1999). It was shown that wortmannin decreased DSB repair and increased cytotoxic effect of etoposide and IR (Boulton et al., 1996; Boulton et al., 2000; Hashimoto et al., 2003). Moreover, potentiation of etoposide cytotoxicity in a DNA-PK-proficient, but not a DNA-PK-deficient Chinese hamster ovary cell line (deletion mutation in the Ku80), suggests that this effect can result in DNA-PK inhibition by wortmannin (Boulton et al., 2000). Additionally, a reduction of DNA-PK activity by wortmannin sensitized human breast cancer cells MCF-7 to cisplatin and inhibited repair of cisplatin-induced ICL (Friedmann et al., 2006). However, no sensitization to cisplatin was observed after Ku86 antisense transfection of MO59K cells (Belenkov et al., 2002). There is no report about wortmannin sensitization to cisplatin and etoposide combined treatment in human glioma cells.

Here, for the first time we investigated the effect of wortmannin on the cytotoxic mechanisms of etoposide and cisplatin combination in MO59K cells. Cell growth, cell cycle phase distribution, DSB formation and repair were selected as the endpoints for this study. In order to answer the question whether any effect of wortmannin could be specifically related to DNA-PK_{cs} inhibition, its effects were also studied in MO59J cells.

2. Materials and methods

2.1. Cell culture

The malignant glioma cells MO59K and MO59J were obtained from American Type Culture Collection (Rockville, MD, USA). MO59K and MO59J cells were maintained in DMEM medium (Sigma-Aldrich, Poznan, Poland), and all cells were supplemented with 10% (v/v) fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). All cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Drugs

Cisplatin was dissolved in water as 2 mM stock and stored at −20 °C. Etoposide was made up as 2 mM stock dissolved in methanol. Wortmannin was dissolved in 1% anhydrous dimethyl sulfoxide (DMSO) as 1 mM stock, stored in aliquots at −20 °C and protected from light. All drugs were purchased from Sigma–Aldrich (Poznan, Poland).

2.3. XTT assay

Exponentially growing MO59K and MO59J cells (5×10^3/well in 200 μl) were seeded into 96-well plates. Drug(s) (6 replicates) were added to the Plates 24 h later. After a 7-day incubation growth inhibition was determined by a sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) cell proliferation kit (Sigma-Aldrich, Poznan, Poland) according to the manufacturer's instructions. Plates were read on a Bio-Rad 550 plate reader (Hercules, CA, USA) at 450 nm and the absorbance at a reference wavelength of 690 nm was subtracted from the 450 nm measurement. Results were obtained as a percentage of controls. Then the concentrations of drug(s) required to produce 50% growth inhibition (IC_{50}) were estimated. Wortmannin (5 μM) was found to have a small inhibitory effect on cell growth (5% after 10 min pretreatment of cells) and these samples were used as controls in experiments with drug combinations. Reduction factor (R) values were calculated from the ratios of the IC_{50} of the drug(s) without wortmannin to the IC_{50} of the drug(s) with wortmannin. Data were averaged from at least 3 independent experiments ± SEM.

2.4. Combination index for synergism and antagonism determination

For combined treatments, the ratio of cisplatin dose to etoposide dose was kept constant based on the IC_{50}, and cells were treated with increasing total doses. The combination index (CI) equation (Berenbaum, 1992):

$$\mathrm{CI} = \frac{a}{A} + \frac{b}{B}$$

was used for data analysis of two-drug combinations, where "a" is the IC_{50} of etoposide in combination, "b" is the IC_{50} of cisplatin in combination, "A" is the IC_{50} of etoposide alone, and "B" is the IC_{50} of cisplatin alone. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively.

2.5. Flow cytometric analysis

Cells were treated with drug(s) for 24 h, fixed with 70% ethanol and stained with propidium iodide (40 μg/ml) and DNase-free