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CYTOTOXIC ACTION OF MALEIMIDE DERIVATIVE 1-(4-CL-BENZYL)-3-CHLORO-4-(CF_3-PHENYLAMINO)-1*H*-PYRROLE-2,5-DIONE TOWARD MAMMALIAN TUMOR CELLS AND ITS CAPABILITY TO INTERACT WITH DNA

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Development of chemical compounds capable to supress tumor progression is a perspective strategy of cancer treatment. Heterocyclic compounds possess a broad spectrum of biological activities, including anticancer one. According to the previous results of in silico modeling maleimide derivative 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dione (MI-1) has a potential effect as an inhibitor of tyrosine protein kinases. The present study was aimed at in vitro evaluation of MI-1 cytotoxic effects toward tumor cells of various lines. The viability of tumor cells after incubation with MI-1 was measured by means of 3,4,5-dymetyltiazol-2-yl-2,5-diphenyl-tetrazolium bromide (MTT) test. The MI-1 compound was shown to be toxic for a majority of studied tumor cell lines with IC_{50} value ranging from 0.8 to 62.2 μg/ml depending on the tissue origin of cells. The most prominent effect of MI-1 towards human cervix carcinoma (KB3-1 and KBC-1) cells with six times higher toxicity towards the multidrug resistant sub-line KBC-1 cells comparing with the action of Doxorubicin was demonstrated. MI-1 inhibited the viability of human pancreatic, hepatocarcinoma, and colon carcinoma cells only in high doses, while human and rat glioblastoma cells were not sensitive to MI-1. Thus, the MI-1 anticancer activity dropped in the following rank of tumor cells: cervix > breast > pancreatic carcinoma > liver carcinoma > colon carcinoma > glioblastoma. Experiments on replacement of methyl green dye from DNA-methyl green complex showed that MI-1 intercalated into DNA molecule structure. The increase of the amount of the additional band of super-spiral DNA in the presence of MI-1 was revealed by means of DNA retardation at electrophoresis in the agarose gel and this effect was more pronounced than the effect of doxorubicin. The data presented indicate a new DNA-targeting mechanism of maleimide derivative 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dione anticancer action.

K e y w o r d s: 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dione, anticancer activity, MTT.

Cancer is a worldwide health problem, and it is the second leading reason of human mortality after the cardiovascular diseases. A development of chemical compounds capable of specific blocking key regulatory proteins of tumor progression is a perspective strategy of cancer treatment [1, 2].

Heterocyclic compounds possess a broad spectrum of biological activities, including anticancer one. The maleimide derivate 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1*H*-pyrrole-2,5-dione (MI-1) was designed *in silico* [3] 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) [4, 5]. MI-1 demonstrated anti-proliferative activity on colorectal cancer cells *in vitro* [6] and *in vivo* [7], as well as adenocarcinoma colon cells of SW620 line [8]. Besides, this compound demonstrated an anti-inflammatory activity in case of experimental chronic ulcerative colitis of

rats [9] and possessed low general toxicity in laboratory animals [7, 10, 11].

Protein kinases are enzymes that regulate different cellular processes including cell proliferation, differentiation and migration, cell cycle regulation, and metabolism [2, 12]. A deregulation of these processes via mutations or over-expression of kinases leads to various diseases [13, 14]. Kinases play an important role in the carcinogenesis and metastases of different types of cancer [2, 15]. Thus, a development of protein kinases inhibitors is of great interest for the cancer treatment. A number of protein kinases inhibitors, such as imatinib [16], benzotriazines, quinazolines, pyrazolopyrimidines, imidazo[1,5-a]pyrazines, pyridopyrimidinones and other heterocycles, ATP-phospho-peptide conjugates have been developed and investigated for the treatment of cancer [12, 17-19].

It is obvious that the multifunctional anticancer agents affecting different bio-molecules and biological processes could be more efficient treatment remedies comparing to such agents with only one bio-target in the tumor cells. The aim of our study was to carry out a search for novel biological targets at the cytotoxic action of maleimide derivative MI-1, namely evaluating a possibility that DNA molecule could also be such target. Besides, we addressed a potential toxic effect of MI-1 towards mammalian tumor cell lines, specifically the drug-resistant tumor cells not studied earlier.

Materials and Methods

Compounds under study. The synthesis of maleimide derivate 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1*H*-pyrrole-2,5-dione (MI-1, Fig. 1) was carried out by successive chemical transformations, as described previously [6, 11]. The stock solution of MI (10 mg/ml) was prepared in the Dimethyl Sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA), and before adding to the cultured cells, further solutions were prepared using culture medium. Doxorubicin (Actavis S.R.L., Bucharest, Romania) was used as a positive control anticancer drug.

Cell culture and anti-proliferative MTT assay. Human pancreatic ductal adenocarcinoma Capan-1 cells, human epidermoid cervix carcinoma KB3-1 cells and its colchicine-resistant sub-line KBC-1, that is characterized by over-expression of plasma membrane P-glycoprotein, were donated by a Collection of the Institute for Cancer Research at Vienna Medical University (Vienna, Austria). Human breast

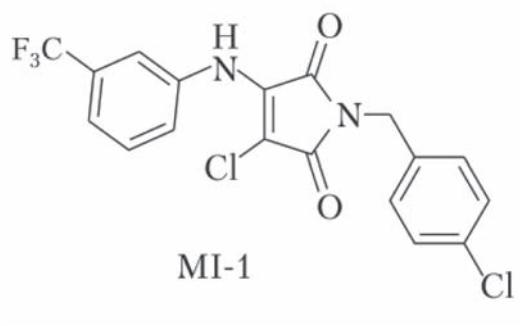

Fig. 1. Schematic structure of the maleimide derivative MI-1 (1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dione)

adenocarcinoma MCF-7 and MDA231, human colon carcinoma HCT116 cells, human hepatocarcinoma HepG2 cells were obtained from the Collection at the Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine). Human glioblastoma cells of U251, U373 and T98G lines, rat glioma C6 cells were obtained from a Collection at the Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine (Kyiv, Ukraine). Cells were grown in RPMI-1640 (Biowest, Nuaille, France) or Dulbecco's-modified Eagle's medium (DMEM, Biowest, Nuaille, France) culture medium supplemented with 10% fetal bovine serum (Biowest, Nuaille, France) at the standard conditions (37 °C in an atmosphere of 5% CO_2).

The MI-1 cytotoxicity towards tumor cells *in vitro* was measured using colorimetric MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich, USA) assay for estimating functional activity of the treated cells. Cells were plated at 5,000 cells/well (substrate-dependent cells) or 15,000 cells/well (suspension cells) in 100 µl of 96-well plates and allowed to grow overnight. The MI-1 or Doxorubicin at 1, 10, and 100 µg/ml was added in 100 µl of cultural medium, and cells were incubated for the next 72 h. After that, the MTT assay was performed according to the manufacturer's recommendations (Sigma-Aldrich, St. Louis, MO, USA). The absorbance of formazan was measured by an Absorbance Reader BioTek ELx800 (BioTek Instruments, Inc., Winooski, VT, USA). The IC_{50} level of MI-1 was calculated as the drug concentration that reduced cell viability by 50% [20].

Electrophoretic gel retardation assay of plasmid DNA. Plasmid DNA (1 µg) pEGFPc-1 (Clontech, Mountain View, California, USA) was mixed with 2 µl of MI-1 (1; 10; 100 µg/ml), and Dox (1 and

10 μg/ml) in 18 μl of 0.9% sodium chloride (Arterium, Lviv, Ukraine) at room temperature for 1 h. Prepared mixture was analyzed by electrophoresis in 1% agarose gel (Lachema, Brno, Czech Republic) with 1× Tris acetate (TAE) buffer containing 1 μg/ml of Ethydium bromide (Sigma-Aldrich, St. Louis, Missouri, USA) for approximately 1 h at a constant voltage of 70 V. The trans-illuminator (MacroVue UV-20, Hoeffer, Troy, Michigan, USA) was used for visualization of plasmid DNA [21].

DNA intercalation assay using methyl green replacement. Methyl green binds to DNA with an absorption maximum at 642 nm. Free methyl green does not show absorption at this wavelength, while the compounds that bind/intercalate with DNA replaced methyl green from the complex of methyl green–DNA and decreased the optical density at 642 nm wave length. 485 μl of salmon sperm DNA (50 μg/ml, Sigma-Aldrich, St. Louis, Missouri, USA) were incubated for 1 h at 37 °C with 15 μl of methyl green (Sigma-Aldrich, St. Louis, Missouri, USA) solution (1 mg/ml in water). 500 μl of MI-1 (1 and 10 μg/ml), or Dox (10 μM) were added to methyl green-DNA complex and incubated for 2 h at 37 °C in the dark. The Ethydium bromide (EtBr, 1 and 10 μg/ml) was used as a positive control. Absorption of methyl green was measured at 630 nm [22] using a fluorescence plate reader (Absorbance Reader BioTek ELx800, BioTek Instruments, Inc., Winooski, Vermont, USA).

Data analysis. The results were analyzed and illustrated using Origin software (version 7). All data are presented as the mean (M) ± standard deviation (SD) of three independent replications. A t test was used for statistical analysis. Statistical significance was identified at $P \leq 0.05$.

Results and Discussion

Cytotoxicity study. In this study, human tumor cells of different tissue origin, namely leukemia, cervix, breast, glioblastoma, and pancreas, were subjected to the action of the MI-1 compound applied in three doses - 1, 10, and 100 μg/ml. The MTT assay was used for evaluation of the antineoplastic activity of the MI-1. It was found that the MI-1 inhibited growth of human breast adenocarcinoma MCF-7 cells with the IC_{50} of 9.7 μg/ml (Fig. 2, Table), while the Doxorubicin demonstrated higher cytotoxicity towards these tumor cells (IC_{50} = 1.9 μg/ml). However, human breast adenocarcinoma cells of the MDA231 line were relatively resistant to the action of MI-1 in dose up to 50 μg/ml (Fig. 2, Table).

Rapid development of the multidrug resistance is one of the main problems in cancer treatment. Among the main mechanisms of such resistance are the over-expression of the ABC transporters on plasma membrane, mutations in the *BRCA* gene, *DYNLL1* gene and other mechanisms that promote drug inactivation and/or degradation [23, 24]. In this study, we compared the anti-neoplastic activity of the MI-1 towards human epidermoid cervix carcinoma cells of KB3-1 line and its colchicine-resistant KBC-1 sub-line that is characterized by P-glycoprotein over-expression. The MI-1 proved to be six times more effective in growth inhibition of KBC-1 cells (IC_{50} = 0.8 μg/ml), comparing to Doxorubicin (IC_{50} = 4.8 μg/ml), while at targeting wild type KB3-1 line, the picture was opposite and these cells were more sensitive to the Doxorubicin (IC_{50} = 0.3 μg/ml) than to the MI-1 (IC_{50} = 7.5 μg/ml) (Fig. 2, Table). Thus, the MI-1 was shown to be capable of circumventing drug resistance mechanism dependent on P-glycoprotein over-expression.

The MI-1 also inhibited growth of human pancreatic ductal adenocarcinoma Capan-1 cells (IC_{50} = 18.0 μg/ml), human hepatocarcinoma HepG2 cells (IC_{50} = 33.5 μg/ml), and human colon cancer HCT116 cells (IC_{50} = 62.2 μg/ml) (Fig. 3, Table). These concentrations are much higher than those

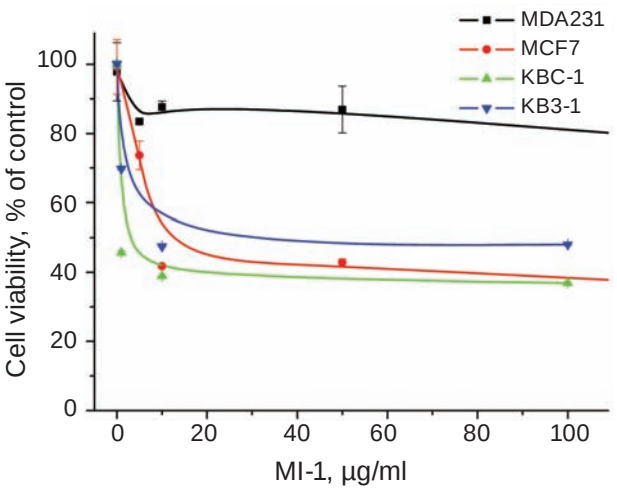

Fig. 2. The anti-proliferative activity of 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dion (MI-1) towards human breast adenocarcinoma (MCF-7 and MDA 231) cells, human epidermoid cervix carcinoma cells of KB3-1 line and its colchicine-resistant KBC-1 sub-line. Cell viability was examined using the MTT assay after 72 h cell exposure to the MI-1 compound

Cytotoxicity indicator (IC_{50}) of 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dion (MI-1) and Doxorubicin (Dox) at targeting human tumor cells of different tissue origin

Cell line	IC_{50}, μg/ml	
	MI-1	Dox
KB3-1	7.5	0.3
KBC-1	0.8	4.8
Capan-1	18.0	0.2
Hep G2	33.5	0.6
HCT 116 wt	62.2	0.8
MCF-7	9.7	1.9
MDA231	>50	1.6
U251	>50	0.4
U373	>50	0.7
T98G	>50	0.4
C6	>50	1.3

mentioned above for inhibition of human epidermoid cervix carcinoma cells of KB3-1 line and its colchicine-resistant KBC-1 sub-line. These doses of the MI-1 compound are also much higher than the cytotoxic doses of the Doxorubicin (Table).

Human glioblastoma cells of U251, U373, and T98G and rat glioma C6 lines were relatively non-sensitive to the MI-1 action, and the IC_{50} level was above 50 μg/ml, while IC_{50} for Doxorubicin indicated high cytotoxic effect of the last chemotherapeutic drug (Fig. 4, Table).

Study of interaction of the MI-1 with DNA. The 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1*H*-pyrrole-2,5-dion (MI-1) was designed *in silico* as an inhibitor of tyrosine kinases [3] that are important targets in the action of many anticancer drugs [4, 5]. However, other biological targets of the MI-1's effect cannot be excluded. In order to address such possibility, we have suggested that the DNA molecule could be another target that is used at the cytotoxic action of the MI-1 compound. It is known that several FDA approved anticancer therapeutics, such as doxorubicin, cisplatin, bleomycin, chlorambucil, etoposide, mephalan can bind the DNA and in such a way affect its structure and functions [22, 25, 26]. Besides, the Reactive Oxygen Species (ROS) induced in treated cells by the above noted anticancer drugs can cause the oxidative damage of DNA molecule, and thus, enhance the efficacy of cytotoxic action of these drugs [27]. The DNA-interacting agents can also affect the cell-cycle checkpoints leading to their block [26].

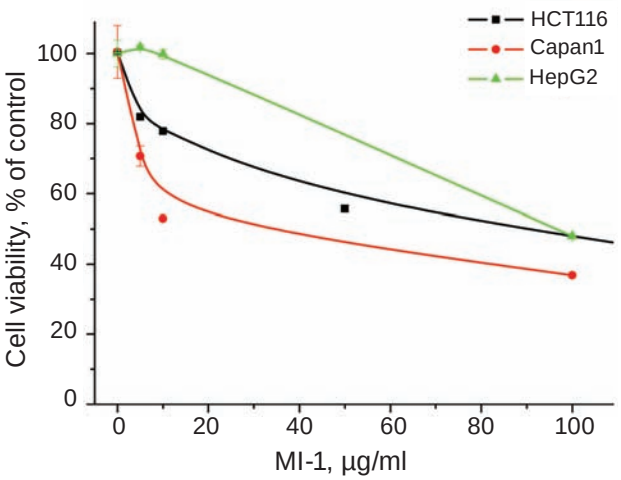

Fig. 3. The anti-proliferative activity of 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dion (MI-1) towards human colon cancer cells of HCT116 line, human pancreatic ductal adenocarcinoma Capan-1 cells, and human hepatocarcinoma HepG2 cells. Cell viability was examined using the MTT assay after 72 h cell exposure to the MI-1 compound

It was shown that the MI-1 used at 1 and 10 μM concentrations replaced the methyl green dye in the DNA-methyl green complex by 21.2 and 16.5%,

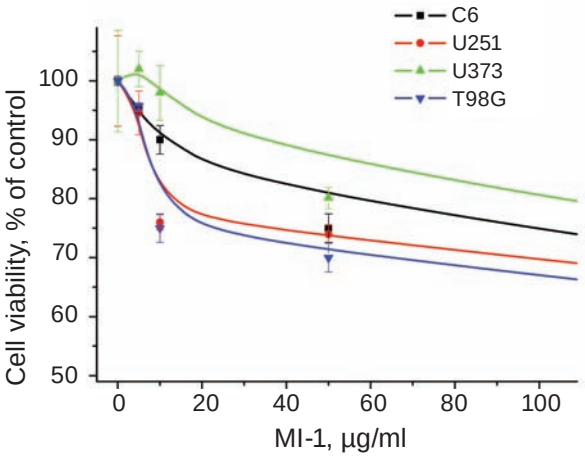

Fig. 4. The anti-proliferative activity of 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dion (MI-1) towards human (U251, 373, T98G) and rat (C6) glioblastoma cells. Cell viability was examined by using MTT assay after 72 h exposure to the compound

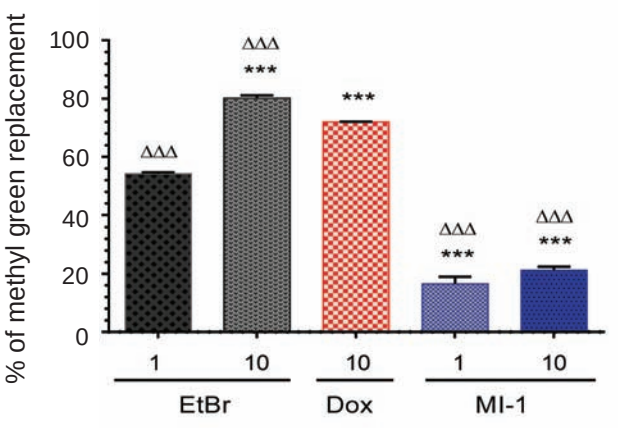

*Fig. 5. The results of replacement of methyl green dye in the DNA-methyl green complex by the MI-1, Doxorubicin and Ethydium bromide (EtBr). ***P < 0.001 (significant changes compared with effect of EtBr (1 μg/ml)); $^{ΔΔΔ}P < 0.001$ (significant changes compared with effect of Doxorubicin (10 μg/ml))*

respectively (Fig. 5). The Doxorubicin (10 μg/ml) also known as the DNA-intercalating agent [26], replaced by 72.1% the methyl green in its complex with the DNA, while the Ethydium bromide (1 and 10 μg/ml), a well-known DNA-intercalating agent, demonstrated 54.1 and 80.1% replacement of the methyl green, respectively (Fig. 5).

As one can see on Fig. 6 (lanes 4-6), plasmid DNA was not retarded at electrophoresis by the MI-1 used in 1, 10 and 100 μg/ml doses, while the Doxorubicin used in 1 and 10 μg/ml doses as a positive control, retarded the electrophoretic mobility in the agarose gel of the supercoiled form of plasmid DNA (Fig. 6, lane 2). It should be noted that three bands of plasmid DNA were detected after its 1 h incubation with the MI-1 compound that suggests its potential to induce supercoiling of the DNA molecule that could cause its functional deterioration. Taken together, the results of the methyl green replacement assay and the results of the electrophoretic retardation study of plasmid DNA definitely indicate a possibility of a direct interaction of the MI-1 with the DNA molecule. However, additional experiments are necessary in order to confirm or exclude a specificity of such binding of the MI-1 to the DNA.

In this study, we detected that the maleimide derivative 1-(4-Cl-benzil)-3-Cl-4-(CF_3-phenylamino)-1*H*-pyrrole-2,5-dione (MI-1 compound) possessed a remarkable cytotoxic activity towards several human tumor cell lines with the inhibition indicator (IC_{50}) ranging from 0.8 to 62.2 μg/ml for different cell lines. The most prominent cytotoxic effect was demonstrated by the MI-1 towards human cervix carcinoma cells of KB3-1 and KBC-1 lines. It should be stressed that the MI-1 showed six times higher toxicity towards the multidrug resistant KBC-1 subline of these cells, comparing with the cytotoxic action of doxorubicin. The MI-1 also inhibited the viability of human pancreatic, hepatocarcinoma, colon carcinoma cells, however, did that at high doses than for the above mentioned human cervix carcinoma cells. Human glioblastoma cells were poorly sensitive to a cytotoxic action of the MI-1. Thus, the anti-neoplastic activity of the MI-1 dropped in the

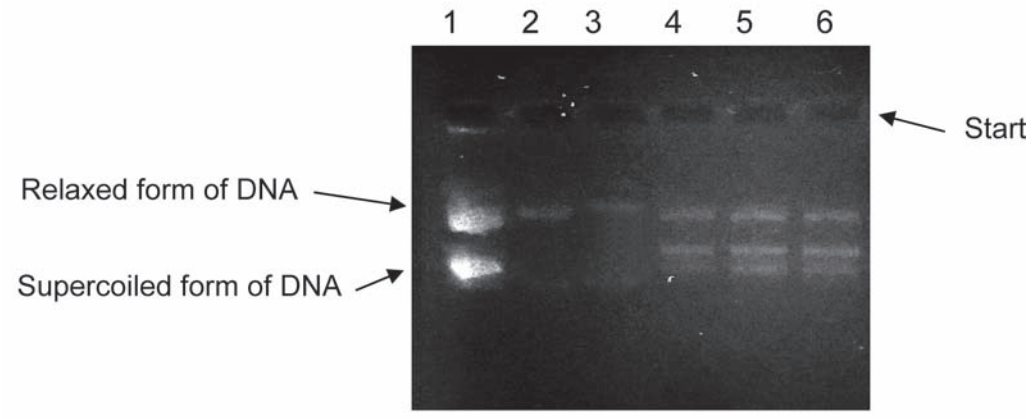

Fig. 6. The results of electrophoretic retardation of plasmid DNA by the MI-1 compound and Doxorubicin (Dox): lane 1 – native plasmid DNA pEGFP c-1 (negative control); 2 – Dox, 1 μg/ml + pDNA; 3 – Dox, 10 μg/ml + pDNA; 4 – MI-1, 1 μg/ml + pDNA; 5 – MI-1, 10 μg/ml + pDNA; 6 – MI-1, 100 μg/ml + pDNA. Studied compounds were added to a sample of plasmid DNA (pEGFPc-1), the mixture was incubated for 1 h at room temperature, and then electrophoresis was conducted in the agarose gel

following rank of cells: cervix carcinoma > breast carcinoma > pancreatic carcinoma > liver carcinoma > colon carcinoma > glioblastoma.

In addition to cytotoxicity study, the investigation of the interaction of the MI-1 with the DNA molecule was carried out using DNA intercalation and DNA retardation tests. The results of both of these tests indicate a possibility of direct interaction of the MI-1 with the DNA molecule. These results allow one to suggest that DNA damaging could be an additional mechanism of induction of tumor cell death, besides earlier proposed mechanism of inhibition of tyrosine-specific protein kinases. However, further investigations are needed to confirm experimentally any or both of these mechanisms of the potential anticancer activity of the synthesized MI-1 compound.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ЦИТОТОКСИЧНА ДІЯ ПОХІДНОГО МАЛЕЇМІДУ 1-(4-Cl-БЕНЗИЛ) -3-ХЛОРО-4-(CF_3-ФЕНІЛАМІНО)-1*H*-ПІРОЛ-2,5-ДІОНУ НА ПУХЛИННІ КЛІТИНИ ССАВЦІВ І ЙОГО ЗДАТНІСТЬ ДО ВЗАЄМОДІЇ З ДНК

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Розробка хімічних сполук, здатних пригнічувати ріст пухлин є перспективною стратегією в лікуванні раку. Гетероциклічні сполуки виявляють широкий спектр біологічної дії, в тому числі протиракову активність. Попередні результати моделювання *in silico* показали, що похідне малеїміду 1-(4-Cl-бензил)-3-Cl-4-(CF_3-феніламіно)-1*H*-пірол-2,5-діон (MI-1) виявляє потенційний ефект як інгібітор тирозин-специфічних протеїнкіназ. Метою роботи було визначити *in vitro* цитотоксичні ефекти MI-1 щодо пухлинних клітин різних ліній. Життєздатність пухлинних клітин після інкубації з MI-1 визначали за допомогою 3,4,5-диметилтіазол-2-іл-2,5-дифеніл-тетразоліум бромід тесту (МТТ-тесту). Показано, що сполука MI-1 є токсичною для більшості досліджуваних пухлинних клітинних ліній із IC_{50} в межах від 0,8–62,2 мкг/мл залежно від тканинного походження клітин. Найвираженіший ефект MI-1 спостерігали на клітинах карциноми шийки матки людини (KB3-1 і KBC-1). Так, MI-1 виявляла в шість разів більшу цитотоксичність щодо медикаментозно стійкої сублінії клітин KBC-1 порівняно з дією доксорубіцину.

Сполука MI-1 пригнічувала ріст клітин раку підшлункової залози, гепатокарциноми і карциноми товстої кишки людини тільки у високих концентраціях. Клітини гліобластоми людини та щурів були нечутливими до дії MI-1. Протипухлинна активність MI-1 знижувалася в такому порядку: рак шийки матки > карцинома молочної залози > рак підшлункової залози > рак печінки > карцинома товстої кишки > гліобластома. Показано, що MI-1 інтеркалює в структуру молекули ДНК, що було підтверджено здатністю цієї сполуки заміщати метиленовий зелений (близько 20%) із комплексу ДНК-метиловий зелений. Методом ДНК електрофорезу в агарозному гелі виявлено збільшення кількості додаткової фракції суперспіральної форми ДНК у присутності MI-1. Такий ефект MI-1 був істотнішим, ніж ефект доксорубіцину. Представлені дані свідчать про новий, асоційований із взаємодією з ДНК, механізм протипухлинної дії похідного малеїміду 1-(4-Cl-бензил)-3-Cl-4-(CF_3-феніламіно)-1*H*-пірол-2,5-діону.

Ключові слова: 1-(4-Cl-бензил)-3-хлор-4-(CF_3-феніламіно)-1*H*-пірол-2,5-діон, протипухлинна активність, МТТ.

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