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Synergistic effect of oxymatrine and 5-fluorouracil on the migratory potential in A549 non-small cell lung cancer cells

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ABSTRACT

Introduction: An interesting research direction is the development of new therapies to reduce metastasis, especially in highly invasive cancer such as lung cancer. One of the commonly used anti-cancer drugs is 5-fluorouracil. Oxymatrine is a natural alkaloid with a wide range of effects. Combined with a cytostatic, it may enhance its action and protect normal cells. Therefore, the study aimed to analyse the effect of oxymatrine and 5-fluorouracil on non-small lung cancer cell line A549.

Material and methods: The study was based on the assessment of the interaction between drugs, cell death, cell cycle phase distribution, fluorescent labelling of F-actin and β-catenin, as well as wound healing and transwell migration assay.

Results: The combined treatment with oxymatrine and the cytostatic in a 1:1 ratio resulted in synergism. Incubation of cells with both substances induced changes in the life processes of A549 cells. In turn, the reorganization of F-actin and β-catenin contributed to the limitation of lung cancer cell migration compared to individual treatment with compounds.

Conclusions: This study demonstrated that the combination of oxymatrine and 5-fluorouracil in the 1:1 ratio may limit the migratory potential of A549 cells. In summary, oxymatrine can support the anti-cancer effect of 5-fluorouracil, but its potential application should be examined in further studies.

Key words: Oxymatrine, 5-FU, non-small cell lung cancer, migratory potential

Introduction

The high incidence and mortality rate of lung cancer makes it one of the leading causes of cancer deaths worldwide [1]. The most common histological subtype of lung cancer is non-small cell lung cancer (NSCLC), a heterogeneous group of tumours with many differences in treatment and patient survival [2]. Despite advances in medicine, radiotherapy, chemotherapy, and targeted therapy, the outcomes are still far from satisfactory. One of the main causes of therapy failure is drug resistance [3, 4].

5-fluorouracil (5-FU) is a cytostatic used in the treatment of many cancers, including NSCLC. The cytostatic belongs to the family of pyrimidine antimetabolite drugs that inhibit the growth of proliferative cells and induce their death [5]. However, drug resistance remains a significant limitation in the clinical use of 5-FU in monotherapy. Therefore, numerous studies are underway to investigate the synergistic anti-cancer effect of combination therapy, which has proved to be an effective strategy for overcoming 5-FU resistance [6, 7].

One such compound may be oxymatrine (OXY), an alkaloid derived from the roots of the Sophora genus plants. Numerous studies determining the therapeutic nature of the compound indicate its anti-inflammatory, antiviral and cardioprotective properties [8]. Additionally, it shows anti-tumour activity by inhibiting proliferation

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and inducing apoptosis in cancer cells. It applies to cancers such as breast [9], colorectal [10], prostate [11], and lung cancer [12]. Research shows the potential of combination therapy based on oxymatrine and chemo- or radiotherapy. The alkaloid not only reduces the toxic effect of conventional methods but also enhances the effect of the cytostatics [13, 14].

This study aimed to analyse the effect of the OXY and 5-FU combination on the basic cellular processes in NSCLC cell line A549.

Material and methods

Cell culture and treatment

The A549 cell line (non-small cell lung cancer cell line; NSCLC) was obtained from the European Collection of Authenticated Cell Cultures (Merck KGaA, Darmstadt, Germany) and grown in Dulbecco's Modified Eagle's Medium (DMEM; Lonza Group, Ltd., Basel, Switzerland). The culture medium was supplemented with 10% foetal bovine serum (FBS; Merck) and 50 μg/mL gentamycin (Merck). The material was grown under standard culture conditions (5% CO_2, 37°C). Cells used for tests were derived from low passages ($p \leq 6$) and tested for Mycoplasma based on the rapid uptake of DAPI (Merck) [15]. During the study, the cells were treated with 5-FU (5-fluorouracil; Merck), OXY (oxymatrine; Symbios, TargetMol), and a combination of compounds in a 1:1 ratio for 24 hours. The control group consisted of cells grown under the same conditions without the 5-FU or OXY addition. The concentrations of compounds used in further studies were selected based on MTT assay. The 1:1 ratio (2 mM OXY/2 mM 5-FU) resulted in synergism, which was estimated from the combination ratio (CI) by the Chou-Talalay method [16].

Interaction between the drugs (MTT assay)

To determine the type of interaction between OXY and 5-FU, the colourimetric MTT assay was used. After a 24 h treatment of A549 cells with a combination of drugs in a 1:1 ratio, cells were incubated with MTT working solution for 3 h. Formazan crystals were dissolved in 2 mL of isopropanol (Avantor, Gliwice, Poland), and the absorbance was read using a spectrophotometer (Spectra Academy, K-MAC, Seoul, Korea) at a wavelength of 570 nm.

The obtained data were analysed according to the Chou-Talalay median effect using the CompuSyn software. The combination index (CI) enables the classification of interactions of the tested compounds into three groups — synergistic ($CI < 1$), additive ($CI = 1$), and antagonistic ($CI > 1$) [16].

Cell death analysis

The Apoptosis Assay Kit containing Annexin V (AV) Alexa Fluor 488 and Propidium Iodide (PI; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to analyse the percentage of viable, apoptotic, and necrotic cells. The experiment was performed following the instructions provided by the manufacturer. Briefly, after removing the culture medium, the cells were resuspended in Annexin-binding buffer (ABB). 5 μL of AV was added to every sample (20 min, dark). The supernatant was removed, and the cells were suspended in the fresh ABB. Then, 1 μL of PI was applied to every probe (5 min, dark). The cells were analysed using Guava®easyCyte™ 6HT-2L Cytometer (Merck) and FlowJo software (version 10.07; FlowJo LLC).

Cell cycle analysis

The cell cycle was analysed by flow cytometry using a Guava®easyCyte ™ 6HT-2L system (Merck). Cells were first fixed in ethanol for 24 h at –20°C and then incubated for 30 min in FxCycle ™ PI/RNase Staining Solution (Life Technologies; Thermo Fisher Scientific, Inc). The obtained results were analysed using FlowJo software (version 10.07; FlowJo LLC).

Fluorescent labelling

The fluorescence labelling of β-catenin (Abcam, Cambridge, United Kingdom) and F-actin (Thermo Fisher Scientific) was performed using the standard protocol described in a previous study [17]. The A549 cells treated with 2 mM OXY, 2 mM 5-FU, and their 1:1 combination were fixed with 4% paraformaldehyde (Thermo Fisher Scientific, 20 min, RT), blocked with 4% bovine serum albumin (BSA, Merck, 20 min, RT). β-catenin was labelled using a primary anti-β-catenin (Sigma-Aldrich, 1 h, RT) and a secondary Alexa Fluor 594 antibody (Thermo Fisher Scientific, 1 h, RT, dark). F-actin was visualized using Alexa Fluor 488 phalloidin (Thermo Fisher Scientific, 20 min, RT, dark). Cell nuclei were stained with DAPI (Merck, 10 min, RT, dark). The preparations were observed under a C1 confocal microscope (Nikon).

Wound healing assay

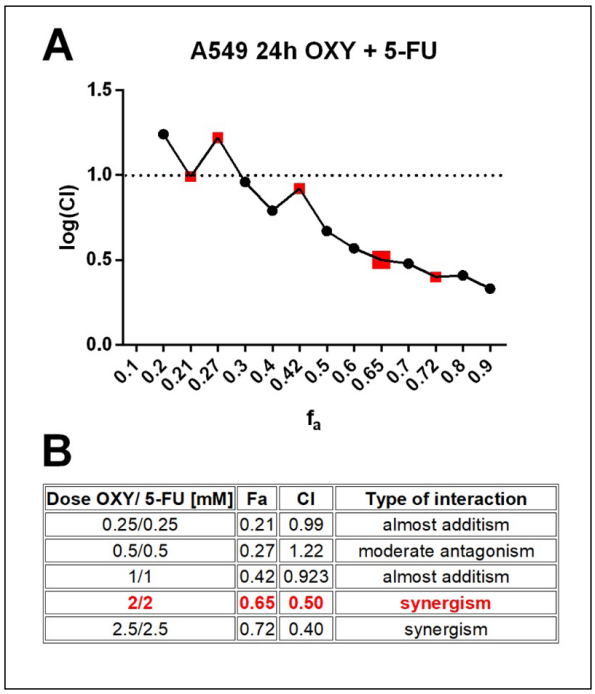
A549 cells migration after treatment with OXY, 5-FU, and their combination was analysed by a wound healing assay. Cells were grown under standard culture conditions to approximately 100% confluency then a "wound" was prepared using a sterile 100 μL pipette tip. The experiment was documented using an inverted Axio Observer Z1 motorized microscope (Zeiss) equipped with a system for live-cell imaging (Pecon).

Transwell migration assay

To analyse the migratory potential of A549 cells, transwell inserts (Corning, NY, USA, 8 μm) were used. The standard protocol described in the previous paper (Hałas-Wiśniewska 2020) was applied in the experiment. The analyses were based on the number of cells from random fields for CTRL, OXY, 5-FU, and their combination. The images were captured using a Nikon Eclipse E800 light microscope (Nikon) with a DS-5Mc-U1 CCD camera and NIS Elements software version 3.30 (Nikon).

Statistical analysis

GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical evaluation. The non-parametric Kruskal-Wallis with Dunn's post hoc test (cell death, transwell migration assay) and two-way ANOVA with Dunnett's multiple comparisons test (cell cycle) were performed. In the case of the wound healing experiment, the non-parametric Kruskal-Wallis with Dunn's post hoc test (wound closure after 24 h) and two-way ANOVA with Dunnett's multiple comparisons test (wound area) were used. For each analysis, statistically significant changes ($p < 0.05$) in comparison to untreated cells (CTRL) were marked with an "*" in all graphs. All data are presented by means ± standard deviation (SD) of three independent experiments (n = 3).



Dose OXY/ 5-FU [mM]	Fa	CI	Type of interaction
0.25/0.25	0.21	0.99	almost additism
0.5/0.5	0.27	1.22	moderate antagonism
1/1	0.42	0.923	almost additism
2/2	**0.65**	**0.50**	**synergism**
2.5/2.5	0.72	0.40	synergism

Figure 1. Analysis of oxymatrine (OXY) and 5-fluorouracil (5-FU) interactions. The interaction analysis was based on the results obtained from the MTT assay. A549 cells were treated with OXY and 5-FU at concentrations from 0.25–2.5 mM for 24 h in a ratio of 1:1. **A.** The combination index plot for OXY and 5-FU co-treatment in A549 cells in the range of f_a from 0.1 to 0.6. CI < 1 — synergism, CI = 1 — additive effect, CI > 1 — antagonism. For real measuring points, the values have been marked in red; **B.** Summary table showing the type of interaction.

Results

Analysis of OXY and 5-FU interactions

One of the research goals was to determine the type of interaction between the tested compounds. The analysis was performed using the Chou-Talalay method based on the results obtained from the MTT assay. Analysis of the data curated for OXY and 5-FU-co-treated A549 cells indicates that 2 mM and 2.5 mM concentrations of both compounds in the 1:1 ratio induced a synergistic effect (Fig. 1A, B). In turn, doses of 0.25 and 1 mM showed almost additivism and 0.5 moderate antagonism (Fig. 1A, B). Based on the obtained results, 2 mM concentrations of compounds were selected for further studies, both in mono-treatment and combination.

Effect of OXY, 5-FU and combination therapy on the cell death induction

The analysis of the percentage of live, apoptotic, and necrotic cells after 24 h treatment with 2 mM OXY, 2 mM 5-FU, and a 1:1 combination was presented in Figure 2. Treatment of cells with 5-FU and the combination of the compounds (COMB) resulted in a statistically significant reduction in the percentage of viable cells (AV-/PI-) compared to untreated control (80.32% and 81.95% respectively, Fig. 2A). In the untreated control (CTRL) and 2 mM OXY-treated cells, were observed 93.81% and 92.07% live cells, respectively. Figure 2B shows an increase in the percentage of early and late apoptotic cells (AV+/PI- and AV+/PI+). As in the case of living cells, only after the use of 5-FU and COMB the obtained results were statistically significant and reached 9.09% and 10.11%, respectively (Fig. 2B).

Necrotic cells (AV-/PI+) were also analysed, but no statistically significant results were obtained (Fig. 2C).

Effect of OXY, 5-FU and their combination on the distribution of cell cycle phases in A549 cells

The cell cycle phase distribution in the control and all study groups is shown in Figure 3. The statistically significant changes compared to the control occurred only after the application of 2 mM 5-FU. It included

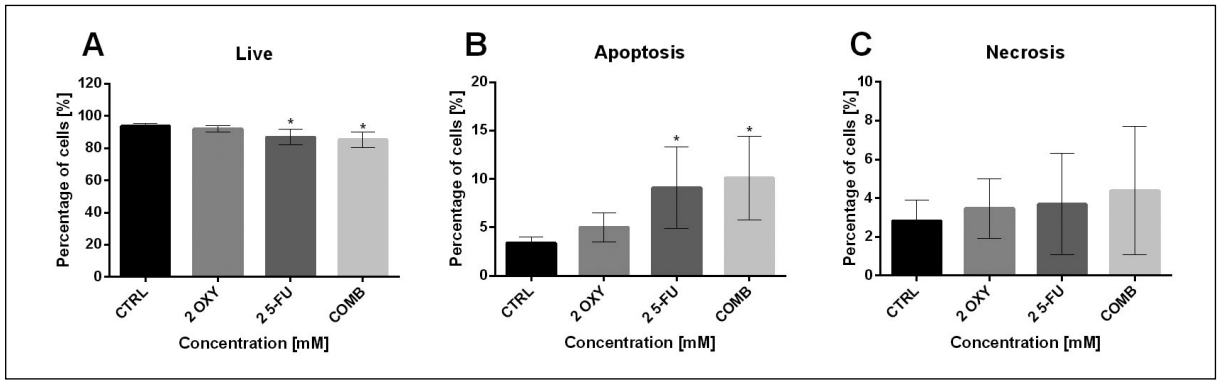


Figure 2. The effect of oxymatrine (OXY) and 5-fluorouracil (5-FU) individually and in combined treatment on cell death of A549 cells. The A549 cells were treated with 2 mM OXY, 2 mM 5-FU, and the combination of both compounds in a ratio of 1:1 and the cell death was analysed using Annexin V and propidium iodide double staining assay. The figure presents the percentage of live, apoptotic, and necrotic cells. '*' indicate statistically significant differences compared to control cells ($p < 0.05$; the non-parametric Kruskal–Wallis with Dunn's post hoc test).

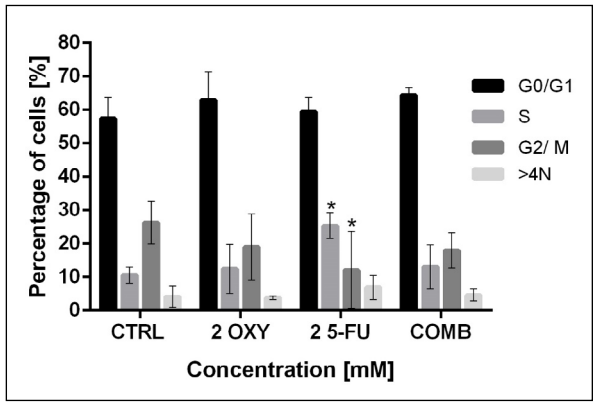


Figure 3. The effect of oxymatrine (OXY) and 5-fluorouracil (5-FU) individually and in combined treatment on the distribution of cell cycle phases of A549 cells. The figure presents the distributions of cell cycle phases (G0/G1, S, G2/M and > 4N) in A549 cells treated with 2 mM OXY, 2 mM 5-FU, and the combination of both compounds in a 1:1 ratio. '*' indicate statistically significant differences compared to control cells ($p < 0.05$; the non-parametric Kruskal–Wallis with Dunn's post hoc test).

an increase in the percentage of cells in the S phase to 25.35% (*vs.* 10.57% in CTRL). There was also a reduction in the percentage of cells in the G2/M phase to 12.11% (*vs.* 26.33% in CTRL). No other statistically significant differences were noted (Fig. 3).

The effect of OXY, 5-FU and their combination on the migration of A549 cells

The main aim of the study was to investigate the effect of OXY, 5-FU, and drug combination in a 1:1 ratio on the migratory abilities of A549 cells. Recommended test methods were used, such as wound healing and transwell migration assays. Additionally, the structure of the actin cytoskeleton and β-catenin were investigated, which are very important in migration and the epithelial-mesenchymal transition (EMT) process.

Figure 4 shows the results obtained in the wound healing assay. After 6 hours, a statistically significant increase in the migration of control cells and after treatment with 2 mM OXY and 2 mM 5-FU was observed. The only exception occurred in cells treated with the combination of compounds, in which migration was significantly reduced (Fig. 4A). Also, after 24 hours, cells treated with OXY/5-FU in a 1:1 ratio were characterized by reduced migratory abilities compared to the rest of the groups (Fig. 4B). Figure 4C presents representative images of the wound-healing assay.

Results obtained from the wound healing assay were supported by the transwell migration test. Figure 5A shows the average number of cells able to migrate on the other side of the membrane. The obtained results suggest that 2 mM OXY alone does not limit the migration of A549 cells. On the other hand, a statistically significant reduction in cell motility occurred after the use of 2 mM 5-FU and the combination of OXY/5-FU in the ratio 1:1 (Fig. 5A). Figure 5B presents representative images from the transwell migration assay for control, OXY, 5-FU and their combination (Fig. 5B).

Fluorescent F-actin labelling indicates an extensive network of actin filaments. Numerous thick actin fibres and cortical arrangements in all groups were observed. However, the application of 5-FU, and most frequently the treatment with the OXY/5-FU combination, resulted in actin clusters in the form of stars. It may indicate strong adhesion to the surface (Fig. 6). Also, the distribution of β-catenin varies depending on the compound

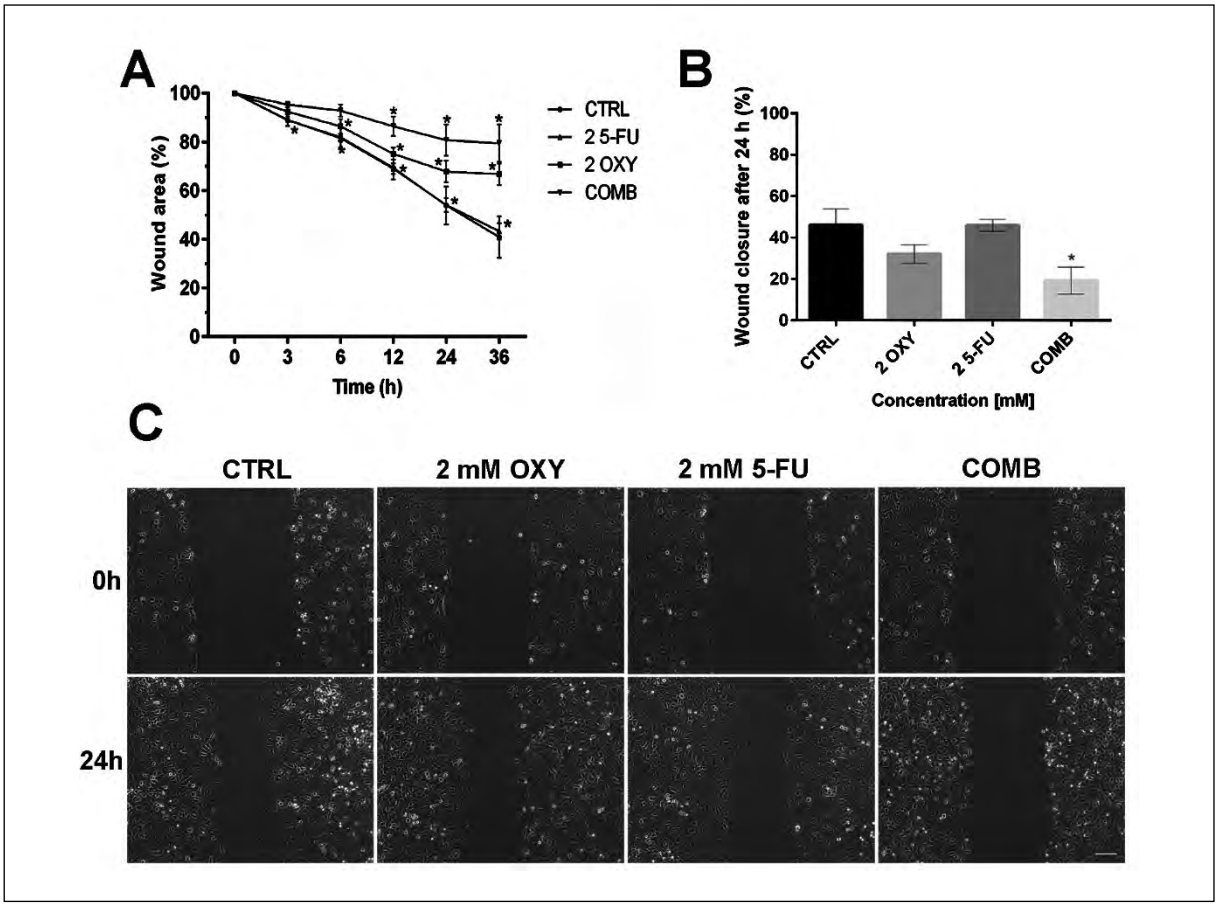


Figure 4. The effect of oxymatrine (OXY) and 5-fluorouracil (5-FU) individually and in combined treatment on the migration of A549 cells — wound healing assay. The A549 cells were treated with 2 mM OXY, 2 mM 5-FU, and the combination of both compounds in a 1:1 ratio for 24 h. **A.** Wound area in timepoints. **B.** Wound closure after 24 h. '*' indicate statistically significant differences in comparison to control cells ($p < 0.05$; 2way ANOVA comparisons test); **C.** The representative images of the results obtained in wound healing assay. Bar = 50 μm.

used. In control cells of the A549 line and cells treated with 2 mM OXY, this protein is localized in the junction areas of the cell and a small amount surrounding the nuclei. On the other hand, under the influence of 2 mM 5-FU and the OXY/5-FU combination, it accumulates mainly in the perinuclear area (Fig. 6).

Discussion

According to the World Health Organization (WHO), in 2020, lung cancer was the second most common neoplasm but the first in terms of mortality. The average 5-year survival in patients diagnosed with lung cancer is still relatively low (10–20%) [18]. Most lung cancers are non-small cell lung cancer (NSCLC) cases treated mainly with surgical resection and/or radiotherapy and chemotherapy. Currently available treatments are not always effective, especially in patients in advanced stages of the disease with metastases. Hence, new methods of treatment based on targeted therapy or the use of combinations of compounds acting synergistically on cancer cells are tested. One of the options is the combination of natural compounds with conventionally used cytostatics. Numerous scientific studies show the anti-cancer properties of OXY. The compound induces cell death in renal cell carcinoma [19], breast cancer [9], and lung cancer [10, 20]. In turn, 5-FU is a conventionally used cytostatic commonly administrated to cancer patients 21. Unfortunately, lung cancer is very often resistant to treatment with the cytostatic. However, there are some methods to overcome drug resistance. Studies by Khakbaz et al. report that the use of a combination of 5-FU with allicin helps to defeat multidrug resistance in gastric carcinoma [22]. Similar results were obtained for the combination of 5-FU with curcumin. This naturally derived compound can inhibit the proliferation of human colon cancer cells and addi-

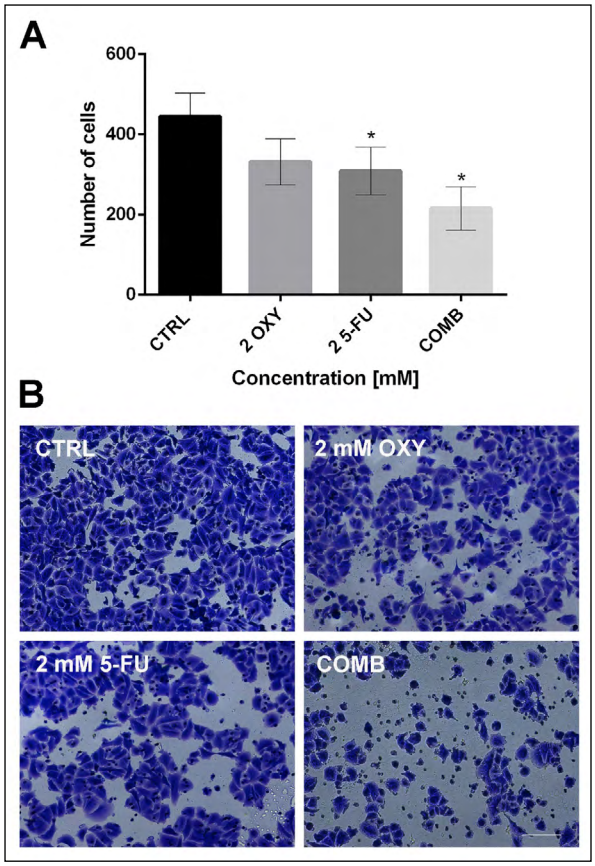


Figure 5. The effect of oxymatrine (OXY) and 5-fluorouracil (5-FU) individually and in combined treatment on the migration potential of A549 cells — transwell migration assay. The A549 cells were treated with 2 mM OXY, 2 mM 5-FU, and the combination of both compounds in a 1:1 ratio for 24 h. **A.** The average number of cells with a high migratory potential to control cells. '*' indicate statistically significant differences in comparison to control cells. ($p < 0.05$; 2way ANOVA comparisons test); **B.** The representative images of the results obtained in the Transwell migration assay. Bar = 50 μm.

tionally can reverse the effects of multi-drug resistance in human colon cancer cell lines [23, 24].

OXY also shows a synergistic effect against cancer cells in combination with cytostatics such as cisplatin and doxorubicin [13, 25]. In addition, the compound may be promising in overcoming chemotherapy resistance. Liang et al. showed that OXY reduces 5-FU resistance in HCT-8 cell line. Additionally, they found that the process involved in chemoresistance was EMT modulated by the activation of the NF-κB signalling pathway [26]. Thus, OXY may also impact EMT, the process favouring metastasis. Another advantage of OXY administration may be the cytoprotective effect in normal cells. Numerous adverse effects are associated with the administration of cytostatics, including 5-FU. Despite the relatively short half-life, the drug can penetrate the blood-brain barrier leading to a phenomenon known as "brain fog" [27]. Another adverse effect is leukopenia [28]. Additionally, in the case of H9c2 myoblasts, OXY reduces the cytotoxic effect of doxorubicin [14]. This effect was associated with the inhibition of reactive oxygen species production in normal cells. Thus, it is possible that in the case of 5-FU, OXY may also prevent negative changes in normal cells. In the presented paper, it is shown that OXY and 5-FU may synergistically reduce the survival and inhibit the migration abilities of the A549 non-small cells lung cancer cell line. It is in accordance with the results presented by Liang et al., indicating that the addition of OXY enhances the anticancer properties of 5-FU [26]. The combination of the compounds was characterized by an increased rate of apoptosis and reduced migration and invasion compared to the treatment with the substances separately. In their previous research, the authors found that a 2 mM dose of 5-FU caused an increase in apoptotic cell percentage to 8% [29]. However, in the combination of 5-FU and OXY was observed an enhancement of this effect. It is in accordance with numerous literature reviews showing that many compounds of natural origin show the proapoptotic effect [30, 31]. Apoptosis induction is a very desirable result, but limiting metastasis is equally important. In this study, the simultaneous use of 5-FU and OXY significantly reduced the migration and invasion of A549 cells. Changes were observed especially in the F-actin pattern. The combination of compounds decreased the number of stress fibres and fluorescence intensity of protein. In addition, the impact of the OXY/5-FU combination on cell motility was manifested by a slower closure rate of the wound and a reduced number of cells able to migrate through the pores of the insert. The only study showing the synergistic effect of OXY and 5-FU on migration was presented by Liang et al. in 2016. Researchers indicated that the use of OXY and 5-FU in colon cancer cells decreased the expression of vimentin, SNAI2 (snail family transcriptional repressor 2), and phosphorylated p65, and increased the expression of E-cadherin. It indicates EMT inhibition, which may prevent metastasis and impact the chemoresistance to 5-FU in colon cancer cells. The researchers showed that the combination of 5-FU and OXY inactivates the NF-κB signalling pathway [26].

Conclusions

In summary, it was demonstrated that oxymatrine enhanced the 5-FU-induced effect in A549 cells such as the reorganization of F-actin and β-catenin. What is more, the present study demonstrated that the combi-

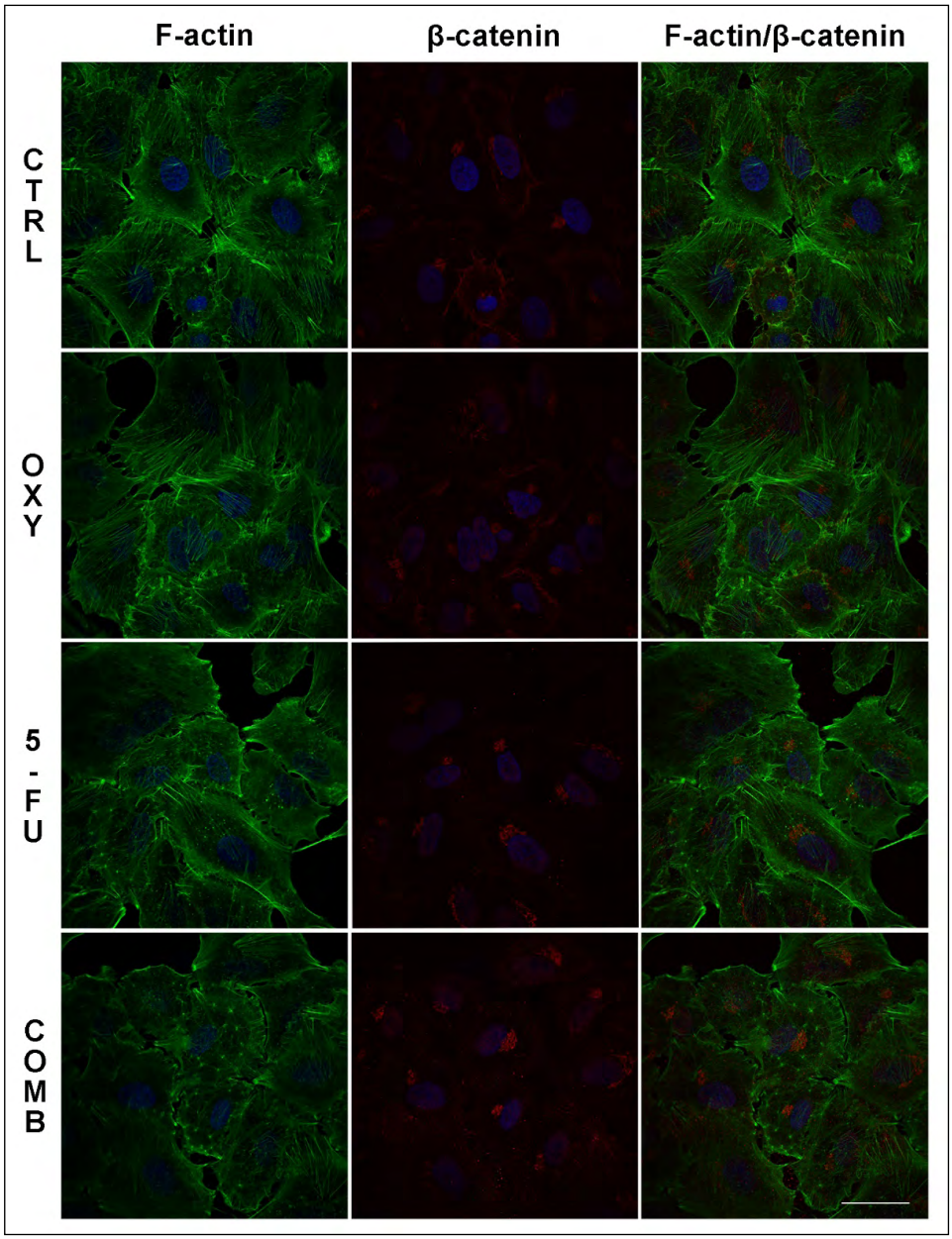


Figure 6. The effect of oxymatrine (OXY) and 5-fluorouracil (5-FU) individually and in combined treatment on the F-actin and β-catenin organization. The A549 cells were treated with 2 mM OXY, 2 mM 5-FU, and the combination of both compounds in a 1:1 ratio for 24 h. Immunofluorescent labelling of F-actin (green), β-catenin (red), and nuclei (blue), Bar = 50 μm.

nation of OXY and 5-FU in the 1:1 ratio may limit the migratory potential of A549 cells. These results suggest the hypothetical benefits of OXY supplementation in lung cancer patients treated with cytostatics, especially in the context of metastasis.

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Conflicts of interest: *None.*

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