Medicinal chemistry

Zhou et al., Med chem 2015, 5:8
DOI: 10.4172/2161-0444.1000284

Research Article Open Access

Design, Synthesis and Biological Evaluation of Novel 1,3,5-triazines Derivatives as Potent Antitumor Agents

Zhixu Zhou, Yalu Zhang, Ning Yan, Yude Wang and Tiemin Sun*

Key Laboratory of Structure-Based Drug Design and Discovery, Shenyang Pharmaceutical University, Shenyang, PR China

Abstract

A series of 1,3,5-triazines derivatives were designed, synthesized and evaluated their biological activity. The preliminary investigation showed that most compounds displayed good to excellent potency against seven tested cancer cell lines as compared with ZSTK474. Compounds 5a, 7a and 7d were further examined for their inhibitory activity against PI3Kα kinase. The most promising compound 7d (PI3Kα half-maximal inhibitory concentration [IC_{50}] = 10.56 nM) showed remarkable cytotoxicity against H1975, A549, PC9, HCT116, BT549, CNE2 and SW480 cell lines with IC_{50} values of 2.83 μM, 4.62 μM, 0.29 μM, 3.92 μM, 0.56 μM, 3.53 μM and 1.27 μM, respectively. The structure-activity relationships (SARs) analyses will guide us to further refine the structure of 1,3,5-triazines derivatives to achieve optimum anticancer activity.

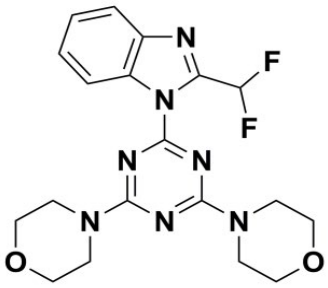
Keywords: Synthesis; ZSTK474; 1,3,5-triazines; PI3K inhibitors; Antitumor activity; Binding model

Introduction

Cancer is a major public health problem in the world [1,2]. Chemotherapy is still one of the primary modalities for the treatment of cancer. However, drug resistance and adverse side effects are still vital problems. This clearly underlines the urgent need for developing novel chemotherapeutic agents with more potent antitumor activities and reduced side effects [2].

The phosphoinositide 3-kinase (PI3K) pathway is a key signal transduction system that links oncogenes and multiple receptor classes to many essential cellular functions, and is perhaps the most commonly activated signalling pathway in human cancer [3]. PI3Ks are a family of three distinct classes (I, II and III) of lipid kinases that play key roles in cell and tissue physiology [4-6]. The three class-Ia PI3Ks (p110α/β/δ) and the sole class-Ib PI3Ks (p110γ) couple growth factor receptors and G-protein coupled receptors respectively to a wide range of downstream pathways [7-11]. The targeting of PI3K with small molecule inhibitors is one of the most promising new approaches to cancer treatment, and a number of programs to develop PI3K inhibitors are currently in progress with several inhibitors in clinical trial [12]. 2-(Difluoromethyl)-1-[4,6-di(4-morpholinyl)-1,3,5-triazin-2-yl]1H-benzimidazole (ZSTK474 1) (Figure 1) is an ATP competitive pan-PI3K class I enzyme inhibitor, and is reported to be in phase I/II clinical trial [13-15]. ZSTK474 has exhibited potent antitumor activity against human cancer xenografts without toxic effects in critical organs [16]. The crystal structure of 1 in complex with p110δ has been obtained and shows that the oxygen of one of the morpholino groups is positioned as a hydrogen bond acceptor from the hinge residue Val828, with the morpholino ring adopting a chair conformation. The benzimidazole group extends into the affinity pocket where its nitrogen acts as a hydrogen bond acceptor for the primary amine of Lys779. The difluoromethyl group points toward Pro758 in the upper wall of the hydrophobic affinity pocket [17]. It was found that the other morpholino group can be used as a modifiable group. Besides that, this conclusion is supported by 1 docked within the p110γ binding site (PDB ID code: 2CHX) (Figure 2) [12].

In this paper we explored the structure-activity relationships for this promising series, focusing initially on changes to one of the morpholinyls of ZSTK474 by different substituents, with one of the aims being the identification of suitable changes that might lead to improve its water solubility and pharmacology activity. Thus, a series of compounds were designed and the synthetic routes to these compounds were explored. The results discussed in this paper reflect our efforts in discovering new potential anticancer chemotherapeutic agents.



ZSTK474 (1)

Figure 1: Structure of ZSTK474.

Experimental Methods

Chemistry

A mass spectrum (MS) was taken in ESI mode on Agilent 1100 LC-MS (Agilent Technologies, USA). HR-TOF-MS data was obtained by using an Agilent Accurate-Mass-Q-TOF LC/MS 6520 instrument (Agilent Technologies, USA). ^{1}H NMR spectra was recorded on Bruker AVANCE-400 MHz NMR spectrometer (Bruker, Germany) with tetramethylsilane (TMS) as an internal standard. All materials were obtained from commercial suppliers and were used without further purification. Reactions' time and purity of the products were monitored by TLC on FLUKA silica gel aluminum cards (0.2 mm thickness) with

***Corresponding author:** Tiemin Sun, Key Laboratory of Structure-Based Drug Design and Discovery, Shenyang Pharmaceutical University, Shenyang, PR China, Tel: 86-13386838360; E-mail: suntiemin@126.com

Received July 21, 2015; **Accepted** August 07, 2015; **Published** August 11, 2015

Citation: Zhou Z, Zhang Y, Yan N, Wang Y, Sun T (2015) Design, Synthesis and Biological Evaluation of Novel 1,3,5-triazines Derivatives as Potent Antitumor Agents. Med chem 5: 345-350. doi: 10.4172/2161-0444.1000284

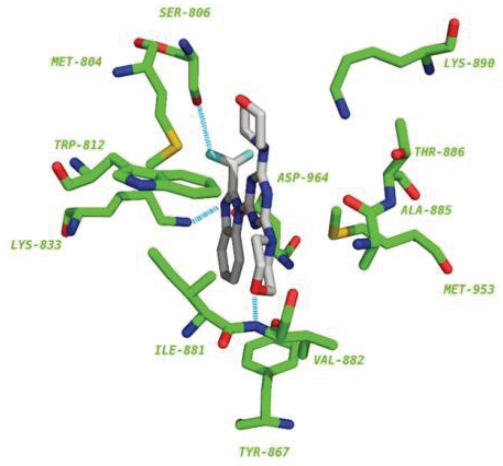


Figure 2: View of ZSTK474 docked in the ATP-binding site of PI3Kγ.

fluorescent indicator 254 nm. Column chromatography was run on silica gel (200-300 mesh) from Qingdao Ocean Chemicals (Qingdao, China).

(4,6-dichloro-1,3,5-triazin-2-yl)morpholine (3)

To a stirred solution of 2,4,6-trichloro-1,3,5-triazine (**2**) (18.44 g, 100 mmol) and Na_2CO_3 (10.60 g, 100 mmol) in H_2O (120 mL) was added dropwise a solution of morpholine in H_2O (25 mL) at 0 °C. The reaction mixture was stirred for 3 h at 0 °C. The resulting precipitate **3** was collected by filtration, washed with water and obtained white solid, yield: 18.54 g, 79%.

(4-chloro-6-(2-(difluoromethyl)-1*H*-benzo[*d*]imidaz ol-1-yl)-1,3,5-triazin-2-yl)morpholine (4)

A mixture of **3** (11.33 g, 48.2 mmol), 2-(difluoromethyl)-1*H*-benzo[*d*]imidazole (8.10 g, 48.2 mmol) and K_2CO_3 (6.66g, 48.2 mmol) in anhydrous DMF (150 mL) was stirred at room temperature for 6 h. The reaction mixture was poured into water (200 mL), the resulting precipitate 4 was collected by filtration, washed with water and obtained white powder, yield: 15.21 g, 86%.

General procedure for preparation of 5a-5h

A mixture of 4 (1.47 g, 4 mmol), Na_2CO_3 (1.27 g, 12 mmol) and primary amines or secondary amines (8 mmol) in THF (50 mL) was heated to reflux for about 1 h until completed (TLC control). The reaction mixture was poured into water, the water phase extracted with dichloromethane and the organic phase was separated, washed with brine, dried ($MgSO_4$), filtered and concentrated the crude product was purified by column chromatography on silica gel with a mixture of hexane/ethyl acetate (3:1) to afford 5a-5h as white powder.

4-(4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-6-(1H-imidazol-1-yl)-1,3,5-triazin-2-yl)-morpholine (5a): Yield: 78%; The theoretical mass of $[M+H]^+$ is 399.14151 amu. The high-resolution mass spectrum shows the $[M+H]^+$ at *m/z* = 398.77556 amu. ^1H NMR (400 MHz, $CDCl_3$) δ ppm 8.59 (s,1H), 8.38 (d, *J* = 8.1 Hz, 1H), 7.90 (d, *J* = 7.9 Hz, 1H), 7.83 (s, 1H), 7.51 (t, J_{HF} = 53.2 Hz, 1H), 7.49-7.41 (m, 2H), 7.21 (s, 1H), 4.07-3.97 (m, 4H), 3.91-3.83 (s, 4H).

4-(4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-6-(piperidin-1-yl)-1,3,5-triazin-2-yl) morpholine (5b): Yield: 73%; The theoretical mass of $[M+H]^+$ is 416.19321 amu. The high-resolution mass spectrum shows the $[M+H]^+$ at *m/z* = 416.19699 amu. ^1H NMR (400 MHz, $CDCl_3$) δ ppm 8.36 (d, *J* = 8.0 Hz, 1H), 7.89 (d, *J* = 7.8 Hz, 1H), 7.62 (t, J_{HF} = 53.60Hz, 1H), 7.47-7.35 (m, 2H), 3.90-3.77 (m, 12H), 1.72 (brs, 2H), 1.66 (brs, 4H).

4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-N,N-dimethyl-6-morpholino-1,3,5-triazin-2-amine (5c): Yield: 70%; MS (ESI) *m/z*: 376.1 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ ppm 8.41 (d, *J* = 7.72 Hz, 1H), 7.88 (d, *J* = 8.1 Hz, 1H), 7.65 (t, J_{HF} = 53.52Hz, 1H), 7.45-7.35 (m, 2H), 3.87 (t, *J* = 4.21 Hz, 4H), 3.78 (t, *J* = 4.21 Hz, 4H), 3.23 (s, 3H), 3.18 (s, 3H).

4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-N-isopropyl-6-morpholino-1,3,5-triazin-2-amine (5d): Yield: 77%; MS (ESI) *m/z*: 390.2 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ ppm 8.43 (dd, *J* = 8.40 Hz, 1H), 7.89 (t, *J* = 8.08 Hz, 1H), 7.68 (t, J_{HF} = 53.64 Hz, 1H), 7.49-7.35 (m, 2H), 4.23 (m, 1H), 3.88 (brs, 4H), 3.80 (brs, 4H), 1.30 (m, 6H).

4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-N,N-diethyl-6-morpholino-1,3,5-triazin-2-amine (5e): Yield: 79%; The theoretical mass of $[M+H]^+$ is 404.19321 amu. The high-resolution mass spectrum shows the $[M+H]^+$ at *m/z* = 404.23557 amu. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 8.37 (d, *J* = 7.9 Hz, 1H), 7.82 (d, *J* = 7.8 Hz, 1H), 7.74 (t, J_{HF} = 53.08 Hz, 1H), 7.49-7.34 (m, 2H), 3.81-3.65 (m, 8H), 3.64-3.52 (m, 4H), 1.22-1.13 (m, 6H).

3-((4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-yl)amino)propan-1-ol (5f): Yield: 48%; The theoretical mass of $[M+H]^+$ is 406.17248 amu. The high-resolution mass spectrum shows the $[M+H]^+$ at *m/z* = 406.17556 amu. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 8.36 (d, *J* = 8.32 Hz, 1H), 7.84 (t, *J* =7.24 Hz, 1H), 7.74 (t, J_{HF} = 52.88 Hz, 1H), 7.47-7.37 (m, 2H), 4.55 (dt, *J* = 19.4 Hz, 2H), 3.76 (brs, 4H), 3.69 (brs, 4H), 3.52-3.51 (m, 2H), 1.78-1.69 (m, 2H).

N-(4-chlorophenyl)-4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-amine (5g): Yield: 78%; The theoretical mass of $[M+H]^+$ is 458.12294 amu. The high-resolution mass spectrum shows the $[M+H]^+$ at *m/z* = 458.17977 amu; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 10.09 (s, 1H), 8.61 (s, 1H), 8.05-7.92 (m, 1H), 7.84 (d, *J* = 8.0 Hz,1H), 7.73 (brs, 2H), 7.56-7.31 (m, 4H), 3.81 (brs, 4H), 3.72 (brs, 4H).

N-(4-bromophenyl)-4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-amine (5h): Yield: 78%; The theoretical mass of $[M+H]^+$ is 504.07243 amu. The high-resolution mass spectrum shows the $[M+H]^+$ at *m/z* = 504.13928 amu;^1H NMR (400MHz, DMSO-d_6) δ ppm 10.09 (s, 1H), 8.59 (brs, 1H), 8.05-7.92 (m, 1H), 7.82 (d, *J* = 8.0 Hz, 1H), 7.67 (brs, 2H), 7.52-7.46 (m, 2H), 7.48-7.36 (m, 2H), 3.79 (s, 4H), 3.71 (s, 4H).

4-(4-(2-(difluoromethyl)-1*H*-benzo[*d*]imidazol-1-yl)-6-(piperazin-1-yl)-1,3,5-triazin-2-yl)morpholine (5i)

A mixture of 4 (1.47 g, 4 mmol), Na_2CO_3 (1.27 g, 12 mmol) and BOC-piperazine (1.49 g, 8 mmol) in THF (50 mL) was heated to reflux for 1 h. The reaction mixture was poured into water, the water phase extracted with dichloromethane and the organic phase was separated, washed with brine, dried ($MgSO_4$), filtered and concentrated to afford tert-butyl-4-(4-(2-(difluoromethyl)-1*H*-benzo[*d*]imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-yl)piperazine-1-carboxylate as white powder, yield: 0.83 g, 40%.

A solution of tert-butyl-4-(4-(2-(difluoromethyl)-1*H*-benzo[*d*]imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-yl)piperazine-1-carboxylate (0.80 g, 1.55 mmol) in DCM:TFA (1:1, 15 mL) was stirred

at room temperature for 30 minutes. The mixture was evaporated under reduced pressure, diluted with H_2O, neutralized with 2 mol L^{-1} NaOH, the resulting precipitate **5i** was collected by filtration, washed with water and obtained white powder, yield: 0.62 g, 97%. MS (ESI) *m/z*: 417.2 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 8.33 (d, *J* = 8.0 Hz, 1H), 7.84 (d, *J* = 8.3 Hz, 1H), 7.72 (t, J_{HF} = 53.8, 1H), 7.53-7.38 (m, 2H), 7.42 (s, 1H), 3.82-3.77 (m, 4H), 3.77-3.72 (m, 4H), 3.31 (brs, 4H), 2.78 (brs, 4H).

2-chloro-1-(4-(4-(2-(difluoromethyl)-1*H*-benzo[*d*] imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-yl)piperazin-1-yl)ethan-1-one (6)

To a stirred solution of 5i (0.83 g, 2 mmol) and TEA (0.56 mL, 4 mmol) in DCM (60 mL) was added dropwise chloroacetyl chloride (0.32 mL, 4 mmol) at 0 °C. The reaction mixture was stirred for 3 h at room temperature. The reaction mixture was poured into water (40 mL), the water phase extracted with dichloromethane (3 × 25 mL) and the organic phase was separated, washed with brine, dried ($MgSO_4$), filtered and concentrated to afford 6 as white powder, yield: 0.79 g, 80%.

General procedure for preparation of 7a-7e

A mixture of 6 (0.70 g, 1.42 mmol), KI (0.01g, 0.06 mmol), Na_2CO_3 (0.31 g, 2.84 mmol) and primary amines or secondary amines (2.84 mmol) in THF (50 mL) was heated to reflux for about 1 h until completed (TLC control). The reaction mixture was poured into water, the water phase extracted with dichloromethane and the organic phase was separated, washed with brine, dried ($MgSO_4$), filtered and concentrated the crude product was purified by column chromatography on silica gel with a mixture of hexane/ethyl acetate (3:1) to afford 7a-7e as white powder.

1-(4-(4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-yl)piperazin-1-yl)-2-(1H-imidazol-1-yl)ethan-1-one (7a): Yield: 85%; MS (ESI) *m/z*: 524.22 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 8.34 (s, 1H), 7.85 (s, 1H), 7.75 (s, 1H), 7.66-7.54 (brs, 1H), 7.53-7.37 (m, 2H), 7.09 (s, 1H), 6.90 (s, 1H), 5.10 (s, 2H), 3.95-3.64 (m, 16H).

1-(4-(4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-yl)piperazin-1-yl)-2-(piperidin-1-yl) ethan-1-one (7b): Yield: 75%; MS (ESI) *m/z*: 541.27 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ 8.33 (d, *J* = 7.9 Hz, 1H), 7.89 (d, *J* = 7.7 Hz, 1H), 7.57 (t, J_{HF} = 53.20 Hz, 1H), 7.48-7.38 (m, 2H), 3.95-3.71 (m, 18H), 2.45 (br s, 4H), 1.64-1.44 (m, 6H).

1-(4-(4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-yl)piperazin-1-yl)-2-(4-methylpiperidin-1-yl)ethan-1-one (7c): Yield: 75%; MS (ESI) *m/z*: 555.29 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 8.38 (d, *J* = 8.0 Hz, 1H), 7.89 (d, *J* = 7.7 Hz, 1H), 7.78 (t, J_{HF} = 52.60 Hz, 1H), 7.51 (m, 2H), 3.95-3.60 (m, 18H), 3.19 (s, 2H), 2.84 (d, *J* = 10.5 Hz, 2H), 2.05 (d, *J* = 12.5 Hz, 2H), 1.58 (d, *J* = 11.3 Hz, 2H), 1.34 (s, 1H), 0.91 (s, 3H).

1-(4-(4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-yl)piperazin-1-yl)-2-(4-methylpiperazin-1-yl)ethan-1-one (7d): Yield: 74%; MS (ESI) *m/z*: 556.28 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ 8.33 (d, *J* = 7.8 Hz, 1H), 7.89 (d, *J* = 7.6 Hz, 1H), 7.56 (t, J_{HF} = 53.50 Hz,1H), 7.47-7.37 (m, 2H), 3.95-3.66 (m, 18H), 2.58 (brs, 8H), 2.30 (s, 3H).

1-(4-(4-(2-(difluoromethyl)-1H-benzo[d]imidazol-1-yl)-6-morpholino-1,3,5-triazin-2-yl)piperazin-1-yl)-2-((2-hydroxyethyl) amino)ethan-1-one (7e): Yield: 75%; The theoretical mass of $[M+H]^+$ is 518.23614 amu. The high-resolution mass spectrum shows the $[M+H]^+$ at *m/z* =518.23928 amu; ^1H NMR (400 MHz, $CDCl_3$) δ 8.32 (d, *J* = 7.7 Hz, 1H), 7.89 (d, *J* = 7.8 Hz, 1H), 7.55 (t, J_{HF} = 53.40 Hz, 1H), 7.48-7.38 (m, 2H), 3.98-3.59 (m, 20H), 2.88 (s, 2H).

Pharmacology

MTT assay *in vitro:* The anti-proliferative activities of compounds 5a-5i and 7a-7e were evaluated against H1975, A549, PC9, HCT116, BT549, CNE2 and SW480 cell lines using the standard MTT assay *in vitro*, with ZSTK474 as the positive control. The cancer cell lines were cultured in minimum essential medium (MEM) supplement with 10 % fetal bovine serum (FBS). Approximate 4 × 10^3 cells, suspended in MEM medium, were plated onto each well of a 96-well plate and incubated in 5% CO_2 at 37 °C for 24 h. The tested compounds at the indicated final concentrations were added to the culture medium and the cell cultures were continued for 72 h. Fresh MTT was added to each well at a terminal concentration of 5 mg/mL, and incubated with cells at 37 °C for 4 h. The formazan crystals were dissolved in 100 mL DMSO each well, and the absorbency at 492 nm (for absorbance of MTT formazan) and 630 nm (for the reference wavelength) was measured with an ELISA reader. All compounds were tested three times in each of the cell lines. The results expressed as IC_{50} (inhibitory concentration 50%) were the averages of three determinations and calculated by using the Bacus Laboratories Incorporated Slide Scanner (Bliss) software.

PI3Kα kinase assay: The PI3Kα kinase activity was evaluated using homogeneous time-resolved fluorescence (HTRF) assays as previously reported protocol [18,19]. Briefly, 20 μg/mL poly (Glu, Tyr) 4:1 (Sigma) was preloaded as a substrate in 384-well plates. Then 50 μL of 10 mM ATP (Invitrogen) solution diluted in kinase reaction buffer (50 mM HEPES, pH 7.0, 1 mM DTT, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.1% NaN_3) was added to each well. Various concentrations of compounds were diluted in 10 μL of 1% DMSO (v/v), with blank DMSO solution as the negative control. The kinase reaction was initiated by the addition of purified tyrosine kinase proteins diluted in 39 μL of kinase reaction buffer solution. The incubation time for the reactions was 30 min at 25 °C and the reactions were stopped by the addition of 5 μL of Streptavidin-XL665 and 5 μL Tk Antibody Cryptate working solution to all of wells. The plate was read using Envision (Perkin Elmer) at 320 nm and 615 nm. IC_{50} values were calculated from the inhibition curves.

Results and Discussions

Chemistry

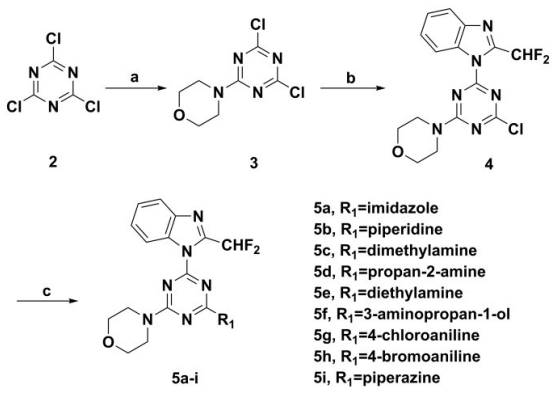
The general route for the synthesis of 1,3,5-triazines compounds 5a-i is depicted in Scheme 1. The synthesis of 4-(4-substituent-6-(2-(difluoromethyl)-1*H*-benzo[*d*]imidazol-1-yl)-1,3,5-triazin-2-yl) morpholine (5a-i) proceeded from a common starting material 2,4,6-trichloro-1,3,5-triazine (2) [12]. Reaction of 2 with morpholine gave 4-(4,6-dichloro-1,3,5-triazin-2-yl)morpholine (3) at low temperature [20]. 3 was subjected to 2-(difluoromethyl)-1*H*-benzo[*d*] imidazole attack on one chlorine atom at room temperature obtained important intermediate 4-(4-chloro-6-(2-(difluoromethyl)-1*H*-benzo[*d*]imidazol-1-yl)-1,3,5-triazin-2-yl)morpholine (4) [21]. The resulting compounds 5a-i were achieved by addition of different primary amines or secondary amines in THF under reflux, since the reaction proceeds under mild reaction conditions which compared to classical methods [12].

In order to further improve its pharmacology activity, we designed and synthesized other target compounds 7a-e based on the structure of compound 5i. The preparation of 4-(4-(2-(difluoromethyl)-1*H*-benzo[*d*]imidazol-1-yl)-6-(piperazin-1-yl)-1,3,5-triazin-2-yl)

morpholine derivatives 7a-e is outlined in Scheme 2. Our starting material, compound 5i, was readily obtained in Scheme 1. On the next step 6 was readily obtained by substitution of 5i secondary amine group with chloroacetyl chloride in ice-salt bath [22]. The resulting compounds 7a-e were achieved by addition of different primary amines or secondary amines in THF under reflux.

In vitro cytotoxicity and structure-activity relationships

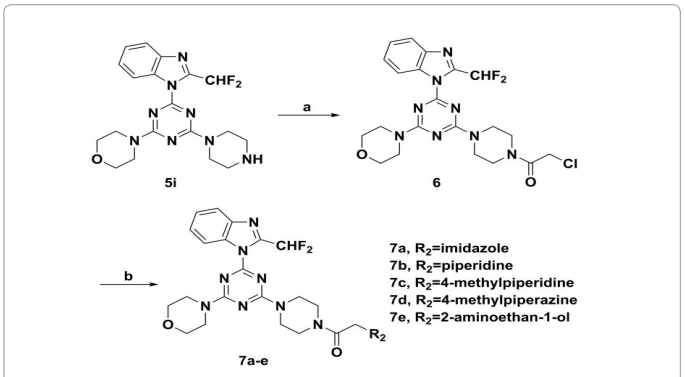
The cytotoxicity of the target compounds were evaluated against cancer cells lines H1975 (human non-small cell lung cancer), A549 (human lung adenocarcinoma epithelial), PC9 (human non-small cell lung cancer), HCT116 (human colcnic carcinoma), BT549 (human breast cancer), CNE2 (human nasopharyngeal carcinoma) and SW480 (colon cancer) by using MTT assay with ZSTK474 as the positive control. The results expressed as IC_{50} values are summarized in Table 1 and Table 2. The IC_{50} values are the average of at least three independent experiments.



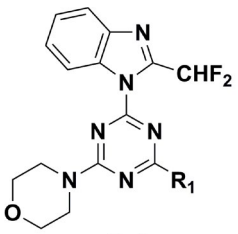
Scheme 1: Reactions and conditions: (a) Na_2CO_3, H_2O, 0 °C, 3 h; (b) 2-(difluoromethyl)-1*H*-benzo[*d*]imidazole, K_2CO_3, DMF, 2 h, r.t.; (c) Na_2CO_3, THF, primary/ secondary amines, reflux, 1 h.

As illustrated in Table 1 and Table 2, all target compounds 5a-5i and 7a-7e showed moderate to significant cytotoxic activities against the different cancer cell lines. Most of these compounds were more potent than ZSTK474 against one or more cancer cell lines. The IC_{50} value of the most promising compound 7d was 2.83 μM, 4.62 μM, 0.29 μM, 3.92 μM, 0.56 μM, 3.53 μM and 1.27 μM against H1975, A549, PC9, HCT116, BT549, CNE2 and SW480 cell lines, respectively.

Preliminary structure-activity relationships (SARs) indicated that substitute fatty amine for one of the morpholinyls of ZSTK474 leaded to a negative effect. For example, compounds 5b (R_1 = piperidine), 5c (R_1 = dimethylamine), 5d (R_1 = propan-2-amine) and 5e (R_1 = diethylamine) demonstrated low potency. The activity of compound 5e was higher than compound 5c suggesting that increases the length of the carbon chains of fatty amine had a positive effect. In contrast, the morpholinyl group was replaced with 4-substituted aniline showed higher selectivity against SW480 cell than against the other six cell lines, such as compounds 5g and 5h. Compound 5h with 4-bromo group displayed higher potency than 5g with 4-chloro group. Compounds 7a-7e showed higher activity in most of tested cell lines than 5a-5i indicated that the inserted N-acetylpiperazine into triazin group



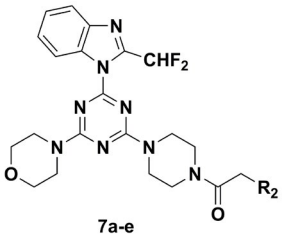
Scheme 2: Reactions and conditions: (a) TEA, chloroacetyl chloride, DCM, 3 h, r.t.; (b) Na_2CO_3, KI, THF, primary/ secondary amines, reflux, 1 h.



Compd.	R_1	IC_{50} (μM) ± SD[a]						
		H1975	A549	PC9	HCT116	BT549	CNE2	SW480
5a	imidazole	4.24±0.41	11.27±1.20	4.89±0.51	**3.18±0.33**	3.27±0.28	**1.40±0.16**	54.00±5.89
5b	piperidine	21.32±2.08	60.27±5.75	11.92±1.26	11.83±1.30	5.92±0.61	7.09±0.67	**3.09±0.29**
5c	dimethylamine	15.94±1.63	>100	26.43±2.88	15.32±1.48	5.98±0.53	7.83±0.72	15.87±1.94
5d	propan-2-amine	18.72±1.72	>100	13.09±1.25	**5.67±0.50**	5.88±0.57	6.71±0.63	**6.62±0.59**
5e	diethylamine	4.27±0.45	35.84±3.27	8.49±0.83	27.70±2.86	2.00±0.18	4.14±0.37	14.46±1.42
5f	3-aminopropan-1-ol	**3.32±0.30**	21.70±2.08	6.71±0.68	15.44±1.52	17.85±1.71	**1.06±0.09**	**3.52±0.36**
5g	4-chloroaniline	22.42±2.16	78.17±8.23	22.41±2.35	24.68±2.56	8.91±0.82	9.52±0.91	**6.11±0.58**
5h	4-bromoaniline	14.56±1.32	29.03±3.14	58.14±5.63	26.69±2.74	9.97±1.03	6.40±0.61	**0.19±0.02**
5i	piperazine	94.20±9.65	39.89±3.76	17.50±1.68	11.77±1.33	21.86±2.27	12.81±1.16	15.04±1.46
ZSTK474[b]		3.37±0.31	10.60±1.14	0.25±0.02	6.42±0.62	0.72±0.06	2.84±0.27	8.17±0.83

Bold values show the IC_{50} values of target compounds lower than the values of positive control. [a] IC_{50}: concentration of the compound (μM) producing 50% cell growth inhibition after 72 h of drug exposure, as determined by the MTT assay. Each experiment was carried out in triplicate.[b] Used as the positive control.

Table 1: Structures and cytotoxicity of compounds **5a-i** against H1975, A549, PC9, HCT116, BT549, CNE2 and SW480 cell lines.



7a-e

Compd.	R_2	IC_{50} (μM) ± SD[a]						
		H1975	A549	PC9	HCT116	BT549	CNE2	SW480
7a	imidazole	3.62±0.34	**7.06±0.66**	0.77±0.07	**3.37±0.30**	2.53±0.24	3.81±0.35	28.19±3.04
7b	piperidine	10.14±0.92	23.93±2.17	4.51±0.40	8.09±0.74	1.41±0.13	3.29±0.35	**2.31±0.21**
7c	4-methylpiperidine	9.32±1.03	19.46±1.86	3.38±0.31	**4.54±0.47**	1.16±0.11	3.88±0.26	**2.13±0.18**
7d	4-methylpiperazine	**2.83±0.26**	**4.62±0.34**	0.29±0.02	**3.92±0.36**	**0.56±0.06**	3.53±0.23	**1.27±0.14**
7e	2-aminoethan-1-ol	3.76±0.39	17.86±1.82	0.84±0.09	15.37±1.61	0.81±0.07	**2.25±0.20**	9.52±0.91
ZSTK474[b]		3.37±0.31	10.60±1.14	0.25±0.02	6.42±0.62	0.72±0.06	2.84±0.27	8.17±0.83

Bold values show the IC_{50} values of target compounds lower than the values of the positive control. [a] IC_{50}: concentration of the compound (μM) producing 50% cell growth inhibition after 72 h of drug exposure, as determined by the MTT assay. Each experiment was carried out in triplicate.[b] Used as the positive control.

Table2: Structures and cytotoxicity of compounds **7a-e** against H1975, A549, PC9, HCT116, BT549, CNE2 and SW480 cell lines.

Compd.	IC_{50} on PI3Kα (nM)
5a	53.84
7a	21.72
7d	10.56
ZSTK474	7.15

Table 3: PI3Kα kinase activity of **5a**, **7a**, **7d** and ZSTK474 *in vitro*

and R_2 exhibited an obvious positive effect on the cytotoxic activity. Among them, 4-methylpiperazine group of R_2 was the most favorable substituent for activity.

In vitro enzymatic assays

As shown in Table 3, the three tested compounds exhibited excellent PI3Kα enzymatic potency, indicating that the inhibition of PI3K may be a mechanism for the antitumor effect. Compound 7d showed the most potent activity with an IC_{50} value of 10.56 nM, which was comparable to that of the positive control, ZSTK474 (IC_{50} = 7.15 nM), indicating that this compound deserves further study with regard to its application in the treatment of cancer.

Binding model analysis

To further elucidate the binding mode of compounds, a detail docking analysis was performed. In our study, the crystal structure of p110γ was selected as the docking model (PDB ID code: 2CHX). The docking simulation was conducted using Glide XP (Schrödinger 2014), since Glide uses a hierarchical series of filters to search for possible locations of the ligand in the active-site region of the receptor. The shape and properties of the receptor were represented on a grid by several different sets of fields that provide progressively more accurate scoring of the ligand poses. The image files were generated using Accelrys DS visualizer 4.0 system. The binding model was exemplified by the interaction of compound 7d with p110γ (Figure 3). Ten conformations were generated. In order to take into account protein flexibility, the conformation with the best score was selected. The dashed green lines represent hydrogen bonds. Furthermore, the distance between ligands and proteins were listed, and they all less than 2.60 Å. Compared with the binding model of ZSTK474 (Figure 2) [12], the same trio of hydrogen bonding interactions between morpholine oxygen and Val882, benzimidazole 3-nitrogen and the amino groups of Lys833 and one of the fluorine atom and SER806 were observed. Moreover, three other hydrogen bonds between the other fluorine atom and SER807,

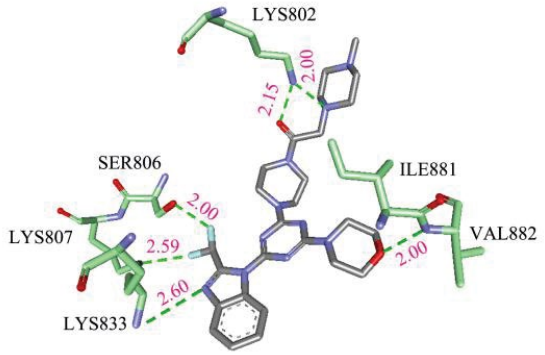


Figure 3: The PI3Kγ active site in complex with compound 7d. The H-bond interaction was shown in green dotted lines.

the oxygen atom of carbanyl group and LYS802 and the nitrogen atom of 4-methylpiperazine and LYS802 were also detected.

Conclusion

In summary, a novel series of (4-substituent-6-(2-(difluoromethyl)-1H-benzo[*d*]imidazol-1-yl)-1,3,5-triazin-2-yl)morpholine derivatives were designed based on ZSTK474. All the target compounds 5a-5i, 7a-7e were synthesized and screened for their cytotoxicity against seven human cancer cell lines (H1975, A549, PC9, HCT116, BT549, CNE2 and SW480) by standard MTT assays. The pharmacological results indicated that most compounds exhibited moderate to excellent activity. The preliminary SARs showed that substituted N-acetylpiperazine can improve cytotoxic activity. Moreover, this encouraging research provides a valuable leading compound 7d with IC_{50} values of 2.83, 4.62, 0.29, 3.92, 0.56, 3.53 and 1.27 μM against tested cell lines respectively and highlights the potential for further development of 1,3,5-triazine derivatives. Further SARs studies and mechanism of action of these compounds are in progress, and the results will be reported in the future.

Acknowledgements

This work was supported by the Program for Innovative Research Team of the Ministry of Education and Program for Liaoning Innovative Research Team in University.

References

1. Siegel R, Ma J, Zou Z, Jemal A (2014) Cancer statistics, 2014. CA Cancer J Clin 64: 9-29.
2. Mohareb RM, El-Sayed NN, Abdelaziz MA (2012) Uses of cyanoacetylhydrazine in heterocyclic synthesis: novel synthesis of pyrazole derivatives with anti-tumor activities. Molecules 17: 8449-8463.
3. Liu P, Cheng H, Roberts TM, Zhao JJ (2009) Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov 8: 627-644.
4. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2: 489-501.
5. Cantley LC (2002) The phosphoinositide 3-kinase pathway. Science 296: 1655-1657.
6. Shepherd PR (2005) Mechanisms regulating phosphoinositide 3-kinase signalling in insulin-sensitive tissues. Acta Physiol Scand 183: 3-12.
7. Shepherd PR, Withers DJ, Siddle K (1998) Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem J 333 : 471-490.
8. Liu P, Cheng H, Roberts TM, Zhao JJ (2009) Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov 8: 627-644.
9. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B (2010) The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol 11: 329-341.
10. Rewcastle GW, Gamage SA, Flanagan JU, Kendall JD, Denny WA, et al. (2013) Synthesis and biological evaluation of novel phosphatidylinositol 3-kinase inhibitors: Solubilized 4-substituted benzimidazole analogs of 2-(difluoromethyl)-1-[4, 6-di (4-morpholinyl)-, 3, 5-triazin-2-yl]-1H-benzimidazole (ZSTK474). Eur J Med Chem 64: 137-147.
11. Knight ZA, Gonzalez B, Feldman ME, Zunder ER, Goldenberg DD, et al. (2006) A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. Cell 125: 733-747.
12. Rewcastle GW, Gamage SA, Flanagan JU, Frederick R, Denny WA, Baguley BC, et al. (2011) Synthesis and biological evaluation of novel analogues of the pan class I phosphatidylinositol 3-kinase (PI3K) inhibitor 2-(difluoromethyl)-1-[4, 6-di (4-morpholinyl)-, 3, 5-triazin-2-yl]-1 H-benzimidazole (ZSTK474). J Med Chem 54: 7105-7126.
13. Kong D, Yamori T (2007) ZSTK474 is an ATP-competitive inhibitor of class I phosphatidylinositol 3 kinase isoforms. Cancer Sci 98: 1638-1642.
14. Joshi MC, Kumar K, Kumar V1 (2014) Potent phosphatidylinositol 3-kinase inhibitors and their biology. Curr Drug Discov Technol 11: 113-126.
15. Zhu J, Hou T, Mao X (2015) Discovery of selective phosphatidylinositol 3-kinase inhibitors to treat hematological malignancies. Drug Discov Today .
16. Yaguchi S, Fukui Y, Koshimizu I, Yoshimi H, Matsuno T, et al. (2006) Antitumor activity of ZSTK474, a new phosphatidylinositol 3-kinase inhibitor. J Natl Cancer Inst 98: 545-556.
17. Berndt A, Miller S, Williams O, Le DD, Houseman BT, et al. (2010) The p110 [delta] structure: mechanisms for selectivity and potency of new PI (3) K inhibitors. Nat Chem Biol 6: 117-124.
18. Kong D, Dan S, Yamazaki K, Yamori T (2010) Inhibition profiles of phosphatidylinositol 3-kinase inhibitors against PI3K superfamily and human cancer cell line panel JFCR39. Eur J Cancer 46: 1111-1121.
19. Dugar S, Hollinger FP, Kuila B, Arora R, Sen S, et al. (2015) Synthesis and evaluation of pyrrolotriazine based molecules as PI3 kinase inhibitors. Bioorg Med Chem Lett 25: 3142-3146.
20. Miller MS, Pinson JA, Zheng Z, Jennings IG, Thompson PE (2013) Regioselective synthesis of 5- and 6-methoxybenzimidazole-,3,5-triazines as inhibitors of phosphoinositide 3-kinase. Bioorg Med Chem Lett 23: 802-805.
21. Pinson JA, Zheng Z, Miller MS, Chalmers DK, Jennings IG, et al. (2013) L-Aminoacyl-triazine derivatives are isoform-selective PI3KÎ² inhibitors that target non-conserved Asp862 of PI3KÎ² ACS Med Chem Lett 4: 206-210.
22. Van Dort ME, Galbán S, Wang H, Sebolt-Leopold J, Whitehead C, et al. (2015) Dual inhibition of allosteric mitogen-activated protein kinase (MEK) and phosphatidylinositol 3-kinase (PI3K) oncogenic targets with a bifunctional inhibitor. Bioorg Med Chem 23: 1386-1394.