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Effect of pivaloyl-substituted pyrrole containing heterocyclic compounds on DNA repair pathways in Ewing sarcoma cells

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Abstract

Aim. To examine deoxyribonucleic acid (DNA) damage repair and cell cycle regulatory mechanisms of Ewing sarcoma cells exposed to pivaloyl-substituted pyrrole containing heterocyclic compounds.

Methods. The study was performed on A673 Ewing sarcoma cell line. The tumor cells were incubated for 48 h in the presence of pivaloyl-substituted pyrrole containing heterocyclic compounds (compounds №20 and №24). Western blot analysis was utilized to examine expression of the markers of DNA single-strand (phosphorylated forms of ATR and Chk1) and double-strand breaks (phosphorylated forms of H2AX, ATM, DNA-PK, BRCA-1, Chk-2). Analysis of the cell cycle phases was performed by flow cytometry (BD FacsCanto, USA).

Results. Pivaloyl-substituted pyrrole containing heterocyclic compounds substantially increased the expression of histone 2A phosphorylated on serine 138 (γ-H2AX) that indicates DNA damage (double-strand breaks). Under exposure to pivaloyl-substituted pyrrole containing heterocyclic compounds the studied cells increased expression of phosphorylated forms of ATM-kinase and BRCA-1. Also cell cycle disorders leading to substantial G2/M arrest and enhanced apoptosis of tumor cells were observed.

Conclusion. Pivaloyl-substituted pyrrole containing heterocyclic compounds induced DNA double-strand breaks in A673 Ewing sarcoma cell line; in response to DNA damage in tumor cells, the mechanisms of DNA double-strand breaks repair were activated; despite activation of DNA repair mechanisms, A673 cells underwent cell cycle arrest in the G2/M-phase and apoptosis.

Keywords: Ewing sarcoma, DNA damage and repair, cell cycle, pivaloyl-substituted pyrrole-containing heterocyclic compounds.

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Ewing's sarcoma is one of the most common malignant bone tumors in children and adolescents. The disease was first described in 1921 by the American pathologist James Ewing (1866–1943). Ewing's sarcoma is more common in males, with the peak incidence from 10 to 15 years of age [1].

This tumor mainly affects the diaphyses of long tubular bones. In addition, it can be localized in the ribs and pelvic, shield, and collar bones. Clinically, this disease normally manifests with pain and the development of edema around the affected bones [2]. The tumor is characterized by a very aggressive clinical course, and metastases are noted (lungs, bone tissue, and lymph nodes) in 20%–30% of patients at diagnosis [3].

The overall treatment strategy for Ewing's sarcoma includes chemotherapy, followed by surgery and/or local radiotherapy [4]. The main types of chemotherapeutic agents include alkylating agents (ifosfamide and cyclophosphamide), anthracyclines (adriamycin and doxorubicin), etoposide, actinomycin D, and vinca alkaloids (vinblastine) [5, 6]. High doses of busulfan and melphalan are prescribed to patients with a poor histological response to the conventional doses of chemotherapy. Using the combination of chemotherapy and topical treatment, the 5-year survival rate of patients is >70%. Nevertheless, tumor relapses are characterized by rapid progression and poor prognosis in many patients (20% 2-year survival rate after relapse) [7, 8].

Considering this background, the mechanisms of the activity of pivaloyl-substituted pyrrole-containing heterocyclic compounds (PSPHs) synthesized by the authors were investigated in relation to their efficacy against Ewing's sarcoma cells. Previously, we have found that PSPHs depolymerized tubulin protein in cells of gastrointestinal stromal tumors.

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Consequently, tumor cells remained in the M phase of the cell cycle (mitotic catastrophe) and died via apoptosis [9].

The object of the present study was tumor cells derived from Ewing's sarcoma. The cell line A673 was acquired (American Type Culture Collection (ATCC) , USA). The cells were cultured under standard conditions (37°C, 5% CO_2) in the Dulbecco's Modified Eagle Medium (DMEM) culture medium (PanECO) supplemented with L-glutamine (PanECO), fetal bovine serum (HyClone), and antibiotics (PanECO). When 70% confluence was achieved, PSPH No. 20 (PSPH-20) and No. 24 (PSPH-24) at concentrations of 5 μmol each were added to the cell cultures. After 24 and 48 h of incubation, the cells were lysed. The resulting cell lysates were divided by molecular weight using the electrophoresis method and detected by the biochemical "antigen–antibody" reaction using immunoblotting.

Expression levels of repair markers of single- (ATR, Chk-1) and double-stranded (γ-H2AX, ATM, DNA-PK, BRCA-1, Chk-2) lesions of deoxyribonucleic acid (DNA) were studied. Doxorubicin at a dose of 0.25 μg/ml and vinblastine at a dose of 1 nmole (Sigma-Aldrich, USA) were used as reference drugs, which are commonly used in the treatment of Ewing's sarcoma.

Doxorubicin is an antitumor antibiotic of the anthracycline series, which acts as an inhibitor of type II DNA topoisomerase. Vinblastine, a vinca alkaloid, is a type of mitotic poison as well as a PSPH, which depolymerizes tubulin.

The influence of PSPH-20, PSPH-24, and chemotherapeutic agents on the regulation of the cell cycle phases of A673 tumor cells and mechanisms of their death were studied using flow cytometry (BD FacsCanto, USA). The obtained results were statistically analyzed using Microsoft Excel 2007 and Biostatistica (S.A. Glantz, McGraw Hill, USA) software. To assess the statistical significance of the studied samples, Student's t-test was used. At $p < 0.05$, differences were considered statistically significant.

PSPH-20 and PSPH-24 induced histone 2 phosphorylation at serine 139 (γ-H2AX) in Ewing's sarcoma A673 cells (Figure 1). The effect was more pronounced after 48 h of incubation of the cells with the test compounds. A similar effect was also observed for doxorubicin and, to a lesser extent, for vinblastine.

Increased γ-H2AX expression in tumor cells indicates the presence of double-stranded DNA breaks. This was also supported by the activation of the corresponding DNA repair

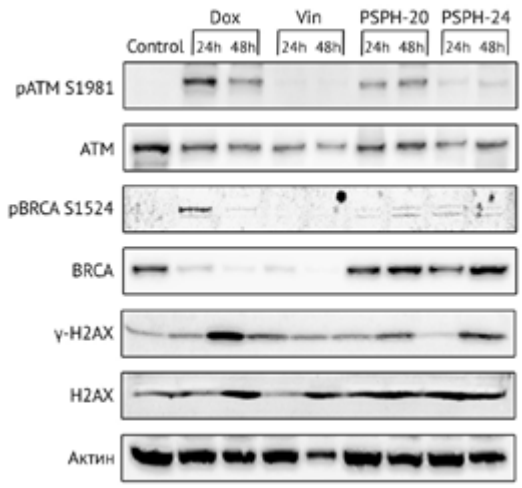

Fig. 1. Ability of chemotherapeutic drugs (doxorubicin and vinblastine) and pivaloyl-substituted pyrrole-containing heterocyclic compounds (PSPHs) to induce double-stranded DNA breaks in Ewing's sarcoma A673 cells and to repair these breaks. Incubation was for 24 and 48 h. γ-H2AX is a marker of double-stranded DNA breaks; pATM S1981 is a marker of repair of double-stranded breaks; pBRBCA S1524 is a marker of the activation of homologous recombination; H2AX, ATM, and BRCA are common (non-activated) forms of proteins; actin signifies the level of protein in the samples. Doxorubicin (Dox), 0.25 μg/ml; vinblastine (Vin), 1 nmol; PSPH-20, 5 μmol, PSPH-24, 5 μmol

pathways, particularly ATM kinase proteins and BRCA1 (see Figure 1). Increased expression of the phosphorylated form of BRCA-1 demonstrates the activation of double-stranded break repair by homologous recombination.

The appearance of single-stranded DNA breaks in cells is accompanied by the activation of the corresponding mechanisms of DNA repair, which may be evidenced by the overexpression of phosphorylated forms of kinases ATR and Chk1 [10]. Our results demonstrated that in Ewing's sarcoma cells, the expression of activated (i.e., phosphorylated) kinases did not increase after 48 h of incubation with the PSPH-20 and PSPH-24 compounds, indicating the explicit ability of PSPH-20 and PSPH-24 compounds to induce double-stranded DNA breaks in A673 cells.

In flow cytometry, a disruption in the regulation of cell cycle phases, manifesting as the arrest of A673 cells in the G2/M phases of the cell cycle (Table 1), was noted following PSPH-20 and PSPH-24 treatments. In addition, PSPH-20 and PSPH-24 induced the death of tumor cells by apoptosis, as demonstrated

Table 1. Effects of doxorubicin, vinblastine, PSPH-20, and PSPH-24 on cell cycle phases of A673 tumor cells

Test substance	Cell cycle phases			Apoptosis, %
	G0/G1, %	S, %	G2/M, %	
Control	77.1 ± 1.0	4.8 ± 1.6	15.4 ± 1.6	2.1 ± 0.9
Doxorubicin, 0.25 μg/ml	11.0 ± 0.8*	4.1 ± 1.0	81.8 ± 2.1*	3.0 ± 0.7
Vinblastine, 1 nmol	14.8 ± 0.9*	1.3 ± 0.6	54.0 ± 1.2*	30.0 ± 1.2*
PSPH-20, 15 μmol	36.6 ± 1.1*	8.0 ± 0.7*	36.0 ± 1.1*	19.4 ± 1.1*
PSPH-24, 15 μmol	40.3 ± 1.2*	9.4 ± 0.7*	33.5 ± 0.9*	16.8 ± 1.0*

Note: Incubation for 48 h; results are presented as arithmetic mean±standard deviation; *differences against control are statistically significant at $p<0.05$; PSPH, pivaloyl-substituted pyrrole-containing heterocyclic compounds

by increased propidium iodide expression, indicating the presence of fragmented DNA.

The present study demonstrated the ability of PSPH-20 and PSPH-24 to induce double-stranded DNA breaks in Ewing's sarcoma cells, leading to the subsequent activation of the ATM-mediated pathway for the repair of these breaks. Despite the activation of this DNA repair system, the tumor cells were arrested in the G2/M phase of the cell cycle, and the subsequent cell death occurred by apoptosis.

CONCLUSIONS

In vitro, Ewing's sarcoma A673 cells are sensitive to PSPHs, which stimulate the formation of double-stranded DNA breaks and subsequent death of tumor cells by apoptosis. Further research is warranted to investigate the cytotoxic effect of these compounds against Ewing's sarcoma cells.

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The authors declare no conflicts of interest on the article presented.

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