Plant Physiology and Biochemistry 96 (2015) 72–82

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

The effects of anti-DNA topoisomerase II drugs, etoposide and ellipticine, are modified in root meristem cells of *Allium cepa* by MG132, an inhibitor of 26S proteasomes

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ARTICLE INFO

Article history:
Received 3 April 2015
Received in revised form
17 June 2015
Accepted 17 July 2015
Available online 23 July 2015

Keywords:
Allium cepa
Apoptosis-like programmed cell death
Chromosome aberrations
DNA topoisomerase II
Etoposide
Ellipticine
Proteasomes

ABSTRACT

DNA topoisomerase II (Topo II), a highly specialized nuclear enzyme, resolves various entanglement problems concerning DNA that arise during chromatin remodeling, transcription, S-phase replication, meiotic recombination, chromosome condensation and segregation during mitosis. The genotoxic effects of two Topo II inhibitors known as potent anti-cancer drugs, etoposide (ETO) and ellipticine (EPC), were assayed in root apical meristem cells of *Allium cepa*. Despite various types of molecular interactions between these drugs and DNA-Topo II complexes at the chromatin level, which have a profound negative impact on the genome integrity (production of double-strand breaks, chromosomal bridges and constrictions, lagging fragments of chromosomes and their uneven segregation to daughter cell nuclei), most of the elicited changes were apparently similar, regarding both their intensity and time characteristics. No essential changes between ETO- and EPC-treated onion roots were noticed in the frequency of G1-, S-, G2-and M-phase cells, nuclear morphology, chromosome structures, tubulin-microtubule systems, extended distribution of mitosis-specific phosphorylation sites of histone H3, and the induction of apoptosis-like programmed cell death (AL-PCD). However, the important difference between the effects induced by the ETO and EPC concerns their catalytic activities in the presence of MG132 (proteasome inhibitor engaged in Topo II-mediated formation of cleavage complexes) and relates to the time-variable changes in chromosomal aberrations and AL-PCD rates. This result implies that proteasome-dependent mechanisms may contribute to the course of physiological effects generated by DNA lesions under conditions that affect the ability of plant cells to resolve topological problems that associated with the nuclear metabolic activities.

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1. Introduction

In Eukaryotes, progression through the cell cycle is accompanied by almost complete dispersion of chromatin during S-phase replication, and by M phase condensation of the nucleoplasm to allow for individualization of chromosomes and segregation of sister chromatids. Many integrated control mechanisms of these alternating processes, based on specific cyclin-dependent kinases (CDKs), cyclins (regulatory subunits of CDK), protein phosphatases and other regulatory factors (De Veylder et al., 2007; Gong and Ferrell, 2010) not only determine the proper sequence of activities critical for interphase and mitotic events, but also organize an apparatus to protect the next generation of cells against errors in the transmission of genetic information (Dissmeyer et al., 2009; Lipavská et al., 2011).

Unrepaired DNA single-strand breaks (SSBs) and other lesions in the genetic material generated both by endogenous processes (replication errors, oxidative stress-induced damage to base pairs) and by exposure to exogenous factors (drugs, xenobiotics, ionizing radiation and UV light) may collide with the transcriptional and replication machinery and may be converted into lethal double-strand breaks (Caldecott, 2007). To preclude such a possibility, any abnormalities or structural damage of the genome activate molecular pathways of the cell cycle checkpoint machinery, which is capable of inhibiting DNA biosynthesis and mitotic chromosome condensation (Jackson and Bartek, 2009; Baranello et al., 2014). Slowing or arrest of the 'nuclear cycle' allow the cell time to express

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http://dx.doi.org/10.1016/j.plaphy.2015.07.016
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specific genes and to activate necessary repair factors (Elledge, 1996; Esnault et al., 2010).

During transcription and DNA replication some genomic aberrations arise as a consequence of inappropriate activity of enzymes called DNA topoisomerases I and II, which are responsible for solving the problems concerning DNA topology (Jackson and Bartek, 2009). Type II topoisomerase (Topo II) is involved in DNA replication, transcription, chromatin remodeling, condensation and chromosome segregation (Mak et al., 2005; Nitiss, 2009a). In an ATP-dependent reaction, by relaxation of positively and negatively supercoiled DNA, Topo II enables the removal of knots, supercoils and catenates (Champoux, 2001; Vos et al., 2011; Lane et al., 2013). Accordingly, its activity is directed mainly toward decatenation of sister chromatids after the S phase is complete (Charbin et al., 2014).

Topo II inhibitors are one of the major groups of cytostatics showing significant anticancer activity. Two categories of chemical agents with different mechanisms of action can block the functions of Topo II: Topo II poisons and catalytic inhibitors (Nitiss, 2009b; Pommier et al., 2010). The first group includes etoposide (VP-16), teniposide (VM-26), and DNA intercalators, such as anthracenedione (mitoxantrone), anthracyclines (doxorubicin and daunorubicin) and aminoacridines (e.g. amsacrine; m-AMSA), which stabilize the cleavable (or 'cleavage') complexes and inhibit DNA religation. Another group of Topo II poisons, including ellipticines, azatoxins, quinolones and isoflavones (genistein), exert their inhibitory actions by enhancing the formation of covalent enzyme-cleaved DNA complexes (Pommier et al., 2010; Balaña-Fouce et al., 2014). Collision of Topo II-DNA cleavable complexes with the advancing replication fork or transcription machinery results in the formation of DNA single- and double-strand breaks (SSBs and DSBs, respectively). Catalytic inhibitors, such as bisdioxopiperazines (ICRF-159, ICRF-193, ICRF-187), fostriecin, aclarubicin, suramin, novobiocin and merbaron (Bassi and Palitti, 2000; Park and Avraham, 2006), block Topo II enzymatic activity without trapping the covalent complexes (Pommier et al., 2010; Balaña-Fouce et al., 2014).

Our current study on *Allium cepa* root meristem cells compares the effects of two DNA Topo II inhibitors – etoposide (ETO; also known as VP-16) and ellipticine (EPC) – on cell cycle progression in primary root meristems of *A. cepa*. Despite distinct modes of action exerted by these drugs and their evident impact on nuclear integrity, no essential changes between ETO- and EPC-treated seedlings were noticed in the frequency of cells at the G1, S and G2/M phases, nuclear morphology, chromosome structures, tubulin-microtubule systems, distribution of mitosis-specific phosphorylation of histone H3, and the induction of apoptosis-like programmed cell death (AL-PCD). However, significant differences between experimental series appeared following incubation of onion roots with ETO and EPC mixed with MG132 (an inhibitor of 26S proteasomes). This result indicates specific dependence of effects formed by Topo II inhibitors on proteasome-mediated degradation of molecular complexes created at the DNA level.

2. Results

2.1. Influence of ETO, EPC, and their mixtures with MG132 on mitotic activity and the incidence of chromosomal aberrations

The final concentrations of Topo II inhibitors, 200 μM ETO and 200 μM EPC, were selected in a series of preliminary tests using 1, 10, 20, 50, 100, 200, 300 μM solutions (on the basis of the available literature) and 0.5, 2, 4, 6, 8, 10, 12, 14, 16, and 18 h treatments, the latter period covering the mean cell cycle time in apical root meristems of *A. cepa* (Navarrete et al., 1987). Exploiting the ability to form fluorescent adducts with DNA, covalent intercalation of 200 μM EPC was recorded in the G1, S, and G2 nuclei of *A. cepa* root meristem cells at all incubation times (0.5, 2, 4, 6, 8, 10, 12, 14, 16, 18 h) by microspectrofluorimetric measurements. As shown in Fig. 1A–K, chromatin fluorescence increased with time, reaching the highest value at the 14 and 16 h treatment time-points, which was followed by a slight decrease of nuclear EPC fluorescence at 18 h of EPC treatment.

A considerable number of studies have implicated that DNA topoisomerases undergo proteasome-mediated degradation in cells treated with Topo II poisons [e.g., (Lyu et al., 2007; Azarova et al., 2010)]. In order to address this problem, some experiments were performed using MG132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal), a membrane-permeable hydrophobic peptide aldehyde which is known to strongly inhibit cellular mechanisms of ubiquitin/26S proteasome-dependent protein degradation (Lee and Goldberg, 1996). Accordingly, two samples of onion roots were pretreated for 30 min with MG132 at a concentration of 100 μM, followed by continuous co-treatment with ETO + MG132 and EPC + MG132 mixtures. Prior to this study, the effects of MG132 applied solely were evaluated.

Despite some increase in mitotic indices (MI, statistically significant with $p < 0.05$ at time points 2 and 4, when compared to the untreated seedlings; Fig. 2A), no structural or functional abnormalities were observed in Feulgen-stained onion root meristems following successive incubations with only MG132. Although successive ETO treatments also did not change MI values (Fig. 2B), a vast number of chromosomal aberrations appeared, with the earliest cases detected as soon as after 2-h incubation. Later, the percentage of mitotic cells with disturbed chromosome structures increased, reaching the highest value (about 25%) at the 8 h time point. Then, the number of aberrant cells gradually decreased until their almost complete disappearance in 18 h (Fig. 2B).

During co-incubation of the MG132-pretreated onion seedlings with the mixture of ETO + MG132 (0.5-to-18 h treatment periods), mitotic activity remained at a level of 4–10% (Fig. 2C). Although evident symptoms of chromosomal aberrations were also observed as soon as after 2-h incubation (Fig. 2C), the highest number of mitotic abnormalities in ETO + MG132-treated root tips (ca. 28% of cells) appeared already after 4 h incubation and were still frequent after 6- and 8-h treatments. Starting from the 12 h time point, the number of cells with aberrant chromatin morphology in root meristems exposed to ETO + MG132 mixture significantly decreased (Fig. 2C).

The changes in MI observed after successive incubation periods of *A. cepa* root meristem cells in 200 μM EPC are shown in Fig. 2D. During the first 8 h of treatment, mitotic activity was similar to the control. At four subsequent time points (10–16 h), MI slightly increased (by about 3–4%). A small number of mitotic cells with chromosomal aberrations were observed in preparations made after a short, 2-h incubation period with EPC (Fig. 2D). With time, similarly as in the case of ETO (Fig. 2B), there was a gradual increase in cells with altered chromatin morphology. The highest amount of the cells showing abnormal mitotic structures (about 23%, as in the case of ETO) was noted after 8-h EPC-treatment. Then, their number gradually declined to about 7% at 18 h of incubation (Fig. 2D).

Compared with onion root meristems incubated with only EPC, the combined influence of EPC + MG132 caused a significant decrease in the number of aberrant M-phase cells (to about 3–5%), with no significant time-related changes in the mitotic activity, except some increase during the earliest incubation periods (Fig. 2E).

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