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Synthesis of novel potential ROCK inhibitors and their antimigratory effects

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Abstract: Rho kinase (ROCK), an enzyme belonging to the serine-threonine kinase family, is involved in the regulation of basic cellular processes such as morphology, movement, division, differentiation and apoptosis. On the other hand, excessively activated ROCK can cause to cardiovascular and neurological disorders or cancer. In recent years, overactivation of Rho kinases has been associated with increased metastasis in various tumor types and has been explored as target for the development of new anticancer drugs. We report here the design and synthesis of five urea derivatives in search of novel inhibitors of cancer cell migration. Compounds evaluated for their cytotoxic activities against breast (MCF-7) cancer cell line. After determination of the ineffective concentrations of compounds on the proliferation of MCF-7 cells, wound healing experiments were conducted to investigate the antimigratory effects of compounds. While compound 4 and 10 had no effect on cell migration, treatment of MCF-7 cells with compound 5, 8 and 9 resulted in significant reduction in cell motility. Taken together our results suggest that the newly synthesized compound 5, 8 and 9 had the potential antimigratory activity through possible ROCK inhibition in cancer cells.

Keywords: ROCK; migration; MCF-7; benzoxazolone; benzimidazole; urea.

1. Introduction

Rho-associated protein kinases are one of the members of AGC family of serine–threonine kinases related the regulation of fundamental cellular processes such as morphology, motility, division and differentiation. ROCK enzymes contain two isoforms, including ROCK I and ROCK II (known as ROKβ and ROKα, respectively). Two isoforms are highly similar in their kinase domains with 92% sequence identity[1].

It has been discovered that ROCK I and ROCK II are involved in the arrangement contraction of smooth muscle, the formation of stress fibers (F-actin and myosin II bundles) and focal adhesion complexes in cells[2-4]. Over time, ROCK has been shown to play a role in Alzheimer's $A\beta_{42}$ amyloid protein formation[5], blood clotting[6], and cancer-related processes[7] such as cell proliferation, cytokinesis, migration, apoptosis[8-9], senescence[10] and autophagy[11].

ROCKs are required for the migration and invasion of cancer cells. Studies suggest that cell contractility is important in regulating the invasiveness of cancer stem-like cells (CSCs), which are

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responsible for metastasis, and targeting this enzyme represents a new therapeutic strategy by preventing the migration of metastatic cancers [12-14].

The ROCK pathway has been found to be associated with cardiovascular and neurological diseases as well as cancer, and inhibiting ROCK can be potentially beneficial for the treatment of these related diseases[15]. ROCK inhibitors have a high potential to be used in many pathological conditions such as asthma, glaucoma, cancer, neuronal degeneration, erectile dysfunction, kidney failure, insulin resistance, osteoporosis[16]. Currently, there are only two ROCK inhibitors in Japan (Fasudil and Ripasudil), one of them (Fasudil) in China and the other one (Netarsudil) in The United States approved for clinical use (Figure 1). Fasudil is used for the treatment of cerebral vasospasm while Ripasudil and Netarsudil are used in the treatment of glaucoma and ocular hypertension[11]. Although there are very few ROCK inhibitors in clinical use, many ROCK inhibitors with different scaffolds such as substituted isoquinoline, pyridine, indazole, aminofurazan-azabenzimidazoles, pyrimidine, pyrazole, quinazolinone have been developed in the past two decades[16].

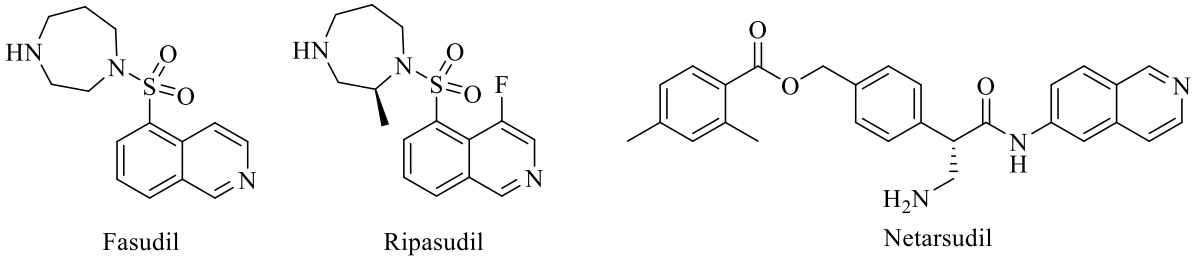


Figure 1. Chemical structures of ROCK inhibitors used in the clinic[17].

Diverse nitrogen and oxygen containing heterocyclic systems are of crucial importance in drug discovery programs. Benzimidazole nucleus have taken a great deal of attention because they exhibit a plethora of biological activities. Furthermore benzimidazole derivative Compound A has been reported as ROCK II inhibitor with an IC_{50} value of 0,25 nM[18]. Benzoxazolone core also has been used in the design of anticancer compounds[19]. Moreover urea linker plays a critical role in ligand interaction at the ROCK active site. Indazole and urea containing Compound B has been found that it inhibits ROCK II with the IC_{50} value of 0.67 µM [20]. Based on the structural information of the indazole-urea type ROCK inhibitor, we hereby designed urea derivatives by replacing the indazole with heterocyclic counterparts such as benzimidazole or benzoxazolone cores. (Figure 2).

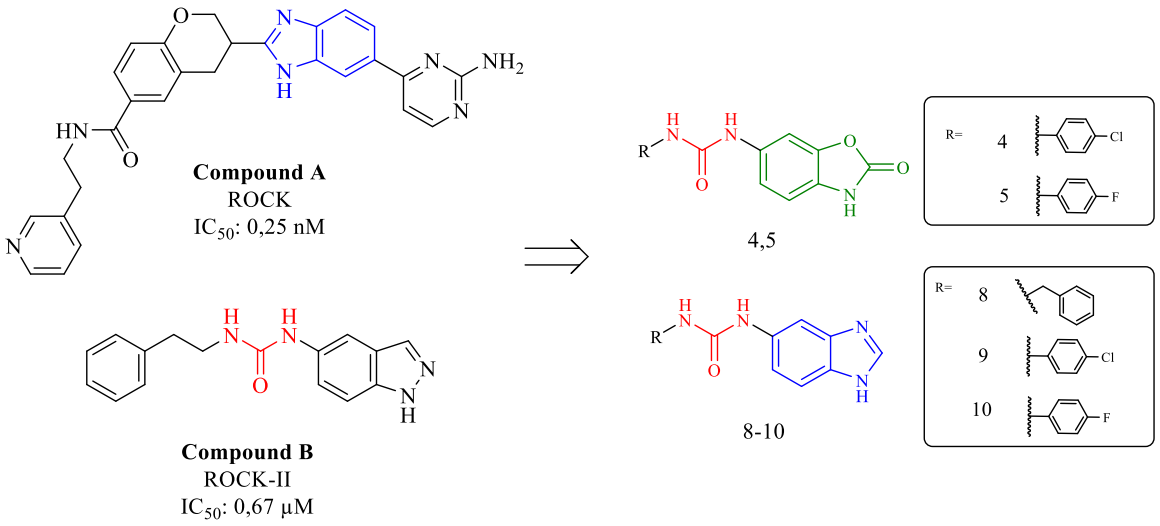


Figure 2. Structures of two ROCK inhibitors and synthesized compounds

2. Experimental

2.1. Chemical Material and Apparatus

The starting materials were obtained from Abcr and Merck and used for the necessary synthesis steps without any purification. ^{1}H and ^{13}C NMR spectra were recorded in Dimethylsulfoxide-D_6 on a Bruker 400 MHz. ^{13}C NMR spectra were documented on a spectrometers Varian 400 (100 MHz) with complete proton decoupling. All coupling constants are reported as Hertz. All chemical shifts expressed in p.p.m. relative to internal tetramethylsilane (TMS). Reaction tracking and resulting product purity were monitored by Analytical thin-layer chromatography (TLC) on precoated Merck silica gel plates (Silica Gel 60Å F254) purchased from Merck. Plates developed with the appropriate solvent system were evaluated using UV lamp (254 nm or 365 nm). Melting points were appointed Stuart SMP40 Digital Melting Point Apparatus. High resolution mass spectra data (HRMS) were collected using Waters LCT Premier XE Mass Spectrometer operating in ESI (+) or ESI (−) method, also coupled to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA) using a UV detector (254 nm). Purity of all final compounds were >95%, according to the ultra-performance liquid chromatography coupled with mass spectroscopy (UPLC-MS) method using (A) water + 0.1% Formic Acid and (B) acetonitrile + 0.1% Formic Acid; flow rate = 0.3 mL/min. Aquity BEH C18 column (2.1 × 100 mm, 1.7 mm, Waters Corporation, Milford, MA, USA) was used as the column. Preparative liquid chromatography was performed on Buchi Prep HPLC Column C18 (250 × 21.2mm, 10μm, Grace, Columbia, Maryland, USA) using Reveleris PREP® purification system.

2.2. Chemistry

*Synthesis of Benzoxazol-2(3H)-one (**1**)*: To a solution of 2-aminophenol (500 mg, 4.58 mmol, 1.0 equiv) in *N,N*-dimetylformamide (DMF) (6 mL) were added carbonyldiimidazole (CDI) (892 mg, 5.5 mmol, 1.2 equiv) and the resulting mixture was heated at 80 °C under nitrogen atmosphere for 2.5 h. Upon completion of the reaction, the reaction mixture was cooled to room temperature (rt) then poured into water and filtered to give the crude product. Yield: 84%, m.p. 139,4-140,9 °C[21]. HRMS (*m/z*): $[M+H]^+$ calculated $C_7H_6NO_2$: 136.0399; found: 136.0395. CAS # 59-49-4

*Synthesis of 6-Nitrobenzoxazol-2(3H)-one (**2**)*: 5 mL 65% of nitric acid was put into reaction bowl, cooled to 0 °C added **1** (500 mg, 3.7 mmol, 1.0 equiv) and stirred at ambient temperature for 4h. The reaction mixture was taken into ice water, the solid was filtered off under vacuum and dried. The crude product was used in the next step without purification. Yield: 70%, m.p. 252,1-253,5 °C[22]. HRMS (*m/z*): $[M+H]^-$ calculated for $C_7H_3N_2O_4$: 179.0093; found: 179.0100. CAS # 4694-91-1

*Synthesis of 6-Aminobenzoxazol-2(3H)-one (**3**)*: **2** (300 mg, 2.0 mmol, 1 equiv) was dissolved in 15 mL of ethanol, $SnCl_2.2H_2O$ (2.256 g, 10 mmol, 5 equiv) was added in five portions into the reaction mixture and the resulting mixture was refluxed for 4h. Upon completion of the reaction, the reaction mixture was neutralized with $NaHCO_3$ solution and transferred to a separatory funnel, diluted with ethyl acetate and extracted. The organic layer was dried, filtered and evaporated. Then crude product was triturated with hexane:ethyl acetate, filtered and dried. Yield: 98%, m.p. 196 °C (decomp.). HRMS (*m/z*): $[M+H]^+$ calculated for $C_7H_7N_2O_2$: 151.0508; found: 151.0505. CAS # 22876-17-1

The reaction mixture was transferred to a separatory funnel and diluted with 20 mL of methylene chloride, washed with water (2 x 25 mL), sat. $NaHCO_3$ (2 x 25 mL), water (25 mL), brine (25 mL), dried over Na_2SO_4, and filtered. The solvent was removed under reduced pressure to afford a brown solid.

*Synthesis of 1-(4-Chlorophenyl)-3-(2-oxo-2,3-dihydrobenzoxazol-6-yl)urea (**4**)*: To a solution of **3** (100 mg, 0.67 mmol, 1 eq) in DMF (2 mL) were added 4-chlorophenyl isocyanate (123 mg, 0.8 mmol, 1.2 eq) and reaction mixture stirred at 80˚C for 15h. The reaction mixture was cooled to room temperature, diluted with ice water, and the solid was filtered off under vacuum and dried. The crude

product which was triturated with methanol, filtered and dried to give gray-white solid. Yield: 62%, m.p. 325.2-331.0 °C. ^{1}H-NMR (400 MHz, DMSO) δ 7.01 (1H, d, J = 8.4 Hz), 7.07 (1H, dd, J = 6.4, 2.0 Hz), 7.33 (2H, d, J= 6.6 Hz), 7.48 (2H, d, J= 6.6 Hz), 7.57 (1H, d, J= 2.0 Hz), 8.74 (1H, s), 8.80 (1H, s), 11.48 (1H, bs). HRMS (*m/z*): $[M+H]^+$ calculated for $C_{14}H_{11}ClN_3O_3$: 304.0489; found: 304.0493. CAS # 1018188-02-7

*Synthesis of 1-(4-Fluorophenyl)-3-(2-oxo-2,3-dihydrobenzoxazol-6-yl)urea (**5**)*: Prepared from **3** and 4-fluorophenyl isocyanate under the same conditions that were applied to **4**. Yield: 76%, m.p. 309.4-310.0 °C. ^{1}H-NMR (400 MHz, DMSO) δ 7.00 (1H, d, J = 8.4 Hz), 7.07 (1H, dd, J = 8.4, 2.0 Hz), 7.12 (2H, d, J = 8.9 Hz), 7.46 (2H, d, J = 8.9 Hz), 7.57 (1H, d, J= 2.0 Hz), 8.68 (1H, s), 8.69 (1H, s), 11.47 (1H, bs). HRMS (*m/z):* $[M+H]^+$ calculated for $C_{14}H_{11}FN_3O_3$: 288.0784; found: 288.0779. CAS # 1189438-64-9

*Synthesis of 5-nitro-1H-benzimidazole (**6**)*: 4-nitro-*o*-phenylenediamine (1 eq), formic acid (2 eq) and 4 M HCl were stirred at 110˚C for 17 h. The reaction mixture was cooled to room temperature, diluted with ice water and pH was adjusted to 7 with $NaHCO_3$. Solid was filtered and dried. Compound **6** was used in the next step without purification. Yield: (85%), m.p. 203.2-205.2˚C[23]. HRMS *m/z* calculated for $C_7H_5N_3O_2$ $[M+H]^+$ 164.0460, found: 164.0454. CAS # 94-52-0

*Synthesis of 1H-benzimidazol-5-amine (**7**)*: Compound **6** was dissolved in methanol, Pd/C was added, and reaction mixture stirred at rt under hydrogen atmosphere overnight. Ethyl acetate was added, and the mixture filtered from Celite pad. Filtrate was evaporated. Compound **7** was used in the next step without purification. Yield: (98%), m.p. 162.4-164.6˚C[24]. HRMS *m/z* calculated for $C_7H_7N_3$ $[M+H]^+$ 134.0718, found: 134.0716. CAS # 934-22-5

*Synthesis of 1-(1H-benzimidazol-5-yl)-3-benzylurea (**8**)*: Prepared from **7** and benzyl isocyanate under the same conditions that were applied to **4.** The solid was purified by preparative liquid chromatography (LC), eluting with a gradient of 0-45% Acetonitrile in H_2O to give **8** as a white powder. Yield: 48 mg, (48%), m. p. 221.8-223.2˚C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.33 (2H, d, J = 5.9 Hz), 6.55 (1H, s), 6.95 (1H, d, J = 8.7 Hz), 7.22-7.25 (1H,m), 7.40 – 7.29 (4H, m), 7.49 (1H, d, J = 8.8 Hz), 7.90 (1H, s), 8.08 (1H, s), 8.56 (1H, s), 12.22 (1H, s); ^{13}C NMR (100 MHz, DMSO-d_6): δ 155.98, 141.46, 140.95, 136.14, 134.08, 128.78, 127.61, 127.17, 119.23, 113.85, 111.56, 100.83, 43.27. HRMS *m/z* calculated for $C_{15}H_{15}N_4O$ $[M+H]^+$ 267.1246, found: 267.1240. CAS # 1791052-17-9

*Synthesis of 1-(1H-benzimidazol-5-yl)-3-(4-chlorophenyl)urea (**9**)*: Prepared from **7** and 4-chlorophenylisocyanate under the same conditions that were applied to **4**. The solid was purified by preparative LC, eluting with a gradient of 0–50% Acetonitrile in H_2O to give **9** as a white powder. Yield: (73%), m. p. 254.0-255.9˚C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.00 (1H, d, J = 8.8 Hz), 7.33 (2H, d, J = 8.9 Hz), 7.52 (2H, d, J = 8.9 Hz), 7.55 (1H, m), 7.91 (1H, s), 8.11 (1H, s), 8.79 (2H, s), 12.28 (1H, s); ^{13}C NMR (100 MHz, DMSO-d_6): δ 153.11, 139.38, 135.09, 134.05, 129.08, 120.10, 119.39, 115.87, 114.30, 111.75, 109.45, 101.56; HRMS *m/z* calculated for $C_{14}H_{12}ClN_4O$ $[M+H]^+$ 287.0700, found: 287.0690. CAS # 1791052-10-2

*Synthesis of 1-(1H-benzimidazol-5-yl)-3-(4-fluorophenyl)urea (**10**)*: Prepared from **7** and 4-fluorophenylisocyanate under the same conditions that were applied to **4**. The crude solid was crystallized in methanol to give **10** as white crystals. Yield: (47%), m. p. 250.1-252.3 ˚C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.09-7.15 (3H, m), 7.46-7.51 (3H, m), 7.88 (1H, s), 8.12 (1H, s), 8.63 (1H, s), 8.65 (1H, s), 12.29 (1H, s); ^{13}C NMR (100 MHz, DMSO-d_6): δ 100.32, 109.67, 113.33, 115.72 (d, $^2J_{C\text{-}F}$ = 22.2 Hz), 120.31 (d, $^3J_{C\text{-}F}$ = 7.9 Hz), 126.23, 135.44, 136.72 (d, $^4J_{C\text{-}F}$ = 2.6 Hz), 147.34, 150.8705, 153.36, 157.71 (d, $^1J_{C\text{-}F}$ = 237.8 Hz); HRMS *m/z* calculated for $C_{14}H_{12}FN_4O$ $[M+H]^+$ 271.0995, found: 271.0985. CAS # 1909184-83-3

2.3. Biological Assay

2.3.1. Cell Culture

MCF-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS (fetal bovine serum) and 1× Penicillin/Streptomycin in an incubator with 5% CO_2 at 37 ºC. The newly synthesized compounds were dissolved in DMSO (0.2%), and an equal amount of DMSO was treated to the control group.

2.3.2. MTT Assay

MTT assay was performed to determine the compound concentrations tested that do not have cytotoxic effect on cells. Briefly, 10000 cell were plated in a 96 well cell culture plate. After 24 h attachment, medium was replaced with fresh comple meidum with phenol red free DMEM. Then cells were treated with increasing logarithmic concentrations of compound 4 (C-4), compound 5 (C-5), compound 8 (C-8), compound 9 (C-9) and compound 10 (C-10) (3-100 µM). After 48 h incubation, MTT solution was added to each well and incubated for 3 h. Formazan crystals were dissolved in DMSO and then the plate was read at 570 nm. Cell viability was calculated as percentage of control[25].

2.3.3. Wound Healing-Migration Assay

When MCF-7 cells reached to 100% confluence in a 12-well plate and the cells were starved over-night. A wound was created by scrating the cells with a yellow tip[26]. The cells were then treated with non-cytotoxic concentrations of compounds in complete medium for 24 h. Images of the scratch were acquired at 0 hours and 24 hours under phase contrast microscopy. Wound closure area was calculated using Image J software.

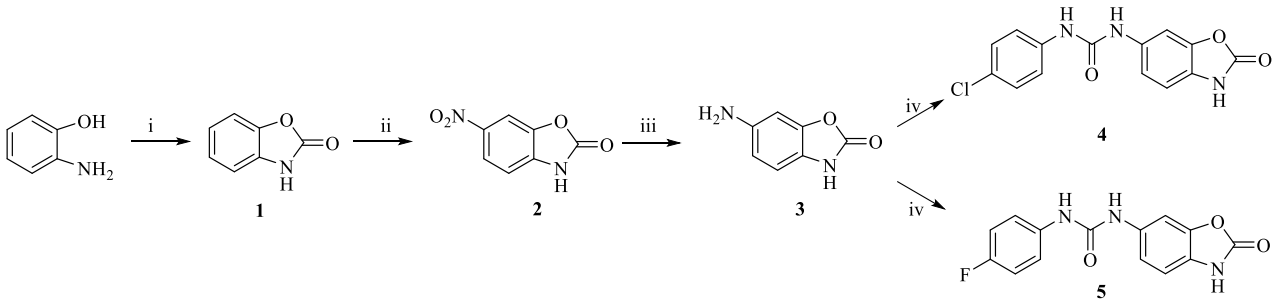
2.4. Statistical Analysis

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

3. Results and Discussion

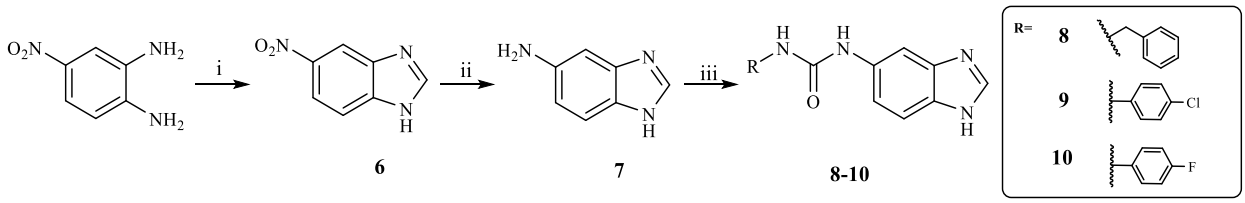
3.1. Chemistry

The synthesis of the resulting benzoxazolone derivatives is outlined in Scheme 1. Benzoxazole was prepared by the reaction of 2-aminophenol and CDI in DMF[27]. Then nitro derivative was obtained by reacting compound 1 (C-1) with nitric acid [28]. Nitro reduction with tin chloride gave starting intermediate amine derivative. Treatment of aminobenzoxazolone with isocyanate derivatives produced urea derivatives C-4 and C-5, with the aim to evaluate to the effects of benzoxazolone core and halogen containing phenyl substitution on the activity[29].



Scheme 1. i. CDI,DMF, 80°C; ii. HNO3, 0°C; iii. SnCl2.2H2O, EtOH, reflux; iv. isocyanate derivative DMF, 80°C

The synthesis of the benzimidazole derivatives is outlined in Scheme 2. The 5-nitro-1*H*-benzo[*d*]imidazole was obtained using 4-nitro-*o*-phenylenediamine in the aqueous acidic medium in the presence of formic acid[30]. Subsequently, catalytic hydrogenation was carried out under hydrogen atmosphere using Pd/C[31]. The resulting amine was reacted with three different isocyanate derivatives in DMF at 80°C [29]. While retaining the urea substitutions common to C-4 and C-5, C-8, C-9 and C-10 were designed and synthesized to evaluate the change in the heretocyclic nucleus on the activity. In addition, with C-8, it was aimed to evaluate the benzyl substitution on the urea group. The final products were purified using preparative LC or crystallization, and were structurally characterized by their spectroscopic data (^{1}H- NMR, ^{13}C-NMR, and HRMS).



Scheme 2. i. formic acid, 4 M HCl, 110°C; ii. Pd/C, MeOH, rt; iii. isocyanate derivative DMF, 80°C

3.2. Biological Assay

It is well known that Rho kinases are involved in the organization of actin cytoskeleton dynamics, and therefore are responsible for cell migration. In order to determine the activity of the candidates of inhibitors of ROCK on cancer cell migration, first we performed MTT assay to evaluate the non-cytotoxic concentrations of compounds tested against MCF-7 cells. Cells were treated with increasing concentrations of C-4, 5, 8, 9 and 10 (3-100 µM). After dissolving the compounds in DMSO, some treated concentrations of C-4, C-5, C-9 and C-10 were incompatible with the medium. Therefore the effect of these concentrations on cell viability was not calculated. Figure 3 shows that none of the treated-concentrations of compounds induced cytotoxicity in MCF-7 cells for 48 h.

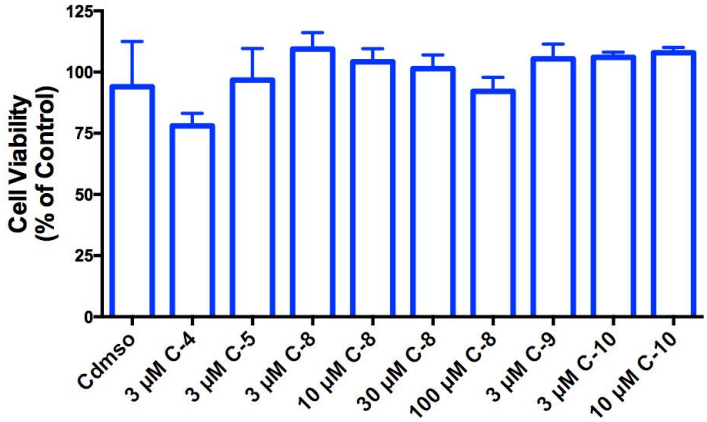


Figure 3. MTT assay results for C-4, C-5, C-8, C-9 and C-10 in 48 h on MCF-7 cell line. Mean±s.d., *n*=4, C_{dmso}: DMSO treated control

To evaluate the effects of synthesized compounds on cancer cell migration in vitro, a wound-healing assay was performed. MCF-7 cells were seeded on 12 well plate, then monolayers were scratched using pipette tip. Images were taken at 0 h and 24 h after the compounds were treated to cells in increasing non-toxic concentrations. Our results showed that while 0,3 µM and 1 µM C-5 treatment inhibited the cell migration in MCF-7 cells significantly compared to control, the decrease in cell migration caused by the treatment of 3 µM C-5 was not found to be significant (Figure 4). Treatment of 3 µM C-8 and 1µM, 3 µM C-9 resulted in significant supression of wound closure up to 24 h (Figure 4). Treatment of C-4 and C-10 treatment had no effect cell migration (Figure 4).

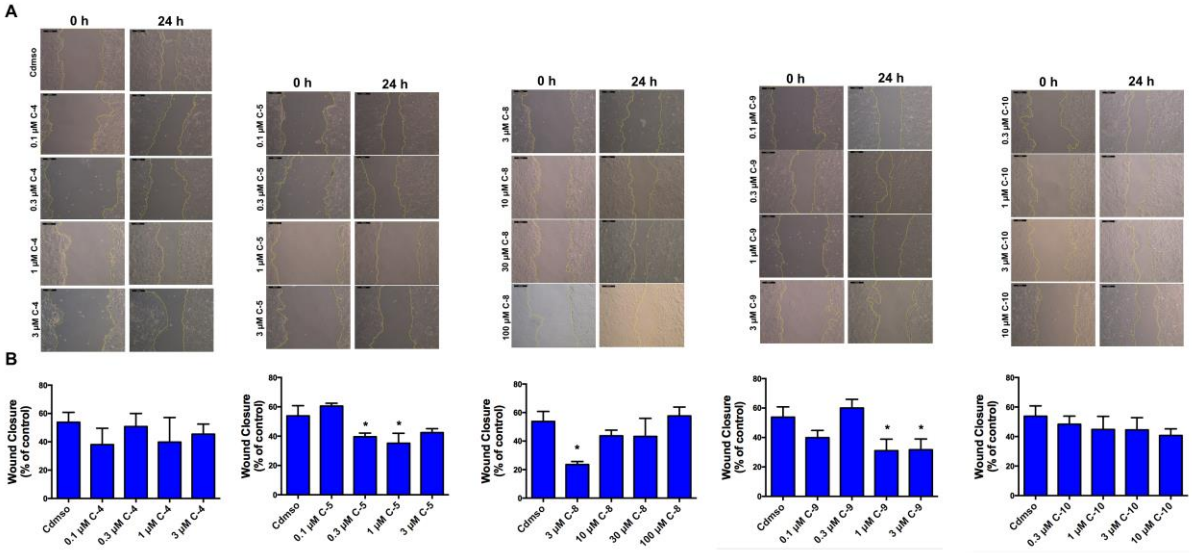


Figure 4 . Effect of C-4, C-5, C-8, C-9 and C-10 on cell migration for 24 h in MCF-7 cells. A. Representative phase contrast images show that the effect of treatment of C-5, C-8 and C-9 resulted in significantly decreased cell migration. Scale bar: 200 µm B. Cells were treated various concentrations of compounds for 24, then relative wound closure was measured. Mean±s.d., *n*=3, $^{*}P<0.05$ compared with control (DMSO) at 24 h. C_{dmso}: DMSO treated control

4. Conclusion

It is well known that ROCK is involved in tumor cell invasion and metastasis via direct induction of cell motility. One of the Rho kinase inhibitor fasudil has shown to reduced the cell migration and invasion in A549 lung cancer cell line and human ovarion cancer cells[32-33]. In this study, C-5, C-8 and C-9, three of the five novel compounds synthesized as potential Rho kinase inhibitors,

showed significant antimigratory effect on MCF-7 cells. With compounds carrying benzoxazolone in the main structure, it was observed that the derivative carrying p-fluorine prevented migration while no antimigratory effect was observed in the corresponding p-substituted chlorine derivative. In compounds bearing the benzimidazole ring, which is the other central structure, the p-substituted fluorine derivative is responsible for the antimigratory effect compared to the p-substituted chlorine-bearing derivative, and the antimigratory effect has continued with the replacement of the p-substituted phenyl with the benzyl fragment. Taken together our results suggest that newly synthesized C-5, C-8 and C-9 have antimigratory potential through possible inhibition of ROCK in cancer cells. These lead compounds may warrant for further development of more potent Rho kinase inhibitors.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/organic-communications

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