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INHIBITOR OF PROTEIN KINASES 1-(4-CHLOROBENZYL)-3-CHLORO-4-(3-TRIFLUOROMETHYLPHENYLAMINO)-1H-PYRROLE-2,5-DIONE INDUCES DNA DAMAGE AND APOPTOSIS IN HUMAN COLON CARCINOMA CELLS

N. S. Finiuk^{1,2}, O. Yu. Klyuchivska^{1,2}, H. M. Kuznietsova^{1,3},
S. P. Vashchuk^{1,4}, V. K. Rybalchenko^{1,3}, R. S. Stoika^{1,2,3*}

¹ Ivan Franko National University of Lviv, 4, Hrushevskiy St., Lviv 79005, Ukraine

² Institute of Cell Biology, NAS of Ukraine, 16, Drahomanov St., 79005 Lviv, Ukraine

³ Taras Shevchenko National University of Kyiv, 64/13, Volodymyrska St., Kyiv 01601, Ukraine

⁴ Lviv Scientific Research Forensic Center of Ministry of Internal Affairs of Ukraine
24, Konyushynna St., Lviv 79000, Ukraine

*Corresponding author e-mail: stoika@cellbiol.lviv.ua

Finiuk N.S., Klyuchivska O.Yu., Kuznietsova H.M., Vashchuk S.P., Rybalchenko V.K., Stoika R.S. Inhibitor of protein kinases 1-(4-chlorobenzyl)-3-chloro-4-(3-trifluoromethylphenylamino)-1H-pyrrole-2,5-dione induces DNA damage and apoptosis in human colon carcinoma cells. **Studia Biologica**, 2020: 14(4); 3–14 • DOI: <https://doi.org/10.30970/sbi.1404.636>

Background. The heterocyclic scaffolds are in the list of key structural blocks used at synthesis of novel biologically active compounds.

Materials and Methods. The present study addressed the evaluation of the mechanisms of the DNA damaging and pro-apoptotic actions *in vitro* of the maleimide derivative 1-(4-chlorobenzyl)-3-chloro-4-(3-trifluoromethylphenylamino)-1H-pyrrole-2,5-dione (MI-1) targeting human colon carcinoma cells of HCT116 line. The Western-blot analysis was used to study changes in apoptosis-associated proteins, DNA comet assay under alkaline conditions was applied for evaluation of the DNA-damaging events, and Barton's assay with diphenylamine was applied for measuring the level of DNA fragmentation in human colon carcinoma cells treated with MI-1 compound.

Results. The results of the Western-blot analysis demonstrated that MI-1 induced the apoptosis in HCT116 cells via mitochondria-dependent pathway. It activated caspase 3 via its cleavage in the treated human colon carcinoma cells. Besides, MI-1 increased the content of mitochondria-specific proteins: endonuclease G (EndoG) and the pro-apoptotic cytosolic protein protease-activating factor 1 (Apaf1). At the same time, MI-1 reduced the level of the anti-apoptotic Bcl-2 protein in HCT116 cells. The

DNA comet analysis under alkaline conditions of the targeted human colon carcinoma cells of HCT116 line demonstrated that MI-1 induced DNA single-strand breaks in line with the olive tail moment of 13.2. The results of the colorimetric diphenylamine assay in HCT116 cells have shown that cell treatment with MI-1 increased the content of fragmented DNA to 14.2 %.

Conclusions. The anti-proliferative action of MI-1 in human colon carcinoma cells of HCT116 line is associated with apoptosis induction via mitochondria-dependent pathway, as well as the DNA damage through single-strand breaks and DNA fragmentation. These data suggest that the 1-(4-chlorobenzyl)-3-chloro-4-(3-trifluoromethylphenylamino)-1*H*-pyrrole-2,5-dione (MI-1) might be a promising agent for suppression of growth of colon tumor cells.

Keywords: 1*H*-pyrrole-2,5-diones, apoptosis, Western-blot assay, comet assay, single-strand breaks, Barton's assay, DNA fragmentation

INTRODUCTION

The heterocyclic structures with specific physicochemical properties are key components of the commercially available pharmaceuticals, including the anti-cancer agents approved by the FDA [19, 24]. The heterocyclic scaffolds are characterized by the lipophilicity, polarity, and bio-isosteric replacements. Besides, they can be easily modified for construction of the targeted pharmaceutical agents [15]. Different heterocyclic scaffolds such as azole, imidazole, pyrazole, indole, pyrrolidine, pyridine, pyrrole, pyrimidines, quinoline, oxadiazole, and others were addressed as key structural blocks for development of the biologically active compounds [19].

Several commercial and experimental anticancer drugs act through the apoptotic signaling pathways [3, 29, 30], targeting cell proliferation, causing DNA damage (cleavage of DNA by endonucleases, strand DNA breaks, changes in chromatin condensation) [13], and microtubule surplus Ca^{2+} [29]. It was reported that the induction of apoptosis is one of the most successful non-surgical approach in cancer treatment [14, 30, 31]. Agents with anti-proliferative action might affect the extrinsic (via death receptors) and intrinsic (mitochondria mediated) pathways of apoptosis [29]. The phosphatidylinositol-3 kinase (PI3K)–protein kinase B (Akt)–mammalian target of the rapamycin (mTOR) and signal transducer and activator of transcription 3 (STAT3) pathways might be involved in the cancer cells growth inhibition [8, 16]. The anticancer agents trigger the TNF receptor superfamily receptors, Fas and/or Fas ligand, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), activate caspase-8 or caspase-10, affect the transcription nuclear factor- κ B (NF- κ B), and mitogen-activated protein kinases (MAPKs) [1, 29]. Mitochondria and mitochondrial proteins are targets for numerous anti-cancer agents that act via the mechanisms of apoptosis [29]. The B-cell-lymphoma protein 2 (BCL-2) family members are key regulators of the intrinsic apoptotic pathway [27]. The agents that possess the anti-proliferative action can also affect the initiator caspases with next activation of the effector caspases that are considered to be the main regulators of apoptosis [1].

The maleimide derivate MI-1 (1-(4-chlorobenzyl)-3-chloro-4-(3-trifluoromethylphenylamino)-1*H*-pyrrole-2,5-dione) was designed and reported based on the *in silico* as an ATP-competitive small-molecule inhibitor of tyrosine kinases EGF-R, FGF-R1, IGF1-R,

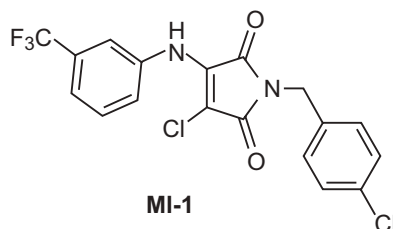
INS-R, SRK, YES, VEGF-R1-3, ZAP70 (type I inhibitor) [7, 28]. It possesses the anti-inflammatory activity in rats with the experimental chronic ulcerative colitis [18]. MI-1 inhibited growth of colorectal cancer cells *in vitro* [6] and *in vivo* [17]. Besides, it demonstrated a toxic action towards cultured cells of different tumors (cervix, colon, breast, liver, pancreatic carcinomas) with the inhibition concentration (IC_{50}) ranging from 0.8 to 18 $\mu\text{g/mL}$ depending on tissue origin of the targeted cells. The most prominent effect was detected towards human cervix carcinoma (KB3-1 and KBC-1) and human colon carcinoma (HCT116) cells [10]. However, the mechanism of the anti-proliferative action of MI-1 was not studied. Here we addressed the investigation of the mechanisms of the pro-apoptotic action and DNA damaging potential of MI-1 in human colon carcinoma cells of HCT116 line.

MATERIALS AND METHODS

Compound under study. The synthesis of maleimide derivate (1-(4-chlorobenzyl)-3-chloro-4-(3-trifluoromethylphenylamino)-1H-pyrrole-2,5-dione) (MI-1, **Fig. 1**) was carried out via chemical transformations, as described previously [6]. Stock solution of MI-1 (10 mg/mL) was prepared in Dimethyl Sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). The working solutions of MI-1 were prepared by its dilution with the culture medium. Doxorubicin (Dox, Actavis S.R.L., Bucharest, Romania) was used as a positive control of the anticancer drug.

Fig. 1. Structure of the maleimide derivative MI-1 (1-(4-chlorobenzyl)-3-chloro-4-(3-trifluoromethylphenylamino)-1H-pyrrole-2,5-dione) [10]

Рис. 1. Структура похідного малеїмиду MI-1 (1-(4-хлоробензил)-3-хлоро-4-(3-трифторометилфеніламіно)-1H-пірол-2,5-діон) [10]



Cell culture. Human colon carcinoma cells of HCT116 line were obtained from the Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine). Cells were cultured in RPMI-1640 (Biowest, Nuaille, France) medium supplemented with 10 % fetal bovine serum (Biowest, Nuaille, France) at standard conditions (37 °C in the atmosphere of 5 % CO_2).

Western-blot analysis. Human colon carcinoma HCT116 cells were treated with MI-1 or Dox at 0.9 $\mu\text{g/mL}$ that corresponds to the IC_{50} of these compounds for HCT116 cells [10]. The final concentration of the DMSO solvent was 0.005 % in the medium with 0.9 $\mu\text{g/mL}$ of MI-1. Cellular proteins were extracted with lysis buffer (20 mM Tris-HCl, pH 8.0, 1 % Triton-X100, 150 mM NaCl, 50 mM NaF, 0.1 % SDS) containing 1 mM of phenylmethanesulfonyl fluoride (PMSF) and 10 $\mu\text{g/mL}$ of protease inhibitors cocktail "Complete" (Basel, Roche, Switzerland). Proteins (30 $\mu\text{g/lane}$) were separated by the SDS/PAGE gelelectrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane using the modified protocol of the Western-blot assay [23].

The primary antibodies against a Cleaved Caspase 3 (Asp175), EndoG, phosphorylated form of MAPK, Apaf1 (Cell Signaling Technology, New England Biolabs GmbH, Vienna, Austria), Bcl-2 (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), beta-actin

monoclonal mouse AC-15 (Sigma-Aldrich, St. Louis, Missouri, USA) were used. Secondary peroxidase-conjugated antibodies (Cell Signaling Technology, New England Biolabs GmbH, Vienna, Austria) were applied at a working dilution of 1:5,000. For protein visualization, the enhanced chemiluminescence (ECL) detection reagents (Sigma-Aldrich, St. Louis, Missouri, USA) were used. The densitometric analysis of protein level was performed using the open source ImageJ software.

DNA comet assay under alkaline conditions. Human colon carcinoma HCT116 cells were treated with MI-1 or Dox at 0.9 $\mu\text{g/mL}$ for 24 h. 100 μL of the treated cells ($n = 35,000$) were mixed with 100 μL of 0.5 % low melting agarose (Promega, Madison, Wisconsin, USA) and transferred to microscopic slides covered with 1% normal molten agarose (Lachema, Brno, Czech Republic). The cells were lysed for 4–6 h at 4 °C in a buffer (2.5 M NaCl; 100 mM EDTA; 10 mM Tris, pH 10.0; 10 % DMSO, 1 % Triton X-100). Electrophoresis was performed at 25 V for 25 min in a buffer (1 mM EDTA; 300 mM NaOH, pH 13.0) at 4 °C. Slides were stained with Ethidium bromide (10 $\mu\text{g/mL}$, Sigma Aldrich, St. Louis, Missouri, USA), and visualization was performed under Zeiss fluorescent microscope with Axiolmager A1 camera. Casplab-1.2.3b2 software (CASPLab, Wroclaw, Poland) was used for calculation of the olive tail moment (OTL). Olive tail moment was calculated as the product of the detectable DNA in the comet tail, and distance between centers of the head mass and tail region ($\text{OTL} = (\text{Tail mean} - \text{Head mean}) \times \% \text{ of tail DNA}$) [9, 20].

Diphenylamine assay of DNA fragmentation. For quantitative determination of DNA fragmentation, diphenylamine assay was applied. Diphenylamine interacts with deoxyribose that results in an increase of blue colored product. Human colon carcinoma cells of HCT116 line were treated for 24 h with MI-1 or Dox at 0.9 $\mu\text{g/mL}$. Cells were lysed in 0.5 mL Tris-EDTA buffer, pH 7.4, supplemented with 0.2 % Triton X-100 and centrifuged for 10 min at 12,000 g at 4 °C. The fragmented DNA (labelled as A) was separated from intact chromatin. The supernatant with fragmented DNA (labelled as B) was transferred into a new tube. 0.5 mL of 25 % Trichloroacetic acid (Sfera Sim, Lviv, Ukraine) was added to tubes A and B, mixed and incubated for 1 h at 56 °C. The DNA sample was centrifuged for 10 min at 14,000 g at 4 °C. 1 mL of freshly prepared diphenylamine reagent (150 mg diphenylamine (Sigma Aldrich, St. Louis, Missouri, USA), 10 mL glacial acetic acid, 150 mL concentrated H_2SO_4 and 50 mL of acetaldehyde solution) was added to pellets A and B and incubated overnight at 37 °C. Afterwards, 200 μL of the colored solution was transferred into a 96-well plate. The percentage of DNA fragmentation was calculated as $\{\text{OD tube B} / (\text{OD tube A} + \text{OD tube B}) \times 100 \%\}$ [2, 11]. The optical density (OD) was measured at 630 nm using Absorbance Reader BioTek ELx800 (BioTek Instruments, Inc., Winooski, Vermont, USA).

Statistical analysis. The results were analyzed and illustrated with GraphPad Prism (version 6; GraphPad Software, La Jolla, California, USA), and presented as a mean \pm standard deviation. ANOVA test (by Dunnett's test) was used for analysis of the statistical data. A P-value <0.05 was admitted as statistically significant.

RESULTS

Recently, we have demonstrated that MI-1 possessed high cytotoxicity towards human colon carcinoma HCT116 cells with the IC_{50} of 0.9 $\mu\text{g/mL}$. The IC_{50} value of Dox for these cells equaled 0.8 $\mu\text{g/mL}$ [10]. The 0.9 $\mu\text{g/mL}$ concentration of MI-1 and Dox

was chosen as a working dose at Western-blot analysis of their pro-apoptotic effects in HCT116 cells.

It was found that MI-1 induced an increase in the content of the cleaved (active) form of caspase 3 in treated HCT116 cells. This effect was comparable with that under the action of Dox (**Fig. 2**).

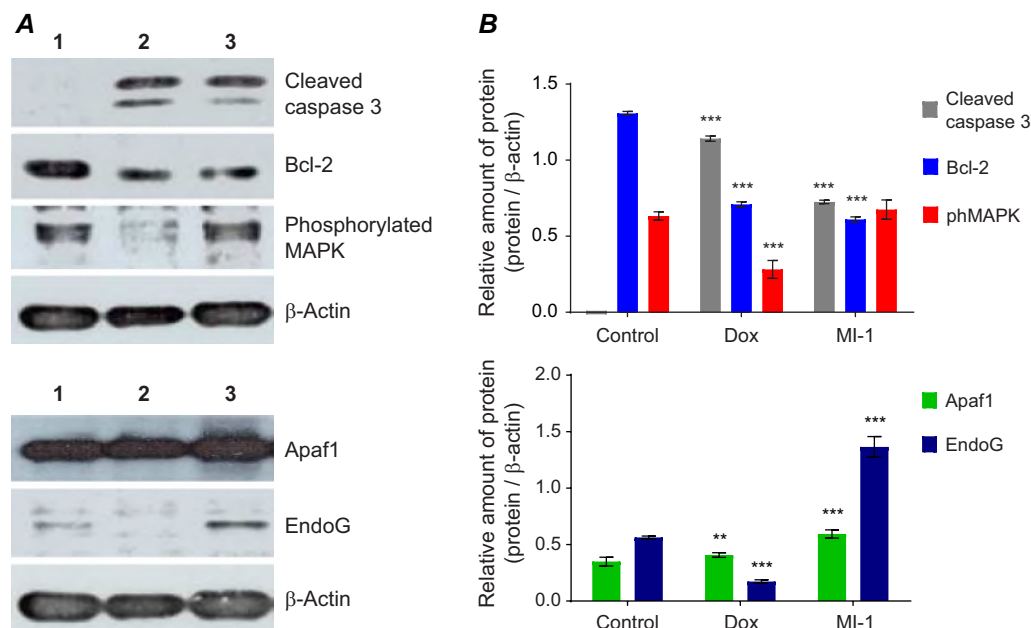


Fig. 2. Results of Western-blot analysis of apoptosis-associated proteins (**A**) and densitometry of the amount of these proteins (**B**) in HCT116 cells after 72 h of treatment with different agents: **1** – control; **2** – doxorubicin (Dox, 0.9 μ g/mL); **3** – MI-1 (0.9 μ g/mL)

Рис. 2. Результати Вестерн-блот аналізу апоптоз-асоційованих білків (**A**) та денситометричний аналіз вмісту цих білків (**B**) у клітинах лінії HCT116 через 72 год дії досліджуваних чинників: **1** – контроль; **2** – доксорубіцин (Dox, 0,9 мкг/мл); **3** – MI-1 (0,9 мкг/мл)

Next, we evaluated the content of Bcl-2 protein – a representative of the Bcl family of proteins that inhibit the development of apoptosis. We also estimated the level of Apaf1 and EndoG proteins known as regulators of apoptosis.

MI-1 reduced the level of anti-apoptotic protein Bcl-2 in human colon carcinoma cell of HCT116 line (**Fig. 2**). The action of Dox led to a similar decrease in the level of Bcl-2 in the studied tumor cells (**Fig. 2**) which is consistent with data on the pro-apoptotic effect of these agents. MI-1 increased the level of the cytosolic protein Apaf1 (apoptotic protease-activating factor 1) and EndoG in HCT116 cells (**Fig. 2**).

MI-1 did not affect the level of the phosphorylated form of MAPK (Mitogen Activated Protein Kinases) in the treated HCT116 cells, while doxorubicin significantly reduced the level of the phosphorylated form of MAPK (**Fig. 2**).

In order to estimate the amount of DNA fragmentation in HCT116 cells under the treatment with MI-1, a colorimetric diphenylamine assay was applied. An increased content of the fragmented DNA was revealed in these cells at such action (**Fig. 3**). At 0.9 μ g/mL dose, MI-1 induced DNA fragmentation at the level of 14.2 %, while Dox at the same dose caused 19.1 % of DNA fragmentation in HCT116 cells (**Fig. 3**).

DNA comet analysis of the targeted cells at their horizontal electrophoresis under alkaline conditions is applied for the detection of single-strand breaks in the DNA molecule and under neutral conditions – for the detection of double-strand breaks in the DNA molecule. This method is widely used for investigation of DNA damage under the action of physical (gamma radiation) or chemical (antitumor drugs) agents [20, 22].

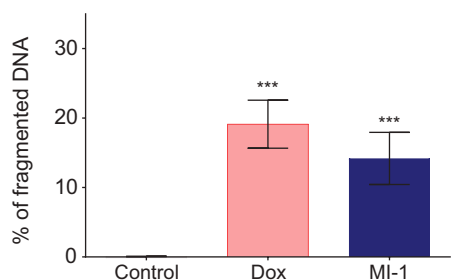


Fig. 3. The level of DNA fragmentation caused by MI-1 and Dox in HCT116 cells. Barton's method with the diphenylamine was used for quantitative identification of DNA fragmentation. *** – $P < 0.001$ (significant changes compared with control – untreated cells)

Рис. 3. Відсоток фрагментованої ДНК у клітинах лінії HCT116 за дії MI-1 і Дох. Для кількісної ідентифікації фрагментації ДНК застосовували метод Бартон з використанням дифеніламіну. *** – $P < 0.001$ (суттєві зміни порівняно з контролем – необробленими клітинами)

We used an alkaline comet assay in order to identify the potential of MI-1 and Dox to induce single-strand DNA breaks in the treated HCT116 cells. At the dose of $0.9 \mu\text{g/mL}$, MI-1 caused DNA damage in HCT116 cells with the OTM (Olive Tail Moment) of 13.2, while Dox induced OTM equal 10.9 (**Fig. 4**). A insignificant DNA damage (OTM = 1.8) was detected in the untreated HCT116 cells (**Fig. 4**).

DISCUSSION

In the previous study, we showed that MI-1 possessed a toxic action towards tumor cells of different origin (cervix, colon, breast, liver, pancreatic carcinomas). The most prominent effect was demonstrated at treatment of human cervix carcinoma (KB3-1 and KBC-1) and human colon carcinoma (HCT116) cells [10]. In this study, we have demonstrated that MI-1 induced the activation of caspase 3 via its cleavage in the treated HCT116 cells. As known, the effector caspases, in particular caspase 3, cleave various intracellular molecular targets that regulate the metabolism of DNA, histones and other proteins with biologically significant functions [25].

The development of antitumor agents that affect the mitochondrial signaling pathway of apoptosis is a promising strategy in the development of targeted antitumor chemotherapy [12, 27]. The Western-blot analysis showed that MI-1 induces the mitochondria-dependent pathway of apoptosis in HCT116 cells. MI-1 reduced the level of the anti-apoptotic protein Bcl-2, increased the level of Apaf1 protein and EndoG in human colon carcinoma cell of HCT116 line. The overexpression of Bcl-2 and Bcl-xL proteins inhibits apoptosis by reducing the production of the reactive oxygen species via stabilization of the mitochondrial membrane potential ($\Delta\psi$) and blocking the release of pro-apoptotic proteins (e.g. cytochrome c) from mitochondria [5]. The Apaf1 is one of central elements in the mitochondrial pathway of apoptosis activated by a wide range of pro-apoptotic stimuli. The formation of the apoptosome of a circular structure that includes Apaf1, cytochrome c, ATP, and initiating pro-caspase-9 is critical for the activation of the caspase cascade in the targeted cells [32].

MI-1 did not affect the content of the phosphorylated form of MAPK in HCT116 cells. MAPK is a group of protein serine-threonine kinases that play an important role in the regulation of cell proliferation and differentiation, as well as the survival and drug resistance of tumor cells [21].

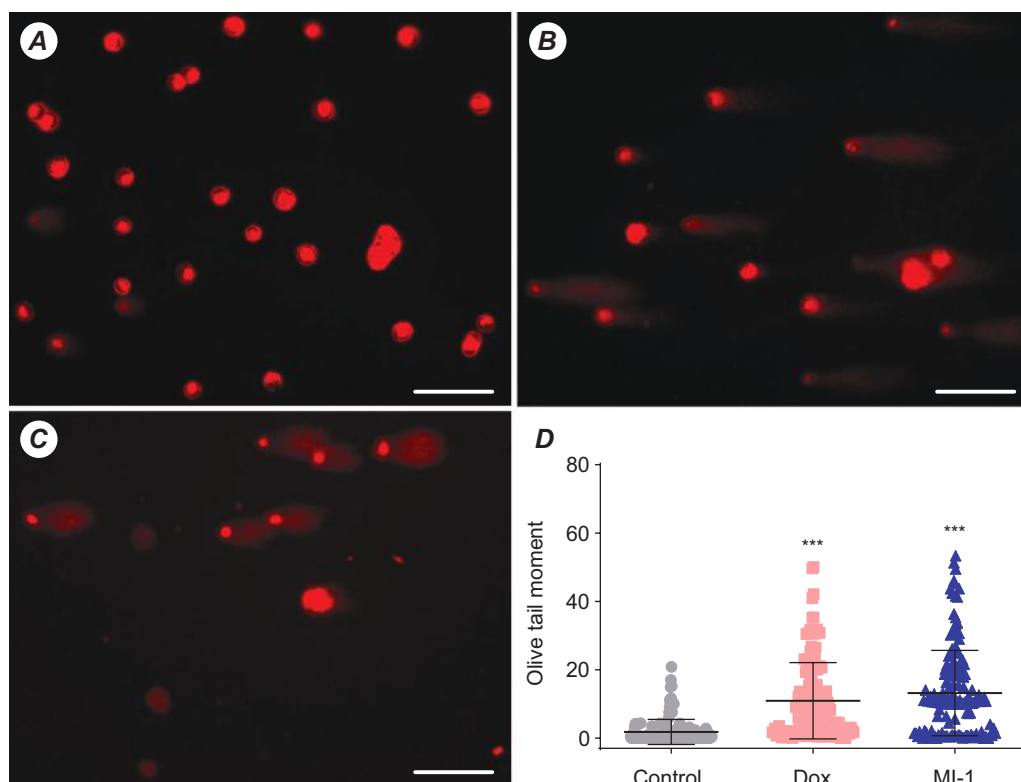


Fig. 4. The DNA damage effect (according to the results of DNA comet analysis under alkaline conditions) of the studied compounds in human colon carcinoma cells of HCT116 line: **A** – control, **B** – Dox (0.9 $\mu\text{g/mL}$), **C** – MI-1 (0.9 $\mu\text{g/mL}$), and **D** – quantitative data of DNA damage (Olive Tail Moment) in HCT116 cells. *** – $P < 0.001$ (significant changes compared with control – untreated cells). Bar equals 20 μm

Рис. 4. Показник ДНК-пошкоджувального ефекту (за результатами ДНК-комет аналізу за лужних умов) досліджуваних чинників у пухлинних клітинах лінії HCT116: **A** – контроль, **B** – Dox (0,9 мкг/мл), **C** – MI-1 (0,9 мкг/мл), **D** – кількісні дані щодо пошкодження ДНК у клітинах на 24-ту годину дії цих чинників. *** – $P < 0,001$ (суттєві зміни порівняно з контролем – необробленими клітинами). Лінійка відповідає 20 мкм

DNA fragmentation is catalyzed by the endonucleases and it is one of the indicators of the catabolic changes in the apoptotic pathway of cell death [29]. MI-1 induces DNA fragmentation and single-strand DNA breaks in the treated HCT116 cells. It is known that antitumor agents induce apoptosis that is accompanied by DNA fragmentation, in particular under the action of EndoG, while the inhibition of the endonuclease activity protects tumor cells from death [4]. As reported, the reactive oxygen species produced in the targeted cells under the action of the antitumor drugs cause the oxidative damage of DNA molecule and, consequently, increase the efficiency of the cytotoxic effects of these drugs [26]. The capability of MI-1 to activate caspase 3 together with the above noted elevation of the EndoG nuclease might be responsible for the degradation of the chromosomal DNA (DNA single-strand breaks and DNA fragmentation) in colon carcinoma cells. We propose a hypothetical scheme of signaling pathways induced by MI-1 during its inhibition of human colon carcinoma cells of HCT116 line (**Fig. 5**).

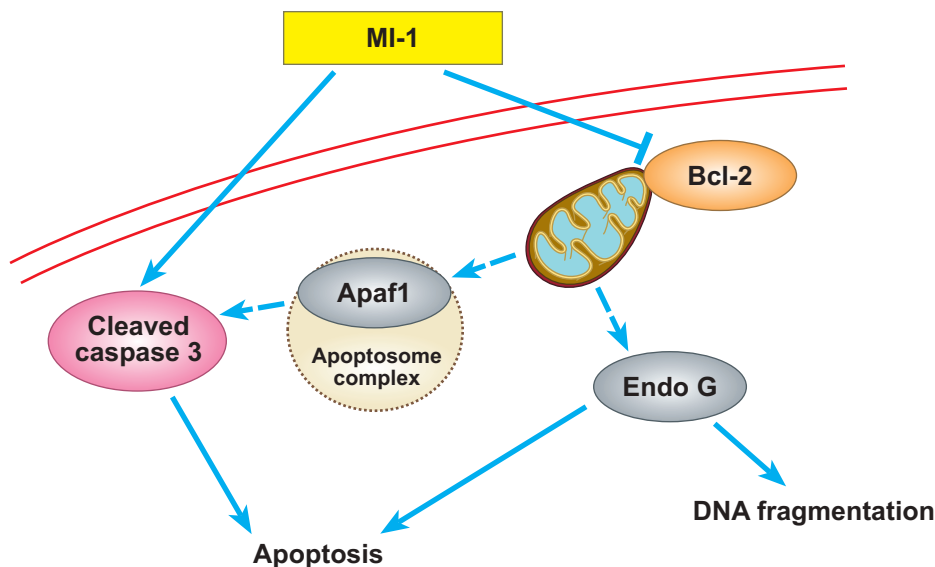


Fig. 5. A general scheme of signaling pathways induced by MI-1 in human colon carcinoma cells of HCT116 line

Рис. 5. Загальна схема сигнальних шляхів, індукованих MI-1 у пухлинних клітинах лінії HCT116

Concluding, we have shown that the anti-proliferative action of protein kinase inhibitor 1-(4-chlorobenzyl)-3-chloro-4-(3-trifluoromethylphenylamino)-1*H*-pyrrole-2,5-dione is associated with apoptosis induction via mitochondria-dependent pathway. Particularly, MI-1 activated caspase 3 and increased the level of mitochondria-specific endonuclease EndoG and Apaf1 protein. At the same time, it reduced the level of Bcl-2 protein in human colon carcinoma HCT116 cells. Besides, MI-1 induced DNA single-strand breaks and DNA fragmentation in these cells. Thus, 1-(4-chlorobenzyl)-3-chloro-4-(3-trifluoromethylphenylamino)-1*H*-pyrrole-2,5-dione (MI-1) might be a promising agent for killing tumor colon cells.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Animal Rights: This article does not contain any studies with animal subjects performed by the any of the authors.

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ІНГІБІТОР ПРОТЕЇНКИНАЗ 1-(4-ХЛОРОБЕНЗИЛ)-3-ХЛОРО-4-(3-ТРИФТОРОМЕТИЛФЕНІЛАМІНО)-1H-ПІРОЛ-2,5-ДІОН ІНДУКУЄ ПОШКОДЖЕННЯ ДНК І АПОПТОЗ У КЛІТИНАХ КАРЦИНОМИ ТОВСТОЇ КИШКИ ЛЮДИНИ

Н. С. Фінюк^{1,2}, О. Ю. Ключівська², Г. М. Кузнєцова³,
С. П. Ващук⁴, В. К. Рибальченко³, Р. С. Стойка^{1,2,3*}

¹ Львівський національний університет імені Івана Франка
вул. Грушевського, 4, Львів 79005, Україна

² Інститут біології клітини НАН України, вул. Драгоманова, 14/16, Львів 79005, Україна;

³ Київський національний університет імені Тараса Шевченка
вул. Володимирська, 64/13, Київ 01601, Україна;

⁴ Львівський науково-дослідний експертно-криміналістичний центр Міністерства
внутрішніх справ України, вул. Конюшинна, 24, Львів, 79000, Україна

*Кореспондуючий автор e-mail: stoika@cellbiol.lviv.ua

Вступ. Гетероциклічні сполуки належать до ключових структурних елементів для синтезу біологічно-активних речовин.

Матеріали та методи. Метою дослідження було вивчення механізмів проапоптотичної і ДНК-пошкоджувальної дії *in vitro* похідного малеїмиду – 1-(4-хлоробензил)-3-хлоро-4-(3-трифторометилфеніламіно)-1H-пірол-2,5-діон (MI-1) у клітинах лінії НСТ116 карциноми товстої кишки людини. Для вивчення зміни кількості апоптоз-асоційованих білків був використаний Вестерн-блот аналіз, для визначення ДНК-пошкоджувальної дії – ДНК-комет аналіз за лужних умов, для оцінювання рівня фрагментованої ДНК – метод Бартонна з дифеніламіном.

Результати. Результати проведеного Вестерн-блот аналізу свідчать про те, що сполука MI-1 активувала ефекторну каспазу 3 завдяки її розщепленню у клітинах НСТ116 карциноми товстої кишки людини. Ця сполука підвищувала вміст специфічних мітохондріальних білків, а саме ендонуклеази G (EndoG) і про-апоптотичного цитозольний протеїну протеазо-активувального фактора 1 (Apaf1). MI-1 знижував кількість анти-апоптотичного протеїну Bcl-2 у клітинах лінії НСТ116. ДНК-комет

аналіз за лужних умов клітин карциноми товстої кишки людини лінії НСТ116 підтвердив, що MI-1 індукував одноланцюгові розриви у молекулі ДНК у клітинах лінії НСТ116 із показником "olive tail moment" на рівні 13,2. Дія MI-1 призводила до зростання до 14,2 % ступеня фрагментації ДНК у клітинах лінії НСТ116, що підтверджено за допомогою колориметричного аналізу з використанням дифеніламіну.

Висновки. Антипроліферативна дія MI-1 у клітинах лінії НСТ116 карциноми товстої кишки людини пов'язана з індукцією опосередкованого мітохондріями апоптозу, і пошкодженням ДНК через одноланцюгові розриви в ДНК і фрагментацію молекули ДНК. Тому 1-(4-хлоробензил)-3-хлоро-4-(3-трифторометилфеніламіно)-1*H*-пірол-2,5-діон (MI-1) може бути перспективним протипухлинним чинником для клітин карциноми товстої кишки.

Ключові слова: 1*H*-пірол-2,5-діони, апоптоз, Вестерн-блот аналіз, комет-аналіз, одноланцюгові розриви, тест Бартона, фрагментація ДНК