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Anti-proliferative Activity of 3,5-Diaminoindazole Analogues

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ABSTRACT

Cancer is the second leading cause of death worldwide. Small-molecule anticancer agents with tolerable safety profiles have been developed to improve survival rates. Indazole analogues have received considerable attention due to their anticancer properties. A series of *N3*-acyl-*N5*-pyrimidinyl-3,5-diaminoindazoles were synthesized and their antiproliferative activities were evaluated in Caki, A549, HepG2, AMC-HN4, and SNU484 human cancer cell lines. Cellular selectivity of 3,5-diaminoindazole with a modified structure of the *N*5-pyrimidinyl substituent was studied. While both compounds **9a** and **9b** showed a high selectivity for the HepG2 cell line, compound **9b** had a higher selectivity than **9a**, with an IC_{50}<<0.1 μM.

Key words : Indazole, 3,5-diaminoindazole, anticancer, *N3*-acyl-*N5*-pyrimidinyl-3,5-diaminoindazoles

Introduction

Cancer is the leading cause of death in industrialized countries. The burden of cancer increases because of the increase in the average life span and the growth of population [1]. According to the GLOBOCAN 2018, about 18.1 million new cancer cases and 9.6 million cancer deaths are estimated to have occurred in 2018. Despite the remarkable progress in cancer therapies, there are still unmet needs in cancer treatments because the conventional methods do not provide substantial improvement in survival rates and show poor safety profiles. The development of anti-cancer therapeutics has been endeavored in a variety of ways. The screening of compound libraries using enzymatic assay in a high-throughput format has been used in the early stage of drug discovery [2,3]. However, the pharmacological effect of a drug is hard to predict based on the enzymatic activity because living systems are extremely complex with a large variety of control and feedback systems, all of which can have an influence on a drug's activity. The cell-based assays have been used as more biologically relevant surrogates than enzyme assay [3-5].

Heteroaromatics are considered as pharmacologically important scaffolds and used widely in drug discovery [6-12]. As a family of the nitrogen containing heteroaromatics, indazole derivatives have received attention because of their biological properties such as anti-inflammatory, anti-HIV, antihypertensive and anti-cancer activities. Therapeutic agents containing indazole scaffold, such as pazopanib, axitinib and niraparib have been approved for the treatment of cancers (Fig. 1) [13]. In this report, we describe the synthesis of 3,5-diaminoindazole derivatives and the evaluation of their anti-proliferative activities.

Experimental

Materials and Instruments

^{1}H- NMR and ^{13}C-NMR spectra were recorded on a Bruker

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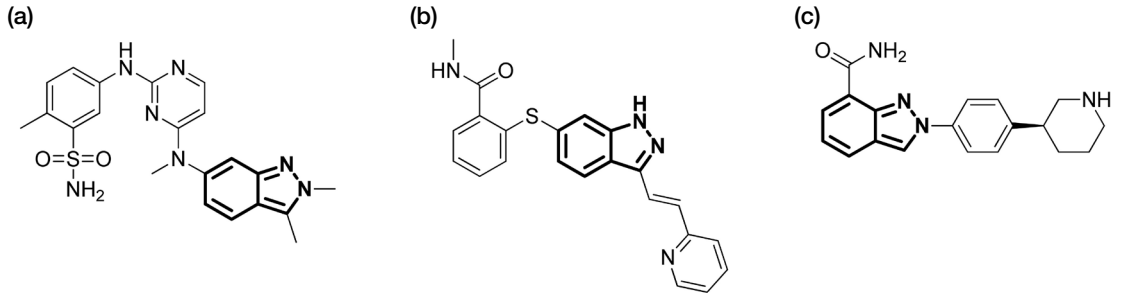


Fig. 1. Indazole derivatives approved for the treatment of cancers. Bold line indicates indazole scaffold: (a) pazopanib, (b) axitinib, and (c) niraparib.

AVANEC 400 (400 MHz) and Jeol ECA500 (500 MHz) spectrometer and chemical shifts (δ) are reported in ppm using tetramethylsilane (TMS) as an internal standard. TLC was performed on E. Merck silica gel 60 F254 plates (0.25 mm). Silica gel column chromatography was performed using Merck silica gel 60 (230∼400 mesh). Unless otherwise noted, all starting materials were obtained from commercially available sources and they were used without further purification. All reactions were performed under a nitrogen atmosphere.

Synthesis

5-Nitro-1*H*-indazol-3-amine (**2**)

A solution of 2-fluoro-5-nitrobenzonitrile (30.3 mmol) and hydrazine hydrate (90.8 mmol) in *n*-butanol (200 mL) was heated at reflux for 4 h. The solvent was removed under reduced pressure, and the remaining residue was dissolved in EtOAc and washed with a saturated aqueous solution of Na_2CO_3. The organic layer was separated, washed with water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The removal of solvent afforded the product 5.14 g in 95.2% yield; ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 12.19 (s, 1H), 8.89 (d, J = 2.0 Hz, 1H), 8.05 (dd, J = 9.2, 2.0 Hz, 1H), 7.34 (d, J = 9.2 Hz, 1H).

3-Bis(*tert*-butoxycarbonyl)amino-5-nitroindazol-1-carboxylic acid *tert*-butyl ester (**3**)

A mixture of **2** (1.78 g, 9.93 mmol), triethylamine (39.9 mmol), catalytic amount of *N,N*-dimethylaminopyridine, and di-*tert*-butyl dicarbonate (39.9 mmol) in tetrahydrofuran (150 mL) was heated at reflux for 3 h. di-*tert*-Butyl dicarbonate (9.9 mmol) and triethylamine (9.9 mmol) were added and the mixture was heated at reflux for 12 h. Tetrahydrofuran was removed under reduced pressure, and the remaining residue was dissolved in EtOAc. The organic layer was washed with a 5% aqueous solution of citric acid, dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was dried under high vacuum to afford 4.63 g (96.9% yield); ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.51 (dd, J = 2 Hz, J = 0.8 Hz, 1H), 8.42 (dd, J = 9.2 Hz, J = 2 Hz, 1H), 8.32 (d, J = 9.2 Hz, 1H), 1.74 (s, 9H), 1.46 (s, 18H).

5-Amino-3-bis(*tert*-butoxycarbonyl)aminoindazol-1-carboxylic acid *tert*-butyl ester (**4**)

A mixture of **3** (9.66 mmol) and catalytic amount of 10% palladium on active carbon in methanol (200 mL) was stirred for 2 h under H_2 atmosphere. Filtration with aid of celite followed by removal of solvent afforded **4** in 84.5% yield; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.92 (d, J = 8.8 Hz, 1H), 6.93 (dd, J = 8.8 Hz, J = 2.4 Hz, 1H), 6.69 (d, J = 2 Hz, 1H), 3.76 (s, 2H), 1.70 (s, 9H), 1.41 (s, 18H).

3-Bis(*tert*-butoxycarbonyl)amino-5-(2-chloro-5-fluoropyrimidin-4-ylamino)indazol-1-carboxylic acid *tert*-butyl ester (**5**)

A mixture of **4** (8.18 mmol), 2,4-dichloro-5-fluoropyrimidine (9.82 mmol), and triethylamine (16.4 mmol) in DMF (150 mL) was stirred at room temperature for 10 h. *N,N*-dimethylformamide was removed under reduced pressure, and the remaining residue was dissolved in EtOAc. The organic layer was washed with water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using hexane/ethyl acetate (1:1, v/v) as eluent to give **5** in a yield of 67.7%; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.14 (d, J = 8.8 Hz, 1H), 8.09 (d, J = 2.8 Hz, 1H), 8.01 (d, J = 2 Hz, 1H), 7.61 (dd, J = 9.2 Hz, J = 2 Hz, 1H), 7.17 (d, J = 2.8 Hz, 1H), 1.72 (s, 9H), 1.45 (s, 18H).

N^5-(2-Chloro-5-fluoropyrimidin-4-yl)-1*H*-indazole-3,5-diamine (**6**)

A mixture of **5** (15.8 mmol) and trifluoroacetic acid (8 mL) in dichloromethane (40 mL) was stirred at room temperature for 20 h. Dichloromethane was removed under reduced pressure,

and the remaining residue was dissolved in EtOAc. The organic layer was washed with a saturated aqueous solution of Na_2CO_3, dried over $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was dried under high vacuum to afford **6** in a yield of 87.4%; ^{1}H NMR (400 MHz, $CDCl_3 + CD_3OD$): δ (ppm) 7.98 (d, J = 3.2 Hz, 1H), 7.88 (d, J = 2 Hz, 1H) 7.54 (dd, J = 8.8 Hz, J = 2 Hz, 1H), 7.33 (d, J = 8.8 Hz, 1H).

2-Biphenyl-4-yl-*N*-[5-(2-chloro-5-fluoropyrimidin-4-ylamino)-1*H*-indazol-3-yl]acetamide (**7**)

A mixture of **6** (2.6 mmol), 4-Biphenylacetyl chloride (2.9 mmol), and triethylamine (6.6 mmol) in tetrahydrofuran (80 mL) was heated at reflux for 3 h and cooled to room temperature. 2N NaOH aqueous solution (50 mL) was added and the reaction mixture was stirred for 30 min at room temperature. All solvents were removed under reduced pressure. Treatment of the residue with EtOAc and saturated aqueous solution of Na_2CO_3 caused precipitation. The precipitate was collected, rinsed with a small amount of water, and dried under high vacuum to afford 1.91 mg (15.3% yield); ^{1}H NMR (400 MHz, $CDCl_3 + CD_3OD$): δ (ppm) 8.02 (d, J = 1.2 Hz, 1H), 7.88 (d, J = 3.6 Hz, 1H), 7.58 (m, 5H), 7.50 (d, J = 8 Hz, 2H), 7.42 (m, 3H), 7.32 (t, J = 7.6 Hz, 1H), 3.70 (s, 2H).

2-Biphenyl-4-yl-*N*-[5-(2-chloro-5-fluoropyrimidin-4-ylamino)-1- trityl-1*H*-indazol-3-yl]acetamide (**8**)

A mixture of **7** (0.36 mmol), trityl chloride (1.1 mmol), and triethylamine (1.1 mmol) in acetonitrile (60 mL) was stirred for 1 h. Additional trityl chloride (1.1 mmol), and triethylamine (1.1 mmol) were added and the mixture was stirred for 2 h. Acetonitrile was removed under reduced pressure, and the remaining residue was dissolved in ethyl acetate. The organic layer was washed with a 5% aqueous citric acid, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using dichloromethane/ethyl acetate (9:1, v/v) as eluent to give 125.6 mg of **8** in a yield of 49.1%; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.95 (d, J = 2.8 Hz, 1H), 7.89 (s, 1H), 7.59 (m, 5H), 7.50 (d, J = 7.8 Hz, 1H), 7.42 (m, 2H), 7.32 (t, J = 7.6 Hz, 1H), 7.28 (m, 17H), 3.68 (s, 2H).

2-Biphenyl-4-yl-*N*-{5-[2-(2-dimethylaminoethoxy)-5-fluoropyrimidin-4-ylamino]-1-trityl-1*H*-indazol-3-yl} acetamide

A mixture of **8** (0.16 mmol), sodium hydride (2.4 mmol), and 2-(dimethylamino)ethanol (0.64 mmol) in chlorobenzene (10 mL) was heated at reflux for 1 h. Chlorobenzene was removed under reduced pressure, and the remaining residue was dissolved in EtOAc. The organic layer was washed with a saturated aqueous solution of Na_2CO_3, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using methanol/dichloromethane (1:9, v/v) as eluent to give 48.5 mg in a yield of 38.5%; ^{1}H NMR (400 MHz, $CDCl_3 + CD_3OD$): δ (ppm) 7.95 (d, J = 2.8 Hz, 1H), 7.89 (s, 1H), 7.59 (m, 5H), 7.50 (d, J = 7.8 Hz, 1H), 7.42 (m, 2H), 7.32 (t, J = 7.6 Hz, 1H), 7.28 (m, 17H), 4.28 (t, J = 6.0 Hz, 2H), 3.71 (s, 2H), 2.62 (t, J = 5.6 Hz, 2H), 2.21 (s, 6H).

2-([1,1′-Biphenyl]-4-yl)-*N*-(5-((2-(2-(dimethylamino)ethoxy)-5-fluoropyrimidin-4-yl)amino)-1*H*-indazol-3-yl)acetamide (**9a**)

A mixture of 2-Biphenyl-4-yl-N-{5-[2-(2-dimethylaminoethoxy)-5-fluoropyrimidin-4-ylamino]-1-trityl-1H-indazol-3-yl} acetamide (0.06 mmol) and trifluoroacetic acid (0.5 mL) in dichloromethane (5 mL) was stirred at room temperature for 1 min. 1N Aqueous HCl (40 mL) was added and the aqueous layer was washed with hexane (30 mL). The aqueous layer was basified to pH 13 using 1 M aqueous NaOH solution and then extracted with ethyl acetate. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using chloroform/methanol/ammonia water (100:10:1, v/v) as eluent to give 96.4 mg of **9** in a yield of 96.4%; ^{1}H NMR (400 MHz, $CDCl_3 + CD_3OD$): δ (ppm) 8.02 (d, J = 1.2 Hz, 1H), 7.88 (d, J = 3.6 Hz, 1H), 7.58 (m, 5H), 7.50 (d, J = 8 Hz, 2H), 7.42 (m, 3H), 7.32 (t, J = 7.6 Hz, 1H), 4.31 (t, J = 6 Hz, 2H), 3.85 (s, 2H), 2.64 (t, J = 6 Hz, 2H), 2.23 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3 + CD_3OD$) : δ (ppm) 171.3, 159.9, 152.1, 143.5, 141.1, 140.6, 139.9, 139.7, 139.5, 139.1, 134.2, 130.6, 129.5, 128.6, 127.0, 126.7, 123.9, 116.4, 114.3, 110.1, 65.0, 57.3, 44.7, 42.3; MS (ESI +) m/z: 526.23 (M + 1)

2-([1,1′-Biphenyl]-4-yl)-*N*-(5-((2-(2-(diethylamino)ethoxy)-5-fluoropyrimidin-4-yl)amino)-1*H*-indazol-3-yl)acetamide (**9b**)

^{1}H NMR (400 MHz, $CDCl_3 + CD_3OD$): δ (ppm) 8.00 (d, J = 1.2 Hz, 1H), 7.91 (d, J = 3.6 Hz, 1H) 7.59 (d, J = 7.6 Hz, 4H), 7.53 (dd, J = 8.8 Hz, J = 2 Hz, 1H), 7.49 (d, J = 8 Hz, 2H), 7.42 (m, 3H), 7.31 (t, J = 7.6, 1H), 4.26 (t, J = 6 Hz, 2H), 3.83 (s, 2H), 2.70 (t, J = 6 Hz, 2H), 2.48 (q, J = 7.2 Hz, 4H), 0.91 (t, J = 7.2 Hz, 6H); MS (ESI +) m/z: 554.43 (M + 1)

2-([1,1′-Biphenyl]-4-yl)-*N*-(5-((2-(3-(dimethylamino)propoxy)-5-fluoropyrimidin-4-yl)amino)-1*H*-indazol-3-yl)acetamide (**9c**)

^{1}H NMR (400 MHz, $CDCl_3 + CD_3OD$): δ (ppm) 8.06 (d, J = 1.6 Hz, 1H), 7.91 (d, J = 3.6 Hz, 1H), 7.59 (m, 4H), 7.51 (m, 3H), 7.42 (m, 3H), 7.31 (t, J = 7.2, 1H), 4.17 (t, J = 6.4 Hz, 2H),

3.83 (s, 2H), 2.41 (m, 2H), 2.17 (s, 6H), 1.80 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) : δ (ppm) 171.4, 160.1, 143.3, 140.9, 140.6, 139.9, 139.7, 139.5, 139.1, 134.5, 130.7, 129.4, 128.5, 127.0, 126.8, 126.5, 123.8, 116.5, 114.1, 110.0, 65.7, 55.8, 43.7, 42.0, 26.1; MS (ESI+) m/z: 540.43 (M+1)

2-([1,1′-Biphenyl]-4-yl)-*N*-(5-((2-(3-(diethylamino)propoxy)-5-fluoropyrimidin-4-yl)amino)-1*H*-indazol-3-yl)acetamide (**9d**)

^{1}H NMR (400 MHz, $CDCl_3+CD_3OD$): δ (ppm) 7.97 (m, 1H), 7.88 (m, 1H) 7.65 (m, 1H) 7.59 (m, 4H), 7.49 (d, J=8 Hz, 2H), 7.42 (m, 3H), 7.35 (m, 1H), 4.22 (t, J=6 Hz, 2H), 3.85 (s, 2H), 2.61 (m, 2H), 2.54 (q, J=7.2 Hz, 4H), 1.86 (m, 2H), 1.02 (t, J=7.2 Hz, 6H); ^{13}C NMR (100 MHz, CD_3OD) : δ (ppm) 171.2, 160.1, 151.9, 143.4, 140.9, 140.6, 139.9, 139.7, 139.5, 139.1, 134.2, 130.6, 129.5, 128.6, 127.1, 126.7, 123.9, 116.4, 114.2, 110.1, 66.0, 49.0, 46.4, 42.3, 25.1, 10.0; MS (ESI+) m/z: 568.70 (M+1)

2-([1,1′-Biphenyl]-4-yl)-*N*-(5-((5-fluoro-2-(2-morpholinoethoxy)pyrimidin-4-yl)amino)-1*H*-indazol-3-yl)acetamide (**9e**)

^{1}H NMR (400 MHz, $CDCl_3+CD_3OD$): δ (ppm) 7.99 (m, 1H), 7.89 (d, J=4 Hz, 1H), 7.60 (m, 5H), 7.49 (d, J=8 Hz, 2H), 7.45 (t, J=4 Hz, 2H), 7.39 (m, 1H), 7.35 (m, 1H), 4.36 (t, J=6 Hz, 2H), 3.86 (s, 2H), 3.64 (m, 4H), 2.70 (t, J=6 Hz, 2H), 2.49 (m, 4H); MS (ESI+) m/z: 590.20 (M+23)

2-([1,1′-Biphenyl]-4-yl)-*N*-(5-((5-fluoro-2-((1-morpholinopropan-2-yl)oxy)pyrimidin-4-yl)amino)-1*H*-indazol-3-yl)acetamide (**9f**)

^{1}H NMR (400 MHz, $CDCl_3+CD_3OD$): δ (ppm) 7.94 (d, J=1.6 Hz, 1H), 7.88 (d, J=3.2 Hz, 1H), 7.59 (m, 5H), 7.50~7.33 (m, 6H), 5.16 (m, 1H), 3.86 (s, 2H), 3.54 (m, 4H), 2.60 (m, 1H), 2.38 (m, 5H), 1.29 (d, J=6.4, 3H); ^{13}C NMR (100 MHz, CD_3OD) : δ (ppm) 166.4, 156.0, 147.9, 139.3, 136.8, 136.6, 136.1, 136.0, 135.8, 135.1, 129.7, 126.3, 125.6, 123.4, 123.2, 122.9, 120.3, 110.9, 106.1, 66.8, 62.6, 59.4, 57.2, 49.8, 38.7, 14.4; MS (ESI+) m/z: 604.24 (M+23)

2-([1,1′-Biphenyl]-4-yl)-*N*-(5-((5-fluoro-2-((1-(4-methylpiperazin-1-yl)propan-2-yl)oxy)pyrimidin-4-yl)amino)-1*H*-indazol-3-yl)acetamide (**9g**)

^{1}H NMR (400 MHz, $CDCl_3+CD_3OD$): δ (ppm) 7.91 (d, J=1.2 Hz, 1H), 7.88 (d, J=3.2 Hz, 1H), 7.60 (m, 5H), 7.51~7.33 (m, 6H), 5.12 (m, 1H), 3.86 (s, 2H), 2.66~2.31 (m, 10H), 2.22 (s, 3H), 1.28 (m, 3H); MS (ESI+) m/z: 617.28 (M+23)

Biological assay

Cell growth inhibition assay were performed with a sulforhodanine B (SRB) assay. The SRB assay was carried out as previously described [13]. Briefly, the cells were plated in 96-well culture plates at a density of 3,000 cells/well in phenol red free-medium and allowed to attach for 10 h. After 24 h or 48 h treatment of compounds, culture media were removed. 0.07 mL of 0.4% (w/v) SRB (Sigma) in 1% acetic acid solution were added to each well and left at room temperature for 20 min. SRB was removed and the plates washed 5 times with 1% acetic acid before air drying. Bound SRB was solubilized with 0.2 mL of 10 mM unbuffered Tris-base solution (Sigma) and plates were left on a plate shaker for at least 10 min. Absorbance was read in a 96-well plate reader at 492 nm subtracting the background measurement at 620 nm. The test optical density (OD) value was defined as the absorbance of each individual well, minus the blank value ('blank' is the mean OD of the background control wells).

Quantification method

The IC_{50} values were determined by the analysis of the SRB assay results with the nonlinear regression using a four-parameter logistic equation shown below.

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(logIC_{50} - X) \times Hillslope}}$$

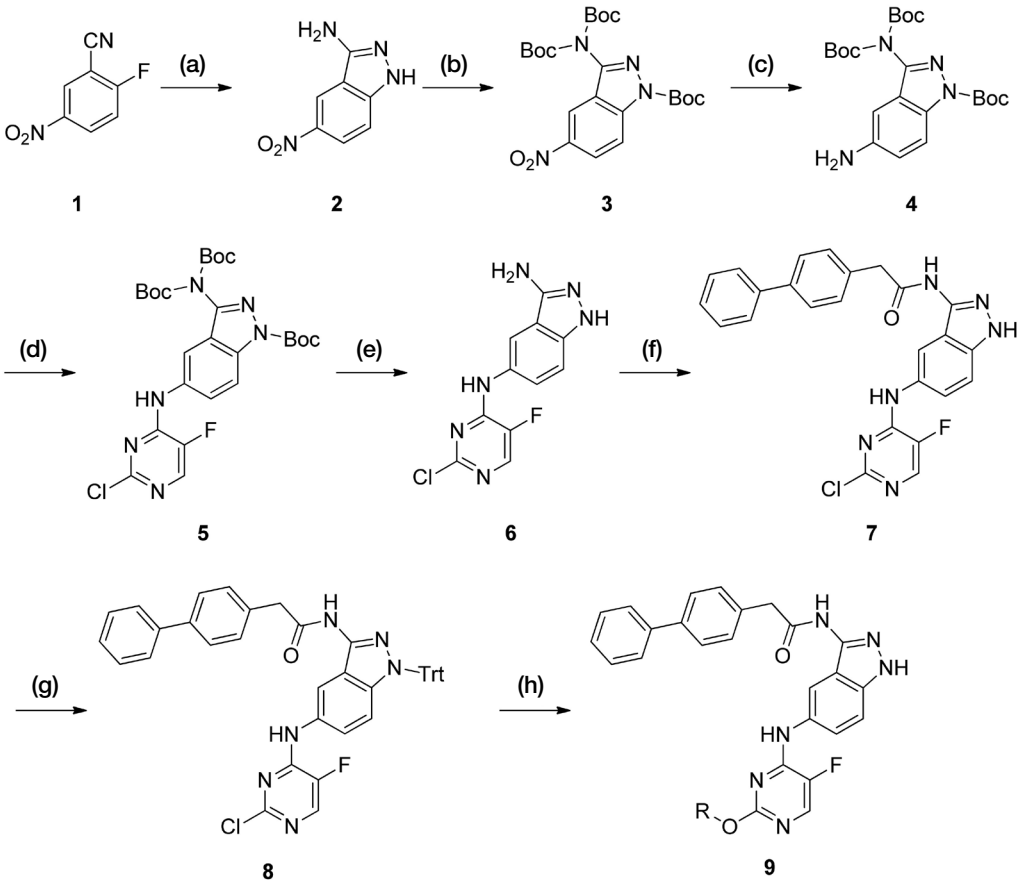
The docking studies of synthesized compounds were performed with the program "AutoDock Vina" [14]. The general functional form of the conformation-dependent part of the scoring function is

$$c = \sum_{i<j} f_{t_i t_j}(r_{ij}),$$

where each atom i is assigned a type t_i, and a symmetric set of interaction functions $f_{t_i t_j}$ of the interatomic distance r_{ij} should be defined.

Results and Discussion

3,5-Diaminoindazole derivatives were synthesized from 2-fluoro-5-nitrobenzonitrile as shown in Scheme 1. Various amines were introduced to the 5-fluoropyrimidine substituent at *N5* position of 3,5-diaminoindazole while keeping 4-biphenylacetyl group at *N3* position. After synthesis of 3-amino-5-nitro-



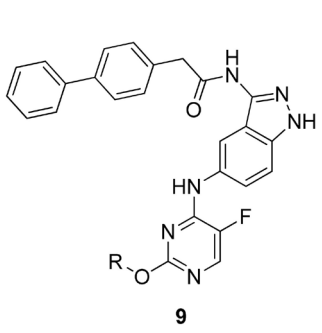
Scheme 1. Reagents and experimental conditions. (a) H_2NNH_2, *n*-BuOH, reflux, (b) $(Boc)_2O$, DMAP, Et_3N, THF, reflux, (c) Pd/C, H_2, MeOH, (d) 2,4-Dichloro-5-fluoropyrimidine, Et_3N, DMF, (e) TFA, DCM, (f) *p*-BiphenylCH_2COCl, THF, Et_3N, reflux, (g) TrtCl, Et_3N, CH_3CN, reflux, (h) i) ROH, Chlorobenzene, ii) TFA, DCM

indazole (**2**), protections of the amino groups of indazole with *tert*-butoxycarbonyl (Boc) group followed by a reduction of the nitro group at position 5 provided compound **4**. Substitutions of 2-chloro-5-fluoropyrimidinyl group at *N5* position and biphenylacetyl group at *N3* position of 3,5-diaminoindazole provided compound **7**. The amino groups were introduced to the 5-fluoropyrimidine substituent using alcohols containing various aminoalkyl groups.

The *in vitro* anti-proliferative activities of the synthesized compounds were evaluated by SRB assay against human cancer cell lines and human fibroblast cell. and the results are shown in Table 1. The nonlinear regression analyses provided the IC_{50} values (Table 1) and confidence intervals (Table 2) for the found IC_{50} values. A 95% confidence interval (CI) is a range of values that one can be 96% certain contains the best-fit values of IC_{50} determined by nonlinear regression.

The modification of substituent at *N5*-position of indazole was performed based on the previous results that the structure vof substituents at *N3* and *N5*-position of indazole had influenced on both potency and selectivity of compounds in the growth of cancer cell lines [15,16]. Five cancer cell lines, Caki (kidney), A549 (lung), HepG2 (liver), AMC-HN4 (head and neck), SNU484 (stomach), and human fibroblast cell were used to evaluate the effects of the synthesized compounds. 5-Fluoropyrimidinyl group was used to study the effects of substituent at *N5*-position of indazole on the growth inhibition of cancer cell lines. Each dimethylamino group, diethylamino group, morpholinyl group, and piperazinyl group was attached to the 5-fluoropyrimidinyl group using either ethyloxy or propyloxy linker. For the dimethylamino group, the cell growth inhibitory activities were decreased for all 5 cancer cell lines when the linker was changed from ethyloxy to propyloxy (**9a** vs. **9c**). However, the same trend was obtained only for A549 and AMC-HN4 when diethylamino group was used (**9b** vs. **9d**). The presence of methyl group in ethyloxy linker did not alter the inhibitory activity (**9e** vs **9f**). Also, there were no noticeable

Table 1. Anti-proliferative activity of the synthesized compounds against human cancer cell lines



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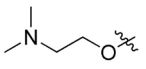
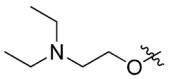
R: **9a**



9b



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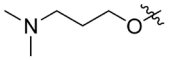

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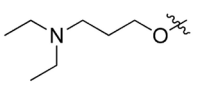
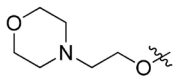
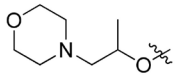


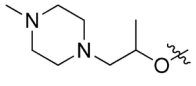
9e



9f



9g



	IC_{50} (μM)					
	Caki	A549	HepG2	AMC-HN4	SNU484	Fibro
9a	1.4	<0.37	<<0.1	<0.1	<0.37	>10
9b	3.3	0.8	<<0.1	<0.37	0.42	>10
9c	>10	2.7	0.24	1.0	1.1	>10
9d	3.2	2.1	ND	0.39	0.36	ND
9e	2.0	2.9	0.36	0.69	0.81	0.84
9f	1.3	2.3	0.51	0.72	0.86	0.76
9g	ND	5.4	0.38	0.65	0.76	0.72

ND: not determined

Table 2. Confidence interval (CI) of the calculated IC_{50} values

	95% CI (μM)					
	Caki	A549	HepG2	AMC-HN4	SNU484	Fibro
9a	1.1∼1.9	NA	NA	NA	NA	NA
9b	2.0∼5.1	0.6∼1.1	NA	NA	0.04∼0.65	NA
9c	NA	2.1∼3.3	0.05∼0.38	0.9∼1.1	0.5∼2.0	NA
9d	1.1∼21	1.4∼3.0	NA	0.31∼0.46	0.35∼0.37	NA
9e	0.8∼4.7	2.3∼3.7	0.22∼0.47	0.59∼0.78	0.70∼0.92	0.40∼1.37
9f	0.3∼3.0	1.7∼3.0	0.41∼0.60	0.60∼0.86	0.79∼0.95	0.25∼1.37
9g	NA	3.7∼7.6	0.31∼0.45	0.53∼0.77	0.60∼0.91	0.21∼1.33

NA: not available

activity difference between morpholinyl and piperazinyl group (**9f** vs. **9g**).

When non-cyclic amino groups were attached, the compounds could not inhibit the growth of fibroblast effectively compared to the cancer cell lines (**9a**, **9b**, **9c**). On the other hand, the growth of fibroblast was inhibited as much as other cancer cell lines when either morpholinyl or piperazinyl group was present (**9e**, **9f**, **9g**). In general, the synthesized inhibitors were more potent for HepG2, AMC-HN4, and SNU484 than Caki and A549. Amongst, compound **9a** and **9b** were very potent for HepG2 with IC_{50} much lower than 0.1 μM.

3-Acetamido-1*H*-indazole derivatives were reported as the inhibitors of various kinases such as glycogen synthase kinase 3 [17-20], cyclin dependent kinase 1 and 2 [15]. The docking studies of compound **9a** were performed with cyclin dependent kinase 2 (CDK2) and mitogen activated protein kinase 14 (MAPK14). The first reason of choosing CDK2 and MAPK14 was that overexpression of CDK2 [21] or MAPK14 were closely related with cancer progression [22]. The second, 3-(2-([1,1′-biphenyl]-4-yl)acetamido)-1*H*-indazole derivatives were reported as highly potent cyclin dependent kinase 2 (CDK2) inhibitor. The last, the fact that the expression level of MAPK14 in HepG2 is higher than both A549 and Caki could be one of the factors for cellular selectivity. Compound **9a** had been docked in to the ATP binding site of both CDK2 and MAPK14. The docking results are shown in Figure 2. Though it may need more studies such as enzymatic assay and cellular functional assay to confirm, the results of docking studies sug-

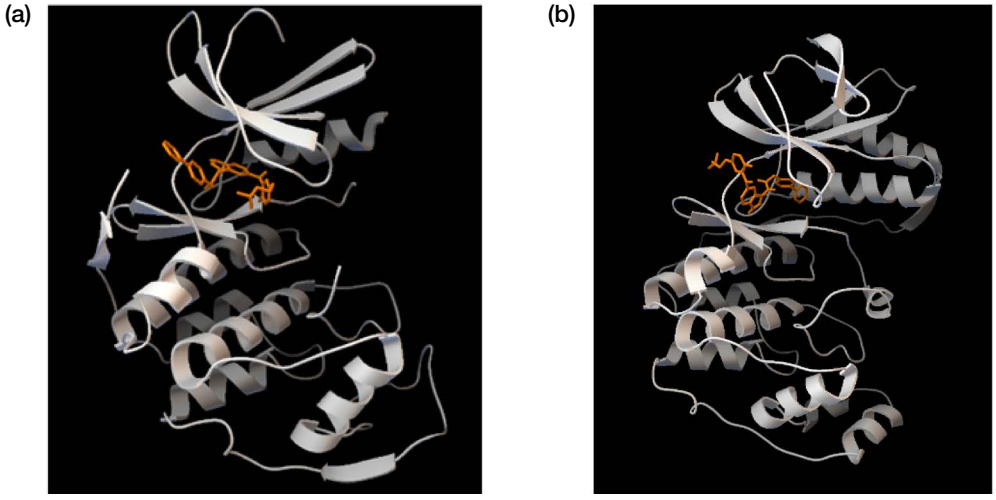


Fig. 2. Suggested binding mode of compound **9a** with CDK2 and MAPK14: (a) CDK2, (b) MAPK14.

gest the possibility that the inhibition of both CDK2 and MAPK14 can contribute to the growth inhibition of cancer cell lines.

In summary, we have designed and synthesized a series of *N3*-acyl-*N5*- pyrimidinyl-3,5-diaminoindazole derivatives, and evaluated their anti-proliferative activity against human cancer cell lines, Caki, A549, HepG2, AMC-HN4, and SNU484. The study of structure and activity relationship showed that the selectivity against cell lines could be achieved by modification of substituents at *N5*-pyrimidinyl group of 3,5-diaminoindazole. The results of high potency of compound **9a** and **9b** against HepG2 cell line suggest that *N3*-acyl-*N5*-pyrimidinyl-3,5-diaminoindazole analogues can be used as hits in the development of anticancer drug.

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