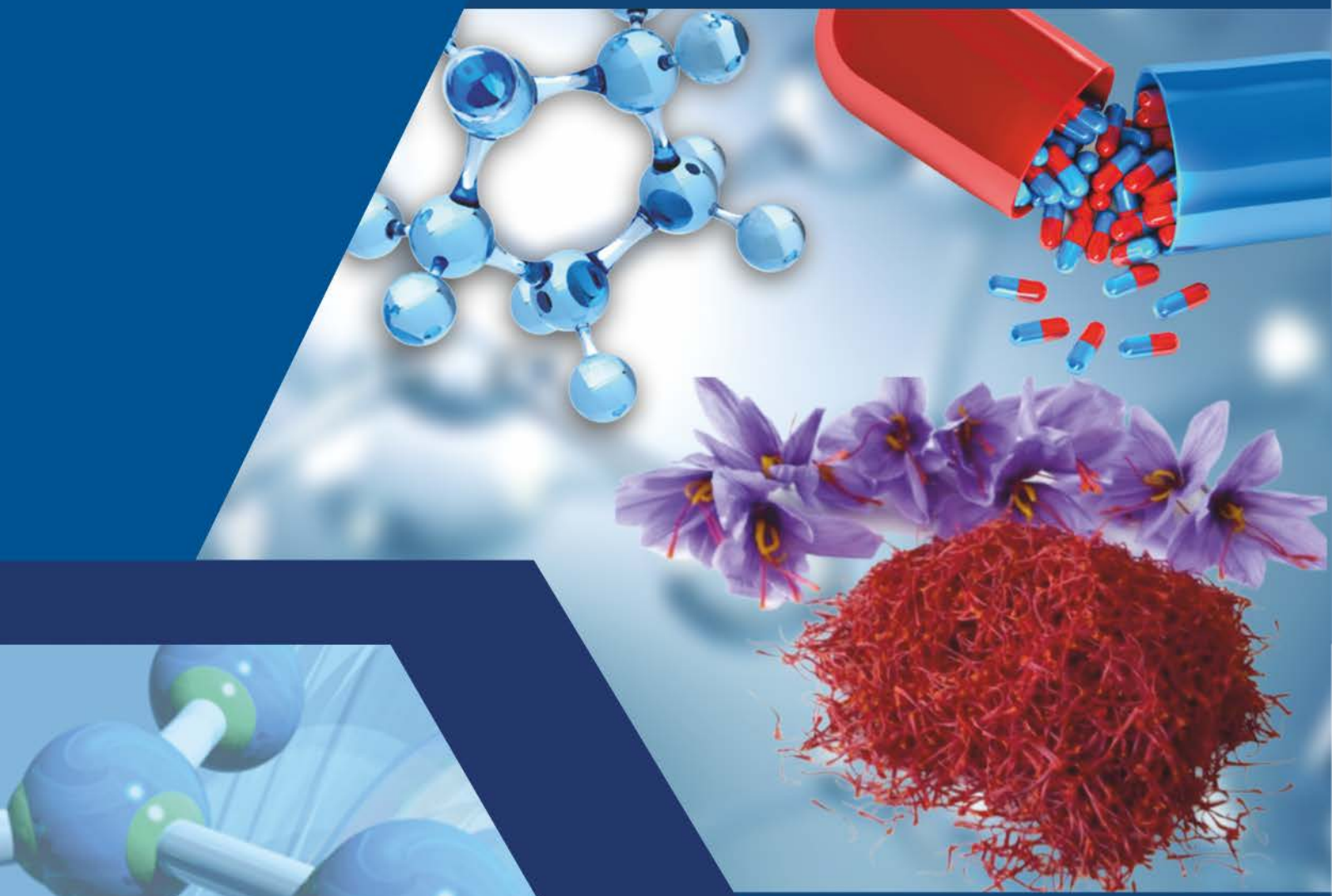
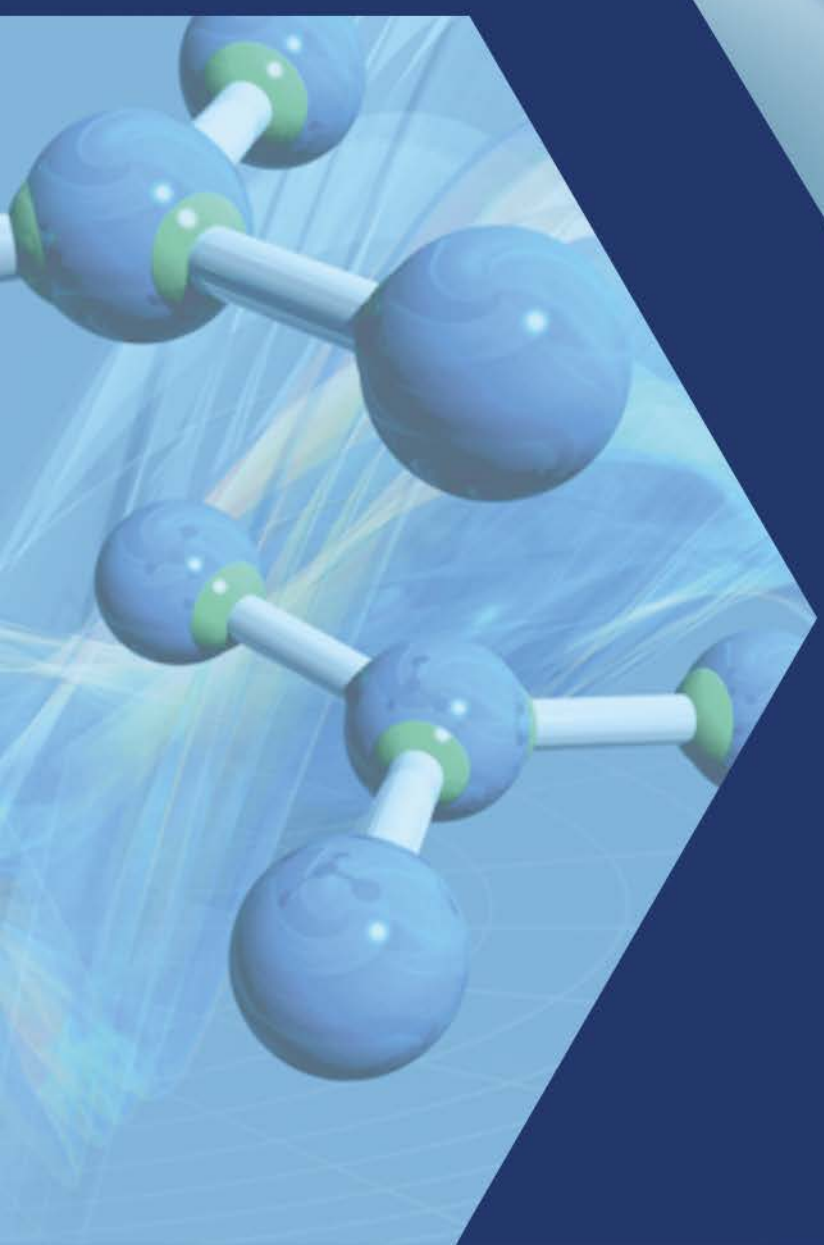
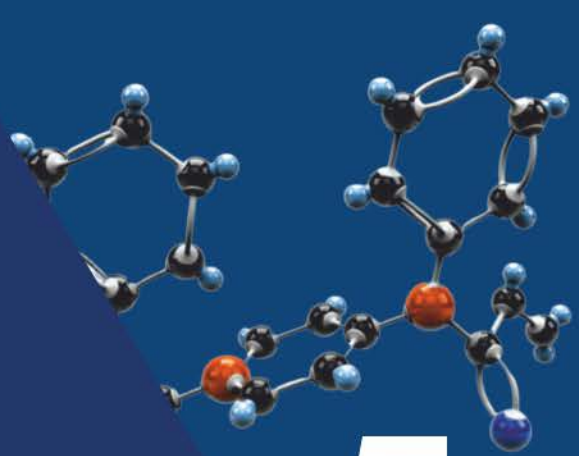
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CYTOTOXICITY OF ARENOBUFAGIN AND ITS DERIVATIVES AGAINST MCF-7 CELLS

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Arenobufagin is a bufadienolide with potent antitumor activity against a variety of human cancer cell lines. In the present study, the cytotoxic activity of arenobufagin and seven newly synthesized derivatives was evaluated against MCF-7 human breast cancer cells using the MTT assay. Cells were exposed to the tested compounds at concentrations of 10, 50, and 300 nM for 24 h. Arenobufagin and the derivatives Ar-004, Ar-007, Ar-008, and Ar-015 exhibited pronounced concentration-dependent cytotoxicity, whereas Ar-014 showed only moderate activity. In contrast, Ar-005 and Ar-011 demonstrated little or no cytotoxic effect under the experimental conditions. The obtained results indicate that structural modification of arenobufagin markedly influences its cytotoxic activity against MCF-7 cells. These findings provide useful preliminary information for the biological evaluation of newly synthesized arenobufagin derivatives and may contribute to the identification of promising lead compounds for further anticancer studies.

Keywords: *arenobufagin; bufadienolides; MCF-7 cells; breast cancer; cytotoxicity; MTT assay.*

Introduction. Breast cancer remains one of the most frequently diagnosed malignancies and a leading cause of cancer-related mortality among women worldwide. Consequently, the identification of novel anticancer agents with improved efficacy and distinct mechanisms of action remains an important objective in cancer research. Natural products have historically served as an invaluable source of biologically active compounds for drug discovery. Among them, bufadienolides have attracted increasing attention because of their potent antiproliferative activity against a wide range of human malignancies. Arenobufagin is one of the major

bufadienolides isolated from *Bufo viridis* and has emerged as a promising lead compound for anticancer drug development. Previous studies have demonstrated potent cytotoxic activity of arenobufagin against a variety of human cancer cell lines, including hepatocellular, colorectal, lung, gastric, esophageal, pancreatic, and breast cancers. [1]. Furthermore, arenobufagin has been reported to induce apoptosis in MCF-7 breast cancer cells through activation of the JNK signaling pathway, leading to multisite phosphorylation of Yes-associated protein (YAP), enhanced interaction with p73, and subsequent activation of pro-apoptotic genes [2].

Growing evidence indicates that relatively small structural modifications to bufadienolides can substantially alter their affinity for biological targets, thereby influencing their pharmacological and toxicological properties [3]. Therefore, the synthesis and biological evaluation of novel arenobufagin derivatives represent a rational strategy for identifying compounds with improved biological characteristics. The MCF-7 cell line is one of the most extensively characterized *in vitro* models of estrogen receptor-positive breast cancer and is widely employed for the preliminary evaluation of anticancer compounds and investigations of apoptosis-related mechanisms [4].

Aim of the study. This study aimed to evaluate the cytotoxic activity of arenobufagin and seven newly synthesized derivatives against MCF-7 human breast cancer cells using the MTT assay. Comparative analysis of these compounds may provide useful information on how structural modification influences the cytotoxic properties of arenobufagin and facilitate the identification of promising candidates for further pharmacological investigation.

Materials and Methods. Arenobufagin and its seven derivatives (Ar-004, Ar-005, Ar-007, Ar-008, Ar-011, Ar-014, and Ar-015) were synthesized in our laboratory. Their chemical structures are presented in Figure 1.

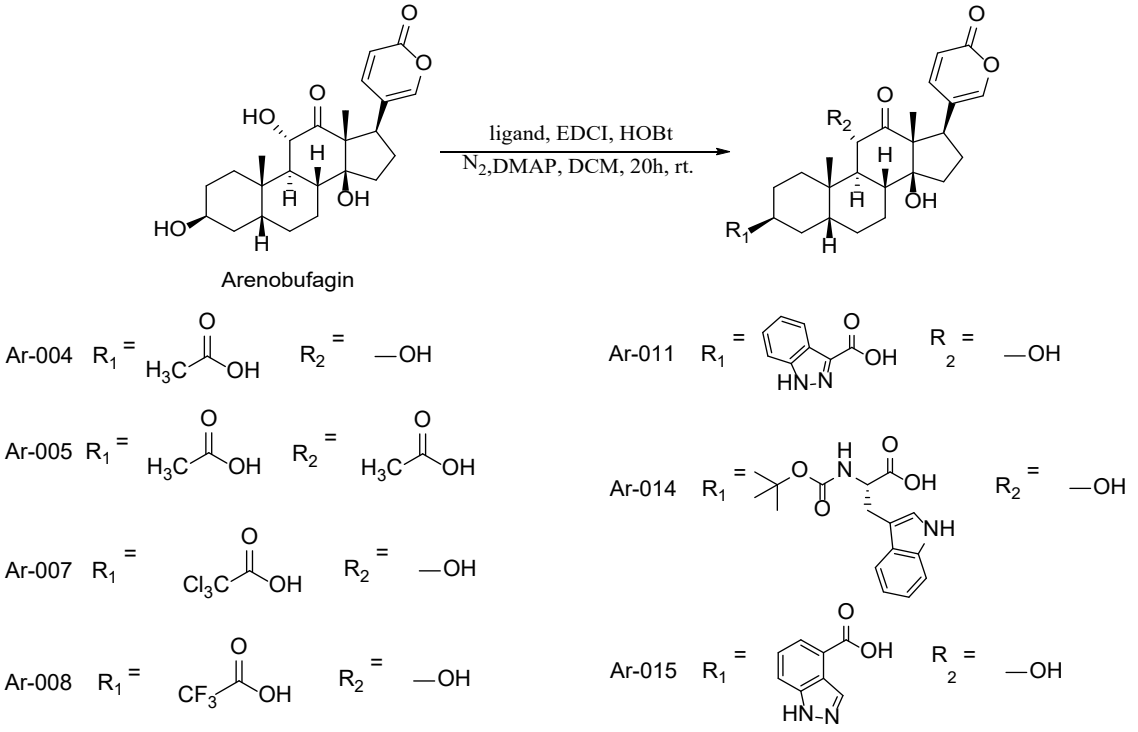


Figure 1. **Structures of arenobufagin and its derivatives.**

Detailed synthetic procedures and structural characterization are beyond the scope of the present study and will be reported separately. Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO) and diluted with culture medium immediately before use. The final concentration of DMSO in the culture medium did not exceed 0.1% (v/v).

The MTT dye 2-4,5-dimethyl-2-thiazolyl-3,5-diphenyl-2H-tetrazolium bromide, Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline (PBS), and trypsin from porcine pancreas were purchased from Sigma-Aldrich (Budapest, Hungary). Penicillin-streptomycin mix, 96-well plates, and cell culture flasks were obtained from Thermo-Fisher (Darmstadt, Germany, CAS number: 156499).

The MTT assay was carried out according to the method described in [5]. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, Budapest, Hungary) supplemented with 1200 mM L-glutamine (Glutamax, Thermo-Fisher, Darmstadt, Germany, CAS: 35050061), 10% fetal bovine serum (FBS), 4.5 g/L glucose, and penicillin–streptomycin (0.1 mg/mL) (Sigma-Aldrich, Budapest, Hungary). Cells were incubated at 37 °C in 5% CO_2 humidified air and maintained by continuous passaging.

Cells were seeded at a 10,000 cells/well density on 96-well plates to achieve complete confluence. After draining the medium, the cells were washed with PBS and incubated with the test solutions for 24 h at 37 °C in 5% CO_2. After removing the test solutions, MTT solution (5 mg/mL) was added to the cells and incubated for 3 h in a light-free chamber. In the presence of living cells, the yellow tetrazolium dye was converted into purple formazan crystals by the action of oxidoreductase enzymes. The purple crystals were dissolved in a 25:1 ratio of isopropanol/hydrochloric acid. The absorbance of the samples was measured by a Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer (USA). All tests were carried out in triplicate.

Statistical analysis. All experiments were performed in triplicate, and the results are presented as mean ± standard deviation (SD).

Results and discussion. The cytotoxic activity of arenobufagin and seven structurally modified derivatives against MCF-7 cells was evaluated after 24 h of exposure using the MTT assay. Cell viability was determined following treatment with 10, 50, and 300 nM of each compound (Figure 2).

At 10 nM, none of the tested compounds reduced MCF-7 cell viability below the control level. In contrast, several derivatives, including Ar-005 and Ar-011, produced viability values exceeding 140%, indicating the absence of detectable cytotoxicity at this concentration. Increasing the concentration to 50 nM resulted in marked differences among the compounds. Arenobufagin, Ar-004, Ar-008, and Ar-015 substantially reduced cell viability, whereas Ar-005 and Ar-011 remained largely inactive. Ar-007 and Ar-014 exhibited intermediate activity.

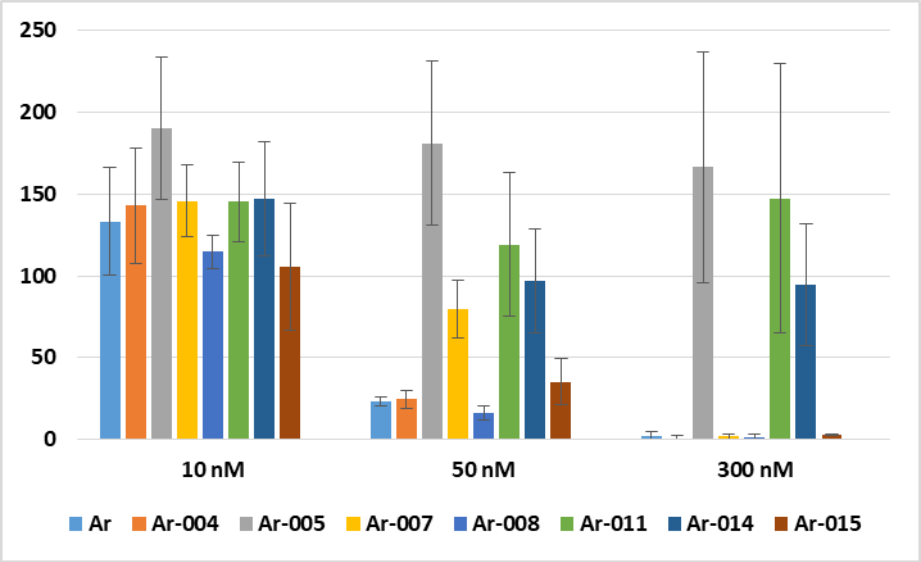


Figure 2. **Results of the study of the effect of arenobufagin and its derivatives on MCF-7 breast cancer cells using the MTT test.**

At 300 nM, arenobufagin, Ar-004, Ar-007, Ar-008, and Ar-015 almost completely abolished MCF-7 cell viability, demonstrating pronounced cytotoxic activity. In contrast, Ar-005 and Ar-011 showed little or no cytotoxic effect, while Ar-014 exhibited only moderate activity.

The present study demonstrated that arenobufagin and its seven derivatives exhibited markedly different cytotoxic activities against MCF-7 human breast cancer cells. While arenobufagin, Ar-004, Ar-007, Ar-008, and Ar-015 produced pronounced concentration-dependent reductions in cell viability, Ar-014 displayed only moderate cytotoxicity, whereas Ar-005 and Ar-011 showed little or no cytotoxic effect within the tested concentration range. These findings indicate that structural modification of arenobufagin substantially influences its biological activity and that relatively small chemical changes may either preserve or attenuate its cytotoxic potential.

The potent activity of arenobufagin observed in the present study is consistent with previous reports describing its broad-spectrum antitumor properties. Arenobufagin has been shown to inhibit the proliferation of various human cancer cell lines, including hepatocellular, colorectal, lung, and breast cancers, through induction of apoptosis, cell-cycle arrest, and inhibition of tumor progression. In particular, studies performed in MCF-7 cells demonstrated that arenobufagin exhibits potent cytotoxicity at nanomolar concentrations, which is in good agreement with the results obtained in the present investigation [2].

Although the present study did not investigate the molecular mechanisms underlying cytotoxicity, several mechanisms have previously been proposed for arenobufagin. As a representative bufadienolide, arenobufagin primarily interacts with Na^+/K^+-ATPase, a membrane protein that functions not only as an ion transporter but also as a signaling receptor regulating cell proliferation, apoptosis, migration, and differentiation. Activation of Na^+/K^+-ATPase-dependent signaling has been implicated in multiple anticancer effects of cardiac glycosides, making this

enzyme an important molecular target for this class of natural compounds [6].

Previous mechanistic studies in MCF-7 cells have further demonstrated that arenobufagin induces apoptosis through activation of the JNK signaling pathway, leading to multisite phosphorylation of Yes-associated protein (YAP). Phosphorylated YAP subsequently interacts with the transcription factor p73, resulting in increased expression of pro-apoptotic proteins, including Bax and p53AIP1, ultimately promoting apoptotic cell death. Although these molecular events were not examined in the present study, they provide a plausible explanation for the marked reduction in MCF-7 cell viability observed after treatment with the parent compound.

A notable finding of the present investigation was the considerable variation in cytotoxic activity among the synthesized derivatives. Whereas several derivatives retained cytotoxicity comparable to that of arenobufagin, others exhibited substantially weaker activity. These observations suggest that the biological activity of arenobufagin is highly dependent on its chemical structure. Since the purpose of the present work was biological evaluation rather than medicinal chemistry, detailed structure-activity relationships cannot be established. Nevertheless, the obtained results clearly demonstrate that structural modification can markedly alter the cytotoxic properties of arenobufagin against breast cancer cells.

Interestingly, Ar-005 and Ar-011 consistently produced MTT values exceeding those of the untreated control. Because the MTT assay quantifies mitochondrial metabolic activity rather than directly measuring cell number, increased absorbance should not be interpreted as evidence of enhanced cell proliferation. Instead, these findings may reflect alterations in cellular metabolism, differences in mitochondrial activity, or assay-specific responses. Therefore, additional studies employing complementary techniques, such as apoptosis assays, cell-cycle analysis, colony formation assays, or direct cell counting, are required to determine the biological effects of these derivatives more precisely.

The present study has several limitations. Only one breast cancer cell line was investigated, and cytotoxicity was evaluated using a single viability assay after 24 h of treatment. Furthermore, the molecular mechanisms responsible for the observed differences among the derivatives were not examined. Despite these limitations, the findings provide useful preliminary information regarding the biological activity of newly synthesized arenobufagin derivatives and identify compounds that warrant further pharmacological investigation.

Conclusion. The present study demonstrated that arenobufagin and its seven derivatives exhibit distinct cytotoxic activities against MCF-7 human breast cancer cells. While arenobufagin and the derivatives Ar-004, Ar-007, Ar-008, and Ar-015 showed pronounced concentration-dependent cytotoxicity, Ar-014 displayed moderate activity, whereas Ar-005 and Ar-011 exhibited little or no cytotoxic effect. These findings indicate that relatively small structural modifications can substantially alter the biological activity of arenobufagin. Although the molecular mechanisms underlying these differences remain to be elucidated, the present results identify several derivatives with promising cytotoxic activity and provide a

basis for further pharmacological, mechanistic, and structure-activity relationship studies.

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ARENOBUFAGIN VA UNI HOSILALARINING MCF-7 HUJAYRALARIGA QARSHI SITOTOKSIKLIGI

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Annotatsiya. *Arenobufagin – bu turli xil inson saraton hujayralariga qarshi kuchli faollikka ega bo‘lgan bufadienolid. Ushbu tadqiqotda arenobufagin va yettita yangi sintez qilingan hosilalarning sitotoksik faolligi MTT tahlili yordamida MCF-7 inson ko‘krak bezi saraton hujayralariga nisbatan baholandi. Hujayralarga 24 soat davomida 10, 50 va 300 nM konsentratsiyalardagi birikmalar bilan ishlov berildi. Arenobufagin va Ar-004, Ar-007, Ar-008 va Ar-015 hosilalari konsentratsiyaga bog‘liq sitotoksikli-*

kni namoyon etdi, Ar-014 esa faqat o‘rtacha faollikni ko‘rsatdi. Aksincha, Ar-005 va Ar-011 eksperimental sharoitlarda sitotoksik ta’sir ko‘rsatmadi. Olingan natijalar shuni ko‘rsatadiki, arenobufaginning strukturaviy modifikatsiyasi uning MCF-7 hujayralariga qarshi sitotoksik faolligiga sezilarli ta’sir qiladi. Ushbu natijalar yangi sintez qilingan arenobufagin hosilalarini biologik baholash uchun foydali dastlabki ma’lumotlarni taqdim etadi va keyingi saratonga qarshi tadqiqotlar uchun istiqbolli birikmalarni aniqlashga hissa qo‘shishi mumkin.

Kalit so‘zlar: *arenobufagin; bufadienolitlar; MCF-7 hujayralari; ko‘krak bezi saratoni; sitotoksiklik; MTT tahlili.*

ЦИТОТОКСИЧНОСТЬ АРЕНОБУФАГИНА И ЕГО ПРОИЗВОДНЫХ ПРОТИВ КЛЕТКИ MCF-7

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Аннотация. *Аренобуфагин – это буфадиенолид, обладающий мощной противоопухолевой активностью в отношении различных линий клеток рака человека. В настоящем исследовании цитотоксическая активность аренобуфагина и семи недавно синтезированных производных была оценена в отношении клеток рака молочной железы человека MCF-7 с использованием анализа MTT. Клетки подвергались воздействию исследуемых соединений в концентрациях 10, 50 и 300 нМ в течение 24 часов. Аренобуфагин и его производные Ar-004, Ar-007, Ar-008 и Ar-015 продемонстрировали выраженную цитотоксичность, зависящую от концентрации, тогда как Ar-014 показал лишь умеренную активность. В отличие от них, Ar-005 и Ar-011 продемонстрировали незначительный или нулевой цитотоксический эффект в экспериментальных условиях. Полученные результаты указывают на то, что структурная модификация аренобуфагина существенно влияет на его цитотоксическую активность в отношении клеток MCF-7. Полученные результаты предоставляют полезную предварительную информацию для биологической оценки недавно синтезированных производных аренобуфагина и могут способствовать выявлению перспективных соединений-кандидатов для дальнейших противораковых исследований.*

Ключевые слова: *аренобуфагин; буфадиенолиды; клетки MCF-7; рак молочной железы; цитотоксичность; MTT-тест.*