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Research Paper

Antimigratory effect of pyrazole derivatives through the induction of STAT1 phosphorylation in A549 cancer cells

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Abstract

Objectives In cancer treatment, it is important to prevent or slow down metastasis as well as preventing the proliferation of cancer cells. In this study, we aimed to find pyrazole compounds with antimigratory properties.

Methods The 'PASSonline' programme was used to determine the possible pharmacological activities of the pyrazole compounds selected from the library, and two pyrazole derivatives were identified as a transcription factor STAT inhibitor with a high probability. There are studies known that JAK/STAT pathway is related to cancer cell migration, thus the possible antimigratory effects of these two synthesized pyrazole compounds were examined in A549 cancer cells.

Key findings Our data demonstrated that compound-2 at different concentrations significantly inhibited cell migration in A549 cells. Then, the effects of these compounds on STAT activation were evaluated. We reported that 10 µM compound-2 induced a significant phosphorylation of STAT1 suggesting that STAT1 activation may be responsible for the antimigratory effect of compound-2.

Conclusions Taken together, the compound-2 is a promising compound with the antimigratory activity for cancer treatment, and further studies are needed to synthesize more active derivatives by evaluating the structure–activity relationship of leading compound-2.

Keywords: Biomedicinal Chemistry; A549 cell line; pyrazole; cancer; antimigratory; bioactivity prediction

Introduction

Drugs used in cancer treatment show their effects mostly by inhibiting the cell proliferation or by inducing cytotoxicity. One of the most common problems in cancer treatment is metastasis. Cancer cell migration is one of the major processes leading to metastatic progression in cancer cases. Therefore, the main purpose of cancer treatment is to eliminate cancer cells as well as to prevent cell migration. Today, new therapeutic approaches are needed to prevent metastasis in cancer treatment. Therefore, drug candidates that suppress cancer cell migration and invasion will be an important potential step for the development of new drugs needed in this field.

Lung cancer, one of the most common malignant tumours, is the most common cause of cancer-related death. In particular, advanced non-small-cell lung cancer (NSCLC) has a 5-year survival rate of less than 10%.[1] While NSCLC treatment is performed surgically in the early stages, it is provided with simultaneous chemo and radiotherapy in locally advanced cases. Palliative chemotherapy is applied for metastatic cases.[2] Due to the difficulty of early diagnosis, the development of resistance to standard therapeutics and the lack of

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effective treatment methods, new therapeutic approaches are needed for effective treatment of lung cancer.

The antipyretic effect of pyrazole-derived compounds was first discovered by Knorr in 1883.[3] Later, the antidepressant, anticonvulsant, antibacterial, anti-inflammatory, vasorelaxant and analgesic effects of pyrazole derivatives were also determined.[4–7] Recent studies have led to the discovery of pyrazole derivatives with cytotoxic effects.[8–11] There are two important aryl-pyrazole derivatives with antitumour activity used in the clinic: Ruxolitinib (Jakavi, Novartis) (3R)-3-cyclopentyl-3-[4-(7H-pyrrolo [2,3-d] pyrimidin-4-yl)pyrazol-1-yl] propanenitrile and Crizotinib (XALKORI, pfizer) 3-[(1R)-1-(2,6-dichloro-3-fluorophenyl)ethoxy]-5-(1-piperidin-4-ylpyrazol-4-yl)pyridine-2-amine.[12] Ruxolitinib inhibits gene expression mediated by cytokine receptor activation via Janus kinase enzyme inhibition, and thus inhibits cell proliferation.[13] Crizotinib (PF-2341066) is used as an inhibitor of anaplastic lymphoma kinase and c-Met (also known as a hepatocyte growth factor receptor).[14] It is also known that the pyrazole-derived compounds show cytotoxic activity through inhibiting a variety of targets, such as topoisomerase II, EGFR, VEGF, B-Raf, m-TOR and HDAC.[15–20] In recent years, especially anti-cancer activities of aryl pyrazole derivatives have been evaluated.[11, 21] On the other hand, few studies evaluating the antimigratory effects of pyrazole derivatives have been found in the literature.[22–24] The increase in the number of these studies evaluating the antimigratory activities of pyrazole compounds is promising for cancer treatment.

We chose five structurally related aryl-pyrazole derivatives from screening chemical libraries. Then, we analysed these aryl pyrazole derivatives for their possible pharmacological effects using PASSonline. This analysis showed that two compounds are potential as transcription factor STAT (signal transducers and activators of transcription) inhibitors with the Pa value more than 0.5.

The JAK/STAT pathway, which is the basic signalling mechanism for cytokines and growth factors, has an important role in cellular events such as cell growth, proliferation, migration and apoptosis.[25] STATs that are activated by phosphorylation by JAK (Janus kinase) and MAP (Mitogen-activated protein) kinases are translocated into the nucleus as dimerized and concomitantly change the expression of related genes. In the STAT family, which consists of seven members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6), each member mediates the effects of different cytokines.[25] STAT5 has been shown to induce cell invasion and migration in prostate cancer cells both *in vivo* and *in vitro*.[26] On the other hand, STAT1 and STAT3 show opposite effects on cancer cell proliferation.[27] The balance between STAT1 phosphorylation activated via interferon gamma and STAT3 phosphorylation activated by IL-6 family members determines the nature of the effect on apoptosis and the cellular cycle.[25] Two different negative regulators of STATs have been identified: 'STAT-induced STAT inhibitors (SSIs) or suppressors of cytokine signaling (SOCS)' that cause inhibition of the JAK/STAT pathway, and 'protein inhibitors of activated STATs (PIAS)', which are nuclear factors binding to phosphorylated STATs.[26–28] It has been shown that PIAS-1 from the PIAS family and SOCS1 from the SOCS family specifically inhibit the activation of STAT1. On the other hand, PIAS-3 from PIAS family and SOCS3 from SOCS family have been shown to specifically inhibit STAT3 activation.[25–32]

Since it is known that activation of JAK/STAT pathway is involved in cell migration by activating metastasis–inducing genes,[33–35] we synthesized these two compounds and aimed to investigate the possible antimigratory activity of the compounds and their effects on STAT1, STAT3 and STAT5A/B in lung cancer cell line A549.

Materials and Methods

Chemistry

1H-NMR spectra were recorded in DMSO-d6 on a Varian Mercury 400 MHz High Performance Digital FT-NMR Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using tetramethylsilane as the internal standard. All chemical shifts were recorded as δ (ppm). All coupling constants were reported as Hertz. High-resolution mass spectra data (HRMS) were collected using Waters LCT Premier XE Mass Spectrometer (high-sensitivity orthogonal acceleration time-of-flight instrument) operating in ESI (+) method, was also accompanied by an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA) using a UV detector monitoring at 254 nm. Flash column chromatography on silica gel was performed on RediSep prepacked disposable silica gel columns using Combiflash (Teledyne-Isco, Lincoln, NE, USA). Melting points were determined with an SMP-II Digital Melting Point Apparatus and were uncorrected (Schorpp Geaete- technik, Germany). Compounds C-1 and C-2 were synthesized according to published procedure.[36]

(5-(4-chlorophenyl)-1H-pyrazol-3-yl)(4-(2-pyridinyl)-1-piperazinyl)methanone (C-1)

Thionyl chloride (0.025 mol) and catalytic amount of DMF were added to the solution of acid derivative (0.01 mol) in CH_2Cl_2 under argon atmosphere and refluxed for 3 h. Then, to the reaction mixture, diisopropylethylamine (0.015 mol) and 1-(pyridin-2-yl)piperazine (0.01 mol) were added and stirred overnight at room temperature. After the reaction was complete, the solvent was evaporated, and the residue was purified by flash chromatography using CH_2Cl_2:MeOH solvent gradient. Yield 69%; Mp 228.8–229.9 °C;^{1}H-NMR ($CDCl_3$): 3.59 (4H, bt), 3.75 (2H, bs), 3.86 (2H, bs), 6.78 (^{1}H, dd, J = 6.8, 5.2 Hz), 6.86 (1H, d, *J* = 8.4 Hz), 7.12 (1H, s), 7.52–7.59 (3H, m), 7.86 (2H, d, *J* = 8.4 Hz), 8.14 (1H, dd, J = 5.2, 2.0 Hz). HRMS (m/z): [M+H]+ calcd for $C_{19}H_{18}ClN_5O$: 368.1278; found 368.1265, CAS # 1093234-47-9

(5-(4-chlorophenyl)-1H-pyrazol-3-yl)(4-(4-methylphenyl)-1-piperazinyl)methanone (C-2)

Prepared from 1-(4-methylphenyl)piperazine under the same conditions that were applied to C1. The crude product was purified by flash chromatography using CH_2Cl_2:MeOH solvent gradient.; Yield 52%; Mp 240.3–240.7 °C 1H-NMR ($CDCl_3$): 2.17 (3H, s), 3.10 (4H, bs), 3.75 (3H, bs), 4.05 (1H, bs), 6.84 (2H, d, J = 8.4 Hz), 7.01 (2H, d, J = 8.4 Hz), 7.13 (1H, s), 7.51 (2H, d, J = 8.0 Hz), 7.79 (2H, d, J = 8.0 Hz). HRMS (m/z): [M+H]+ calcd for $C_{21}H_{21}ClN_4O$: 381.1482; found 381.1493, CAS # 1291018-03-5

Cell Culture

The non-small cell lung cancer cell line (A549) was used to assess the pharmacological activity of aryl pyrazole-derived compounds. The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Sigma) and 1% penicillin (PAA) streptomycin (Sigma). Pyrazole derivative compounds were dissolved in DMSO (dimethyl sulfoxide). Cells were exposed to 0.2% DMSO.

In Silico Bioprediction

In order to predict the pharmacological activities of five aryl pyrazole derivated compounds selected from screening chemical libraries, Prediction of Activity Spectra for Substances (PASSonline) webserver was used.[37, 38] This software evaluates the molecular structure of the studied compounds according to their similarity to the active compounds and creates a spectrum of biological activity. The software lists activities in this spectrum from high probability

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to low probability according to Pa (probability 'to be active') and Pi (probability 'to be inactive') values.

Determination of real-time cytotoxic activity

The xCELLigence Real-Time Cell Analyzer (RTCA) DP system (ACEA, Biosciences Inc.) was used to evaluate the cytotoxic activities of pyrazole derivatives. In this widely used impedance-based system, the increase in cell index indicates increased cell proliferation, and the decrease in cell index indicates cell death.[39–42] The monolayer A549 cells were treated with C-1 and C-2 at increasing concentrations, and real-time cell tracking was performed for 48 h. Cell death was evaluated by comparing the maximum cell index values of the compounds with the control group.

Wound healing assay

The possible antimigratory effects of pyrazole derivative compounds in A549 cells were investigated by wound healing assay. 75×10^4 cells were seeded in each well of the 12-well plate and incubated for 48 h. When the cells reached 100% confluency, a scratch wound was created by scraping the cell layer using a sterile yellow pipette tip. The cells were washed with PBS twice, and fresh medium was added. The cells were preincubated with 0.1 µg/ml mitomycin C for 30 min. Before the treatment of drugs, the pictures of two different areas of each wound were taken randomly at 0 h using a Leica DMIL phase contrast microscope. The cells were exposed to 0.3 µM, 1 µM, 3 µM, 10 µM, 30 µM C-1 and 0.3 µM, 1 µM, 3 µM 10 µM C-2 for 24 h. Images of the migrated cells were captured again at 24 h. Then, the wound size of the images was measured using image j programme, and wound closure % was calculated. All the experiments were repeated seven times.

Determination of STAT activation

15×10^5 A549 cells were seeded in each well of the six-well plate, and the cells were cultured for 48 h to ensure 100% confluency. The wound was formed with a yellow pipette tip under laminar flow. Following the removal of cell debris, 1 ml of fresh medium was added. The cells were incubated with 0.1 µg/ml mitomycin C for 30 min, and then the cells were treated with 10 µM and 30 µM C-1, and 10 µM C-2 for 24 h. In addition to these experimental groups, a negative control group was prepared without any wound and mitomycin C application. After 24 h of incubation, STAT activation in cells was measured using STAT Family Activation (STAT1, STAT3 and STAT5) Human InstantOne ELISA Kit (Invitrogen, catalog no:85-86095). For this purpose, the cells in each group were lysed in homogenization buffer in the kit. An equal amount of total protein was added to each well. Fifty microliters of antibody mixture was added to these wells and incubated in the shaker for 1 h at room temperature. After the incubation, the wells were washed three times. One hundred microliters of detection reagent was added to each well and incubated on the shaker for 20 min at room temperature. One hundred microliters of Stop Solution was then added to each well, and the plate was read at 450 nm.

Statistical analysis

Statistical significance ($P < 0.05$) was assessed using one-way analysis of variance followed by Tukey's tests when analyzing multiple groups. All the values were expressed as the mean ± SEM.

Results

Chemical structure relationship on pharmacological activities of aryl pyrazole derivatives

The chemical formula of aryl pyrazole derivatives was screened in the PASSonline programme, and the possible pharmacological effects of the compounds were listed according to their probability levels (Figure 1). PASSonline analysis showed that compound-1 (C-1) and compound-2 (C-2) as a potential STAT inhibitor with high probability (Pa >0.5) indicating that these compounds might prevent the activation of STAT (Figure 1). Thus, we synthesized C-1 and C-2 for evaluating their cytotoxic and antimigratory activities.

Evaluation of cytotoxic activity of aryl pyrazole derivatives

A549 cells were treated with increasing concentrations of C-1 and C-2 (3, 10, 30 and 100 µM), and cell proliferation was monitored in real time for 48 h. When xCELLigence original traces and maximum cell indexes were evaluated, it was observed that 100 µM C-1-induced cell death significantly and the lower concentrations than 100 µM did not affect the cell proliferation (Figure 2A). On the other hand, 3 µM and 10 µM C-2 had no effect on cytotoxicity, and 30 µM and 100 µM C-2 caused cell death (Figure 2B).

Evaluation of antimigratory activity of aryl pyrazole derivatives

To assess the possible antimigratory effects of aryl pyrazole derivatives C-1 and C-2, wound was created in confluent A549 cells in culture and evaluated. Cell proliferation was prevented by the pretreatment of cells with 0.1 µg/ml concentration mitomycin C for 30 min so that only the migratory activities of the cells were evaluated in the wound healing assay. Then, the cells were treated with increasing concentrations of C-1 (0.3, 1, 3, 10 and 30 µM) and C-2 (0.3, 1, 3 and 10 µM). After 24 h, the image of the cells was captured under phase-contrast microscopy, and the percentage of wound closure was calculated from the images taken at 0 and 24 h. Our results showed that treatment of 1, 3 and 10 µM C-2 resulted in a significantly decreased wound closure compared to the control group in A549 cells suggesting that C-2 has antimigratory effect. On the other hand, C-1 had no effect on cell migration (Figure 3).

Investigation of the possible effects of aryl-pyrazole derivatives on STAT activation

The efficacy of C-1 and C-2 on STAT activation, which was determined to cause high probability of STAT inhibition in PASSonline data, was evaluated using ELISA kit. The STAT Family Activation Human ELISA Kit was used to evaluate the effects of C-1 and C-2 on STAT family activation. A549 cells were preincubated with 0.1 µg/ml mitomycin C for 30 min. Then, the cells were treated with 10 µM C-1 and 10 µM C-2 for 24 h, and the effects of these compounds on STAT1, STAT3 and STAT5A/B phosphorylation leading to activation were investigated using the ELISA kit. Our results indicated that C-1 did not affect any STAT activation tested in A549 cells (Figure 4). While C-2 did not change the phosphorylation of STAT3 and STAT5A/B, it was determined that 10 µM C-2 caused a statistically significant increase in STAT1 activation (Figure 4).

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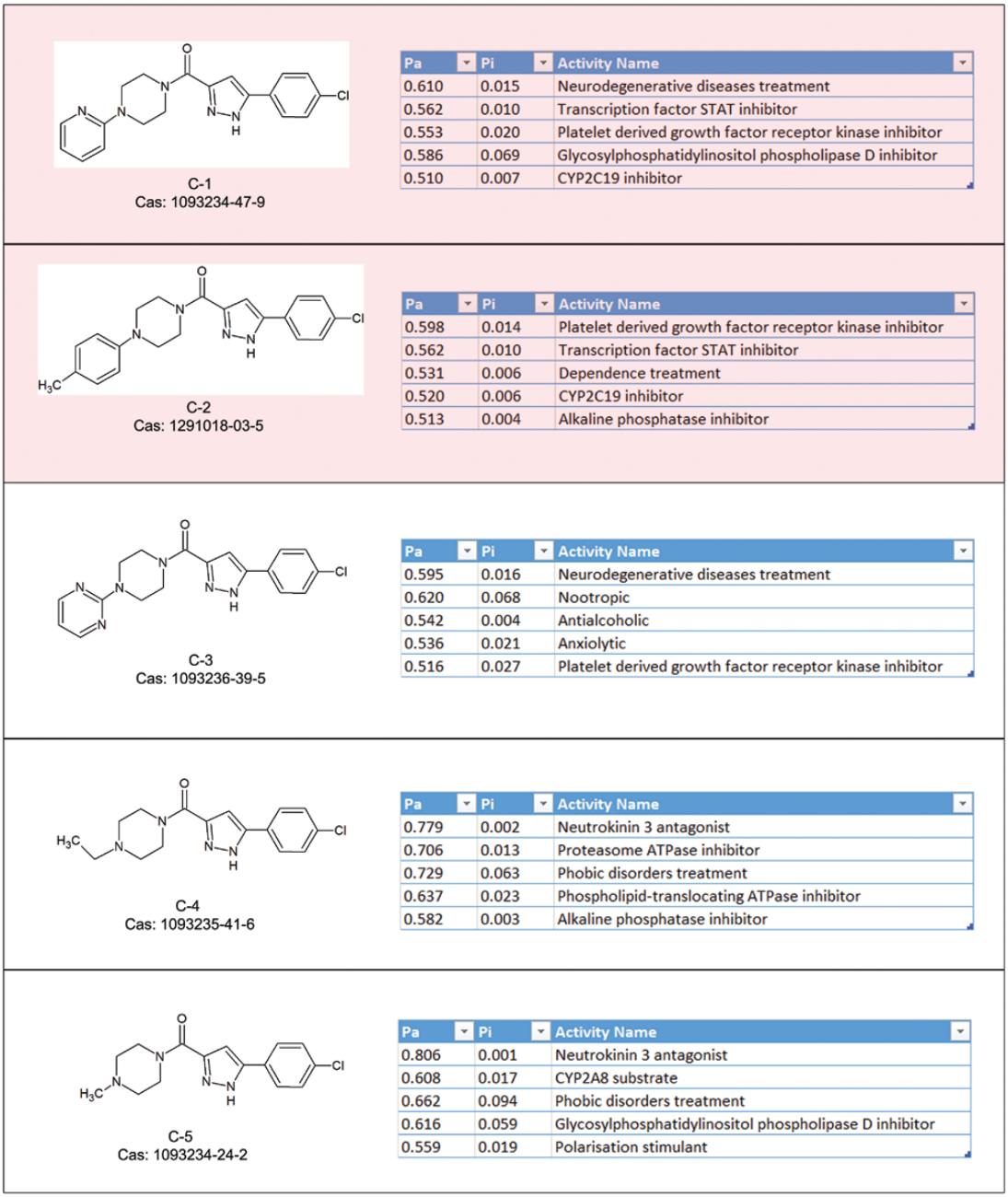

Pa	Pi	Activity Name
0.610	0.015	Neurodegenerative diseases treatment
0.562	0.010	Transcription factor STAT inhibitor
0.553	0.020	Platelet derived growth factor receptor kinase inhibitor
0.586	0.069	Glycosylphosphatidylinositol phospholipase D inhibitor
0.510	0.007	CYP2C19 inhibitor

Pa	Pi	Activity Name
0.598	0.014	Platelet derived growth factor receptor kinase inhibitor
0.562	0.010	Transcription factor STAT inhibitor
0.531	0.006	Dependence treatment
0.520	0.006	CYP2C19 inhibitor
0.513	0.004	Alkaline phosphatase inhibitor

Pa	Pi	Activity Name
0.595	0.016	Neurodegenerative diseases treatment
0.620	0.068	Nootropic
0.542	0.004	Antialcoholic
0.536	0.021	Anxiolytic
0.516	0.027	Platelet derived growth factor receptor kinase inhibitor

Pa	Pi	Activity Name
0.779	0.002	Neutrokinin 3 antagonist
0.706	0.013	Proteasome ATPase inhibitor
0.729	0.063	Phobic disorders treatment
0.637	0.023	Phospholipid-translocating ATPase inhibitor
0.582	0.003	Alkaline phosphatase inhibitor

Pa	Pi	Activity Name
0.806	0.001	Neutrokinin 3 antagonist
0.608	0.017	CYP2A8 substrate
0.662	0.094	Phobic disorders treatment
0.616	0.059	Glycosylphosphatidylinositol phospholipase D inhibitor
0.559	0.019	Polarisation stimulant

Figure 1 Estimation of the potential activity of five pyrazole derivates using PASSonline programme. C-1: compound 1, C-2: compound 2, C-3: compound 3, C-4: compound 4, C-5: compound 5.

Discussion

Pyrazole derivatives have been tested for many biological activities such as anti-cancer, antidepressant, anticonvulsant, antibacterial, anti-inflammatory.[4–6, 10, 11, 21, 43] In recent years, many studies have focused on the anticancer activities of pyrazole derivatives. Liu et al. have shown that pyrazole-derived compounds can be used as anticancer agents since they exhibit antimitotic effects by causing depolarization of tubules.[44] In addition, pyrazole derivatives are known to display anticancer activities by inhibiting various target molecules such as topoisomerase II, EGFR, VEGF, B-Raf, m-TOR and HDAC.[15–20] On the other hand, few studies evaluating the antimigratory effects of pyrazole derivatives have been found in the literature. In the first of these studies, one of the synthesized compounds containing pyrazole, thiazole and naphthalene ring has been shown to inhibit the migration of HeLa cells.[22] In another study, a series of the pyrazole derivative containing benzimidazole skeletons has been shown to inhibit cancer cell migration in MCF-7 cell line.[23] Additionally, Zhang et al. showed a series of pyrazole compounds that inhibit STAT3 phosphorylation. These compounds have been shown to induce apoptosis and inhibit migration in patient-derived high grade glioma cells (HGG).[24] However, the antimigratory mechanism of action of pyrazole derivative compounds has not been fully revealed.

In this study, it was aimed to evaluate the anticancer activity of pyrazole-derived compounds in terms of both cytotoxicity and antimigratory activity. For this purpose, five structurally related

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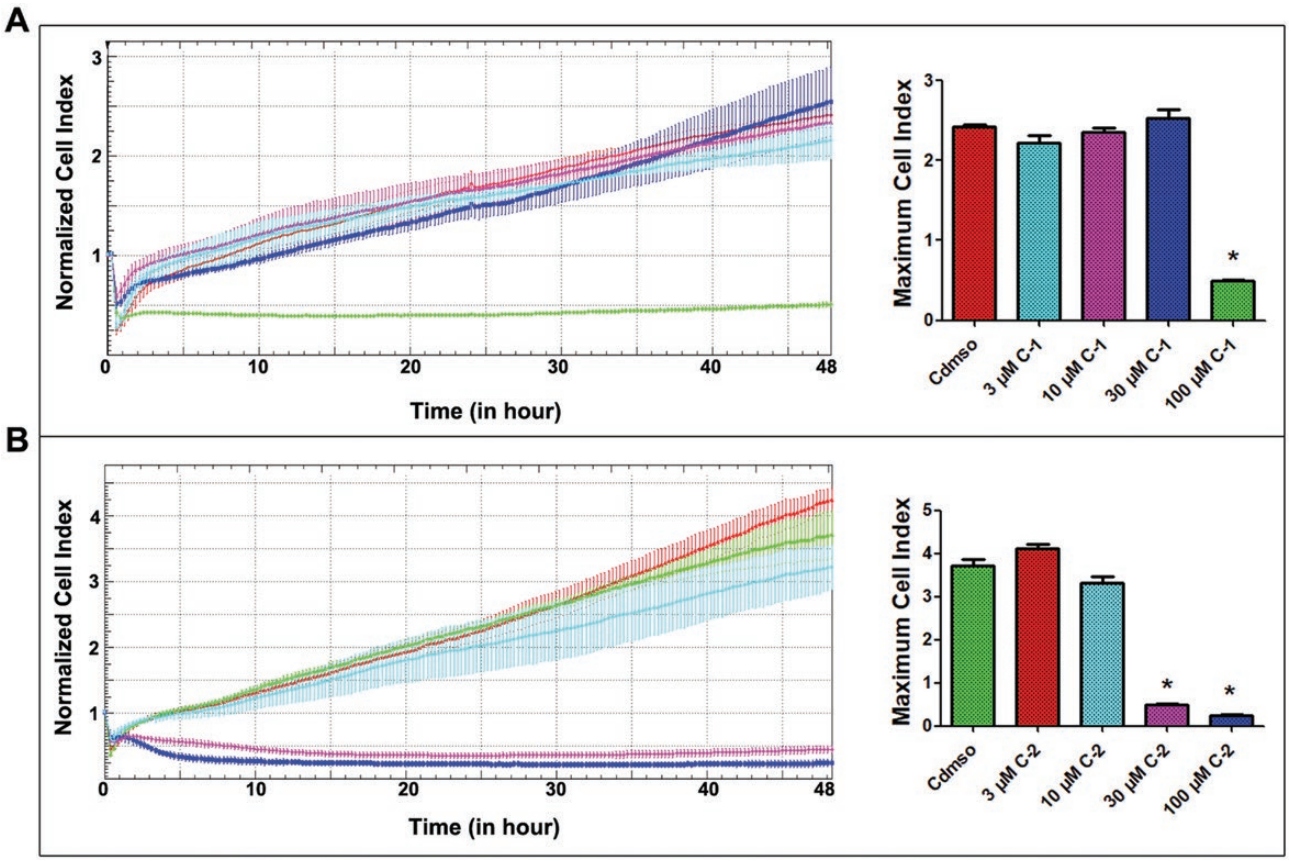

Figure 2 Effect of C-1 and C-2 on A549 cancer cell proliferation. (A) Effect of C-1 on cell proliferation in A549 cells. (B) Effect of C-2 on cell proliferation in A549 cells. Data were analysed by analysis of variance andTukey post hoc test. Results are expressed as means ± SEM. (*) indicates significant difference from control DMSO ($n = 4$, $P < 0.05$). Cdmso: control DMSO group, C-1: compound 1, C-2: compound 2.

pyrazole derivative compounds selected from chemical libraries were evaluated with the PASSonline programme, and C-1 and C-2, which were likely to cause STAT inhibition, were synthesized (Figure 1). The cytotoxic and antimigratory effects of the synthesized C-1 and C-2 in A549 cancer cell line were evaluated. Cytotoxic effects of the aryl pyrazole derivatives of different concentrations were evaluated in A549 cells, and it was determined that C-1 at 100 µM concentration and C-2 at 30 and 100 µM concentrations induced cell death (Figure 2). When the cytotoxic activities of the compounds were compared, it was found that the C-2 was more active because C-2 showed a lower concentration of cytotoxicity. The possible antimigratory effects of the synthesized aryl pyrazole derivatives C-1 and C-2 were investigated. We determined that only C-2 significantly reduced the cancer cell migration. Although C-1 is cytotoxic, it does not show antimigratory activity. These results suggest that antimigratory and cytotoxic effects might develop through different mechanisms.

Our findings implied that both cytotoxicity and antimigratory activity profiles of C-1 and C-2 were different. In order to show whether the difference between the biological activities of these compounds was related to their effects on STAT activation, STAT1, STAT3 and STAT5 activities of the compounds were examined. Our results showed that treatment of C-1 had no effect on STAT1, STAT3 and STAT5A/B phosphorylation. While the treatment of 10 µM C-2 did not affect STAT3 and STAT5A/B activation, it resulted in a significant increase in STAT1 activation due to the increased phosphorylation in A549 cancer cells. This finding indicates that the antimigratory effect of C-2 may be caused by the increase of STAT1 activation, which is known to have antimigratory properties. It has been shown that STAT3 promotes cancer cell migration and invasion while STAT1 suppresses proliferation, tumour growth, angiogenesis and metastasis.[27, 45, 46] Furthermore, STAT1 plays an important role in the IFN-mediated inhibition of monocyte migration.[47] In another study conducted *in vitro* lung cancer cells, it was also shown that STAT1 overexpression significantly reduced the cell migration and invasion ability.[48] However, the only mechanism suggested for the antimigratory mechanism of action of pyrazole derivative compounds in the literature is that it is through STAT3 inhibition.[24] When the findings of these studies and our study are evaluated together, the antimigratory mechanism of action of pyrazole derivative compounds may depend on the change in the activation balance of STAT1 and STAT3.

Conclusions

Our findings have shown that treatment of C-2 increases the activation of STAT1 and does not change the activation of STAT3, causes the balance between STAT1 and STAT3 to change in favour of STAT1. We suggest that the mechanism underlying the specific effect of the C-2 on STAT1 may be the direct activation of STAT1 or the inhibition of the specific STAT1 inhibitors, SOCS1 and PIAS1 proteins. Further investigation of the antimigratory mechanism of action of C-2 and the synthesis of new derivatives with more effective antimigratory activity will be an important step in the treatment of cancer.

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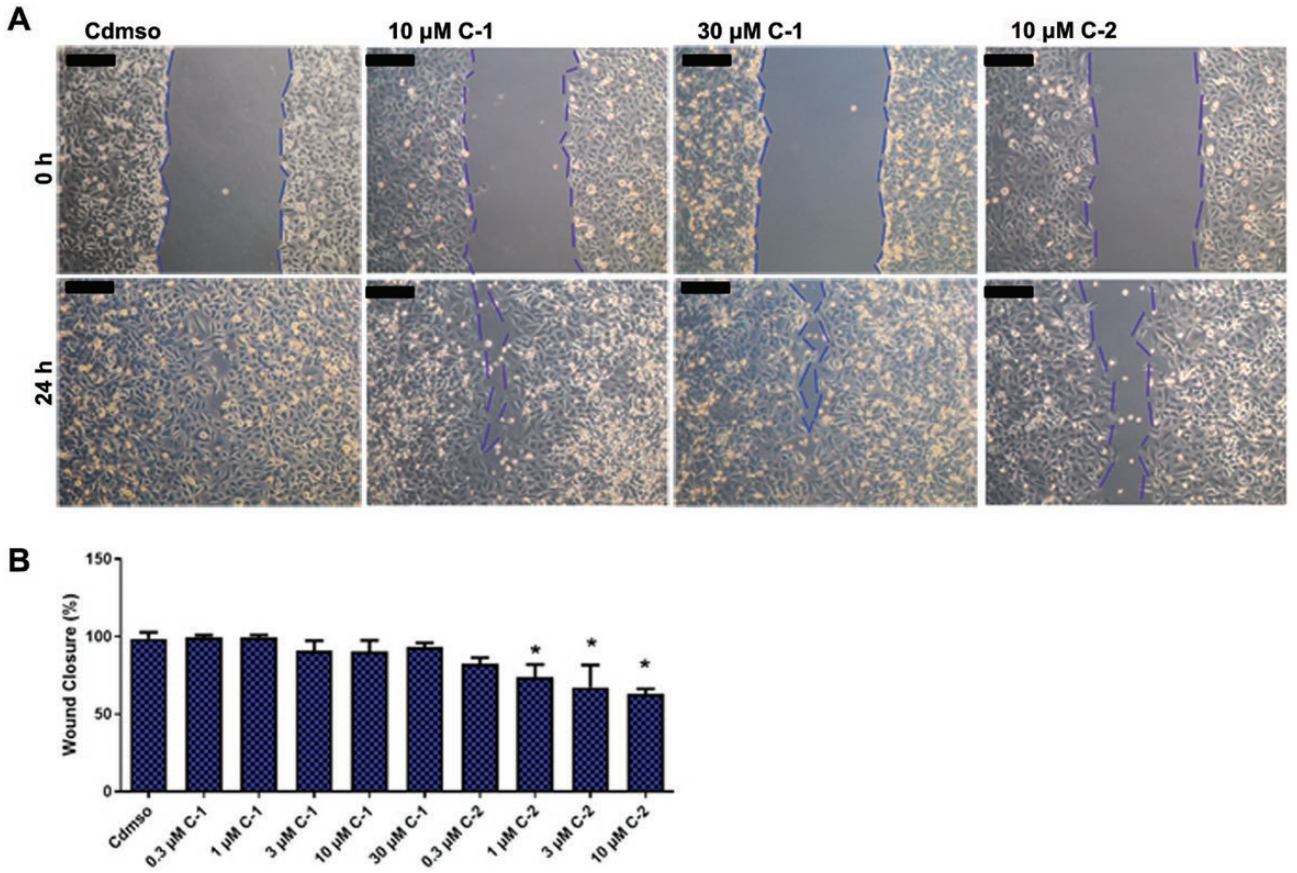

Figure 3 Effect of C-1 and C-2 on wound-healing migration in A549. (A) Representative phase contrast images of cells. Cells were exposed to 10 μM and 30 μM C-1, and 10 μM C-2 for 24 h. (B) Quantification of wound healing closure. Scala bars: 200 μm. Data were analysed by analysis of variance and Tukey post hoc test. Results are expressed as means ± SEM. (*) indicates a significant difference from control DMSO ($n = 7$, $P < 0.05$). Cdmso: control DMSO group, C-1: compound 1, C-2: compound 2.

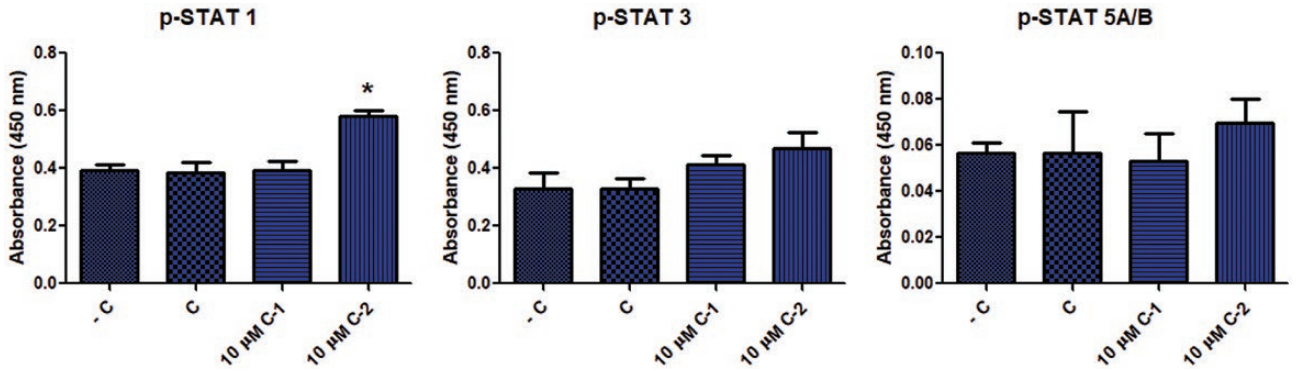

Figure 4 Effect of 10 μM C-1 and C-2 on STAT1, STAT3 and STAT5 phosphorylation in A549 cells. Data were analysed by analysis of variance and Tukey post hoc test. Results are expressed as means ±SEM. (*) indicates significant difference from control group ($n = 4$, $P < 0.05$). -C: negative control group (without any wound and mitomycin C application), C: control group, C-1: compound 1, C-2: compound 2.

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Author Contributions

Y.D.Ş.D. performed cytotoxicity and STAT activity experiments, analysed the data; A.Ö. performed PASSonline scan; R.G.Ö. and S.C.C. carried out the wound healing experiments; B.Ç. synthesis of compounds C-1 & C-2; M.A. provided the study design, study mentorship; Y.D.Ş.D., A.Ö. and M.A. contributed in part to writing and editing the manuscript and approved the final version.

Conflict of Interest

The authors state no conflict of interest.

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