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Synthesis and evaluation of novel 2,4-diaminopyrimidines bearing a sulfoxide moiety as anaplastic lymphoma kinase (ALK) inhibition agents

Feng Wu [a], Han Yao [a], Wei Li [a], Niuniu Zhang [c], Yangyang Fan [b], Albert S.C. Chan [a], Xingshu Li [a,*], Baijiao An [b,*]

[a] School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou 510006, PR China
[b] School of Pharmaceutical Sciences, Binzhou Medical University, Yantai, Shandong Province 264003, PR China
[c] School of Pharmaceutical Sciences, Guilin Medical University, Guilin 541199, PR China

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ABSTRACT

Anaplastic lymphoma kinase (ALK) targeted therapies have demonstrated remarkable efficacy in ALK-positive lung adenocarcinomas. Here we synthesized and evaluated sixteen new 2,4-diaminopyrimidines bearing a sulfoxide moiety as anaplastic lymphoma kinase (ALK) inhibitors. The optimal compound **9e** exhibited excellent antiproliferative activity against non-small cell lung cancer NCI-H2228 cells, which is better than that of Brigatinib and similar to Ceritinib. Mechanism study revealed that the optimal compound **9e** decreased the mitochondrial membrane potential and arrested NCI-H2228 cells in the G0/G1 phase, finally resulting in cellular apoptosis. It is interesting that **9e** could effectively inhibit the migration of NCI-H2228 cells and may be a promising leading compound for chemotherapy of metastatic cancer.

Anaplastic lymphoma kinase (ALK) is a key member of the insulin receptor tyrosine kinase family.[1] The deregulation of ALK has been observed in various cancers, such as non-small cell lung cancer (NSCLC),[2] anaplastic large cell lymphoma (ALCL),[3] diffuse large B-cell lymphoma (DLBCL)[4] and inflammatory myofibroblastic tumor (IMT).[5]

Being a validated target for cancer therapy, the development of ALK inhibitors has received more and more attention. To date, six drugs targeting ALK have been approved by the FDA including Crizotinib,[6] Ceritinib,[7,8] Alectinib,[9] Brigatinib,[10,11] Lorlatinib[12,13] and Entrectinib[14,15] (Fig. 1). However, regardless of the initial clinical benefit demonstrated in ALK inhibitors, the development of drug resistance still remains a major problem.[16,17,18] Therefore, it is still necessary to develop new ALK inhibitors for combating the obstacle.

Sulfoxide moiety is an effective pharmacophore that is included in the structure of many important drugs such as Armodafinil,[19] Sulforaphane,[20] Sulindac,[21] Modafinil,[22] Omeprazole[23] and its S-isomer Esomeprazole[24] (Fig 2). Different from the sulfone moiety, the sulfur atom of sulfoxide is the chiral center and can produce two different chiral isomers. In many cases, two chiral isomers have obvious differences in biological activity. The typical example is omeprazole and esomeprazole, the proton pump inhibitors used in the treatment of gastric ulcers. Although omeprazole (racemic) itself is a widely used drug, its chiral isomer, esomeprazole, which provide better effect than the former, was also approved by the FDA later.

Inspired by the success of drugs containing sulfoxide, and the widely used 2,4-diaminopyrimidines moiety in anti-tumor drugs. A series of novel ALK inhibitors were designed, synthesized and evaluated on the basis of the molecular scaffold of Brigatinib and Ceritinib, the two successful drugs approved by FDA, by replacing the dimethylphosphine oxide moiety or sulfone moiety with sulfoxide and the other structural modifications (Scheme 1).

The synthetic route for the designed compounds (**9a-9c**, **9f-9h**, **9j-9n**) was shown in Scheme 2. Treating **1a-1c** with appropriate iodides in the presence of NaOH or *t*-BuOK gave **2a-2d**,[25] which were oxidized by hydrogen peroxide to afford **3a-3d**. Reaction of **3a-3d** with 2,4,5-trichloropyrimidine in the presence of NaH in DMF provided the intermediates **4a-4d**, which undergone substitution with appropriate aromatic amines **8a-8f** to afford target compounds (**9a-9c**, **9f-9h**, **9j-9n**). Meanwhile, the aromatic amines **8a-8f** were prepared with compound **5**, which was alkylated with MeI to obtain **6**,[26] and then converted to **7a-7f** via the nucleophilic substitution with appropriate amines. Finally, the nitro group of **7a-7f** was reduced with hydrogen in the presence of palladium on activated carbon to give **8a-8f**.[27]

The synthetic route of the designed compounds **9d**, **9e**, **9i**, **9o** was

* Corresponding authors.
E-mail addresses: lixsh@mail.sysu.edu.cn (X. Li), Anbj3@bzmc.edu.cn (B. An).

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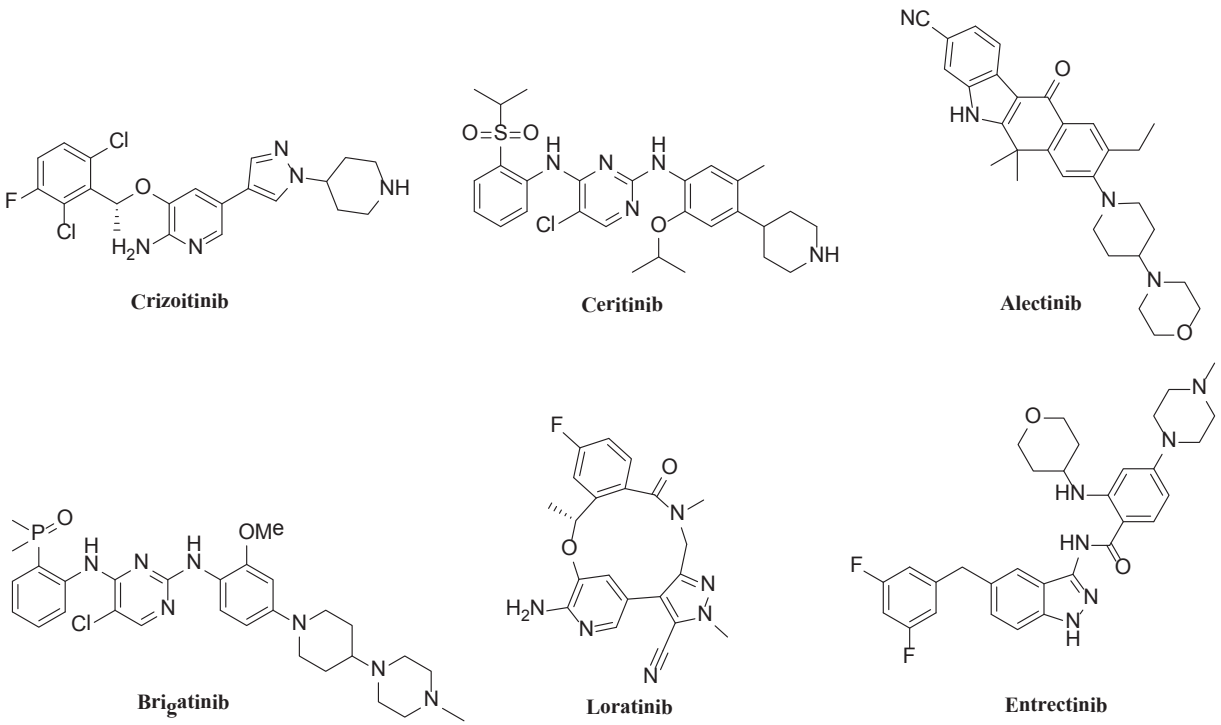


Fig. 1. Representative ALK inhibitors.

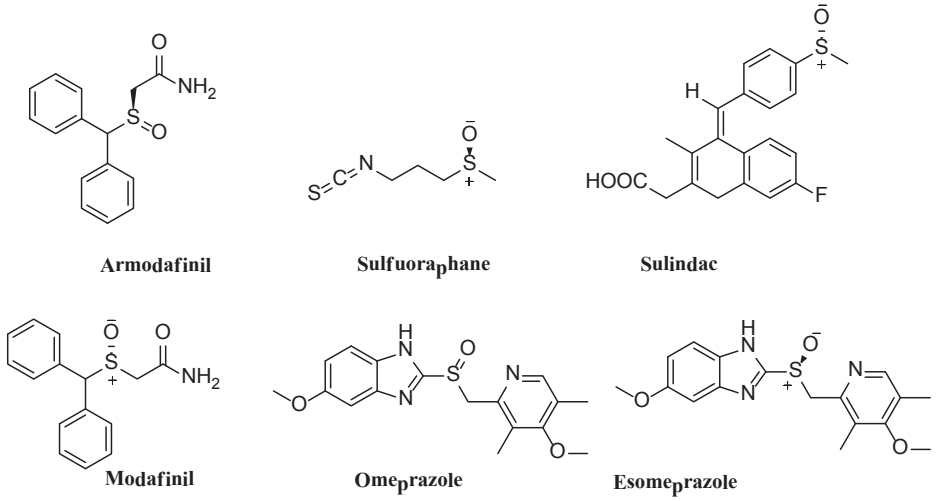
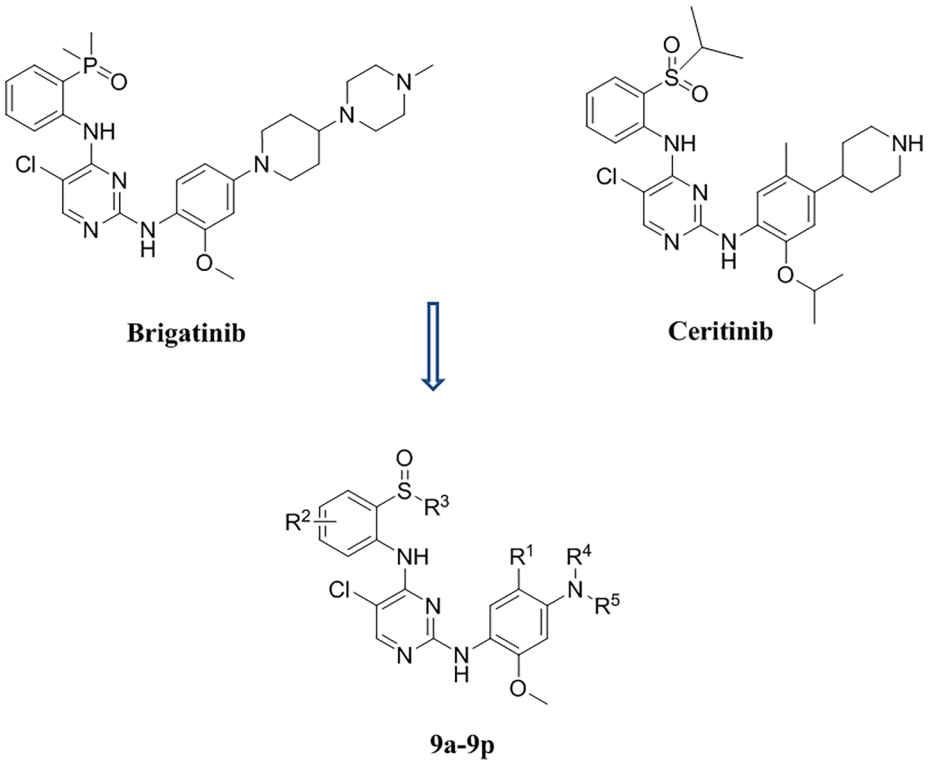


Fig. 2. Representative drugs containing sulfoxide moiety.

outlined in Scheme 3. First, **4a** or **4b** reacted with 4-fluoro-2-methoxy-5-nitroaniline to give compounds **10a and 10b**, which were convert to **11a-11c** by the reaction with various amines. Then, reduction of **11a-11c** with tin (II) chloride dehydrate provided **9o**, **12b** and **12c**, which reacted with acrylic chloride in the presence of $NaHCO_3$ to yield the target compounds **9d**, **9e**, **9i**.

Compound **9p** was prepared according to Scheme 4. Compound **2a** reacted with 2,4,5-trichloropyrimidine in the presence of *N*,*N*-diisopropylethylamine in isopropanol to provide intermediate **13**. Coupling of **13** with **8c** at 80 °C in the presence of trifluoroacetic acid provided the compound **9p**.

To evaluate the antiproliferative activities of the newly synthesized 2,4-diaminopyrimidines derivatives bearing a sulfoxide moiety, CCK8 assay was performed against two cancer cell lines including NCI-H2228 and BAF3-ALK with Ceritinib and Brigatinib as positive controls. The results listed in Table 1 indicated that most of the synthesized



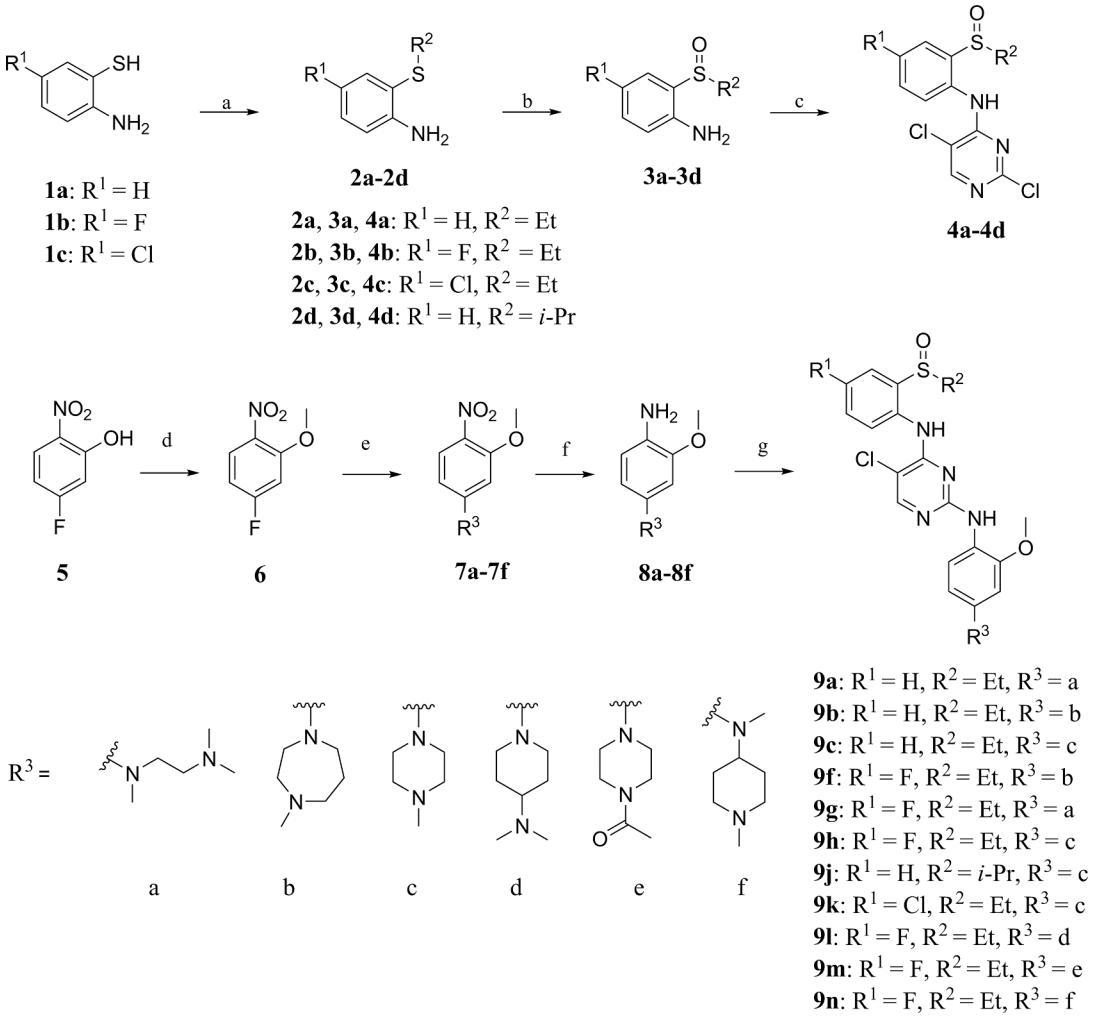
Scheme 1. Design of AlK inhibitors.

compounds exhibited excellent antiproliferative activity with IC_{50} values in sub-micromolar level. Among them, compound **9e**, bearing a propionamido group in C ring (Table 1), exhibited the best results with the IC_{50} values ranging from 8 to 24 nM, which is better than that of Brigatinib and similar to Ceritinib. Further structure–activity studies showed that when the other moiety of the molecule remain unchanged, a cyclic R^3 group seems beneficial for anti-tumor activity than that of chain group. For example, **9b** and **9c**, a homopiperazine or piperazinyl connected to the C ring, provided 51.5 and 35.2 nM of the IC_{50} comparing the 69.3 nM of compound **9a**, which bears a chain R^3 group. When there is a halogen atom such as fluorine or chlorine on the A ring (Table 1), the activity generally decreases (48.1 to 1435 nM of the IC_{50} values of compounds **9f-9h**, **9k-9n** via the 35.2 nM of **9a**). Comparing the isopropyl group in the sulfoxide moiety (**9j**, 82.9 nM of the IC_{50} for H2228 cancer lines), ethyl is more beneficial to the anticancer activity (**9c**, 35.2 nM of the IC_{50}). In general, that the sulfoxide moiety is connected to the ethyl group, the A ring does not contain a halogen compound, and the R^3 group on the C ring is piperazinyl and a propionamido group in its neighbor may be the best scaffold.

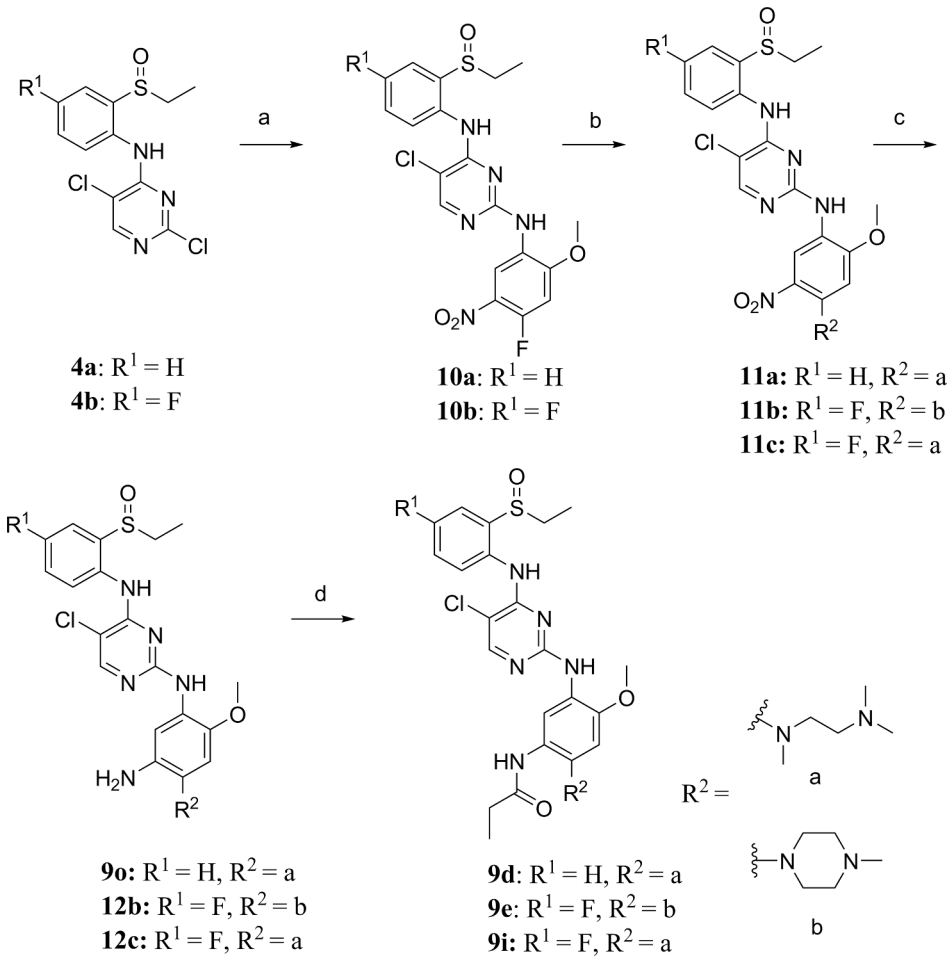
Due to its best antiproliferative activity against cancer cell lines, in the following study, **9e** was selected as the optimal compound for further study on cellular mechanism. The flow cytometry analysis was performed to study its effect on the cell cycle using human NCI-H2228 cancer cells. Treatment of the tumor cells with 20 nM of **9e** for 0, 12, 24 and 48 h, we found compound **9e** induced cell cycle arrest at the G0/G1 phase in a time-dependent manner, with a concomitant change in G2/M or S phase cells. At beginning, the percentage of cells in G0/G1 phase of the cell cycle was 67.7%. When the time was increased from 12, 24 to 48 h, the percentage of cells at the G0/G1 phase increased to 71.23%, 82.13%, and 86.49%, respectively (Fig. 3A). Meanwhile, western blot assay was performed to detect the changes of related proteins (the cell cycle activators CDK4 and Cyclin D1). The results in Fig. 3B exhibited that the levels of CDK4 and Cyclin D1 were down regulated in a certain extent, especially the level of Cyclin D1 which was significantly decreased after the treatment of compound **9e**.

The results of the cell cycle analysis hypothesized that compound **9e** induced the apoptosis of cancer cells. To evaluate the effect of apoptosis progression, flow cytometry assay was performed using propidium iodide (PI) and fluorescent immunolabeling of the protein annexin-V (V-FITC). Brigatinib, with high selectivity and potency to ALK, was chosen as a positive drug. After NCI-H2228 cells were treated for 24 h with **9e** at indicated concentrations, the cells were harvested, stained with Annexin V-FITC and PI, and analyzed by flow cytometry (Fig. 4A). The early and late apoptosis cells at the concentrations of 5, 25 and 50 nM or DMSO (0.01%) were founded to be 6.58%, 23.53%, 23.44% and 4.30%, respectively. When the incubation time was extended to 48 h (Fig. 4A), the early and late apoptosis cells increased to 22.3%, 57.0%, 85.4% and 1.81%, respectively. These results indicated that **9e** induced cell apoptosis in a concentration- and time-dependent manner. To study the morphological alterations of cancer cells caused by compound **9e**, NCI-H2228 cells were exposed to different concentrations of **9e** (5, 25 and 50 nM) for 48 h, stained with Hoechst 33342, and then photographed with an Olympus inverted fluorescence microscope. As shown in Fig. 4C, the cell membrane of NCI-H2228 cells is intact and the nucleus is uniform in size in the control group. After the treatment of **9e** under different concentrations (5, 25 and 50 nM), the nucleus of the NCI-H2228 cells gradually pyknosis into a uniform dense mass and then breaks into fragments of varying size.

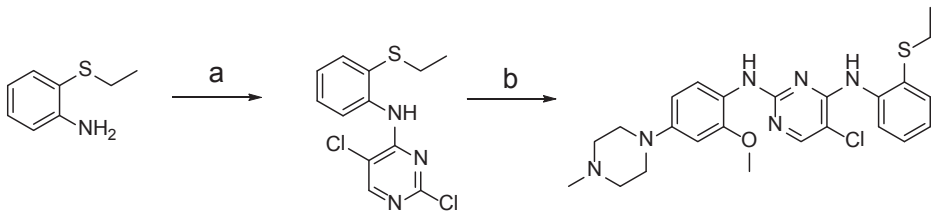
A prelusion of cell apoptosis is the mitochondrial membrane depolarization, which usually leads to the decrease of mitochondrial



Scheme 2. Reagents and conditions: (a) appropriate iodides, *t*-BuOK or NaOH, C_2H_5OH or CH_3OH, rt; (b) H_2O_2, AcOH, 0 °C to rt; (c) 2,4,5-trichloropyrimidine, NaH, DMF, 0 °C to rt; (d) CH_3I, K_2CO_3, DMF, 60 °C; (e) appropriate amines, K_2CO_3, DMF, 110 °C; (f) Pd/C, H_2, MeOH, rt. (g) **4a**-**4d**, TFA, *i*-PrOH, 80 °C;



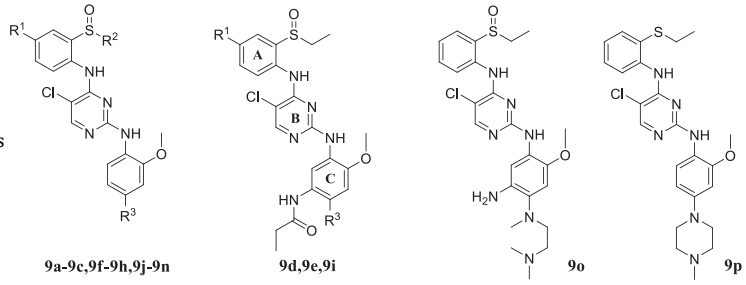
Scheme 3. Reagents and conditions: (a) 4-fluoro-2-methoxy-5-nitroaniline, 37% hydrochloride, 2-butanol, 80 °C; (b) appropriate amines, DIEA, dioxane, 100 °C; (c) tin (II) chloride dehydrate, HCl (aq) , DCM, MeOH, 50 °C; (d) acryloyl chloride, saturated $NaHCO_3$ (aq) , THF, 0 °C.



Scheme 4. Reagents and conditions: (a) 2,4,5-trichloropyrimidine, DIEA, *i*-PrOH, 80 °C; (b) **8c**, TFA, *i*-PrOH, 80 °C.

Table 1

Evaluation of antiproliferative activity of the synthesized compounds



Comp.	R^1	R^2	R^3	Cell type (IC_{50} /nM) [a]	
				NCI-H2228	BAF3-ALK
9a	H	Et		69.3 ± 0.05	26.3 ± 0.09
9b	H	Et		51.5 ± 0.07	23.5 ± 0.08
9c	H	Et		35.2 ± 0.03	13.1 ± 0.06
9d	H	/		55.4 ± 0.11	35.4 ± 0.02
9e	F	/		24.4 ± 0.10	8.11 ± 0.01
9f	F	Et		64.5 ± 0.01	26.7 ± 0.02
9g	F	Et		84.3 ± 0.11	34.7 ± 0.11
9h	F	Et		104.1 ± 0.03	48.9 ± 0.03
9i	F	/		48.1 ± 0.02	47.2 ± 0.04
9j	H	^{i}Pr		82.9 ± 0.13	32.3 ± 0.08
9k	Cl	Et		134.1 ± 0.14	128.1 ± 0.11
9l	F	Et		337 ± 0.58	79.4 ± 0.23
9m	F	Et		704 ± 0.77	85.7 ± 0.72
9n	F	Et		1435 ± 1.89	82 ± 0.57
9o	/	/	/	2632 ± 2.77	75 ± 0.45
9p	/	/	/	981 ± 1.38	78 ± 0.33
Ceritinib				22.1 ± 0.08	7.11 ± 0.02
Brigatinib				31.2 ± 0.02	8.49 ± 0.01

[a] The anti-proliferative activities of the compounds were assessed by the CCK8 assay. IC_{50} values are indicated as the mean ± SD (standard error) of three independent experiments.

membrane potential. The decreased mitochondrial membrane potential (MMP, ΔΨm) is thought to be a signal of apoptosis. To explore whether compound **9e** could reduce mitochondrial membrane potential in cancer cells, NCI-H2228 cells were treated with **9e** at different concentrations (5, 25, and 50 nM) or DMSO (0.01%) for 24 h, followed by incubation with the fluorescence probe JC-1 for 30 min, and then analyzed by fluorescence microscopy. As the concentration of **9e** increased, a shift from aggregates (red fluorescence) toward the form of monomers (green fluorescence) occurred, which indicated that **9e** can induce MMP collapse, and eventually trigger apoptotic cell death (Fig. 5A).

Tumor cell migration is one of the most important factors leading to the death of tumor patients. To evaluate the ability of **9e** to inhibit the tumor cell migration, NCI-H2228 cells were suspended in 1640 medium containing **9e** for 12 h or 24 h, and then photographed under a phase contrast microscope. As showed in the Fig. 5B, NCI-H2228 cells incubated with **9e** (25 nM) for 12 h exhibited obvious inhibition of tumor

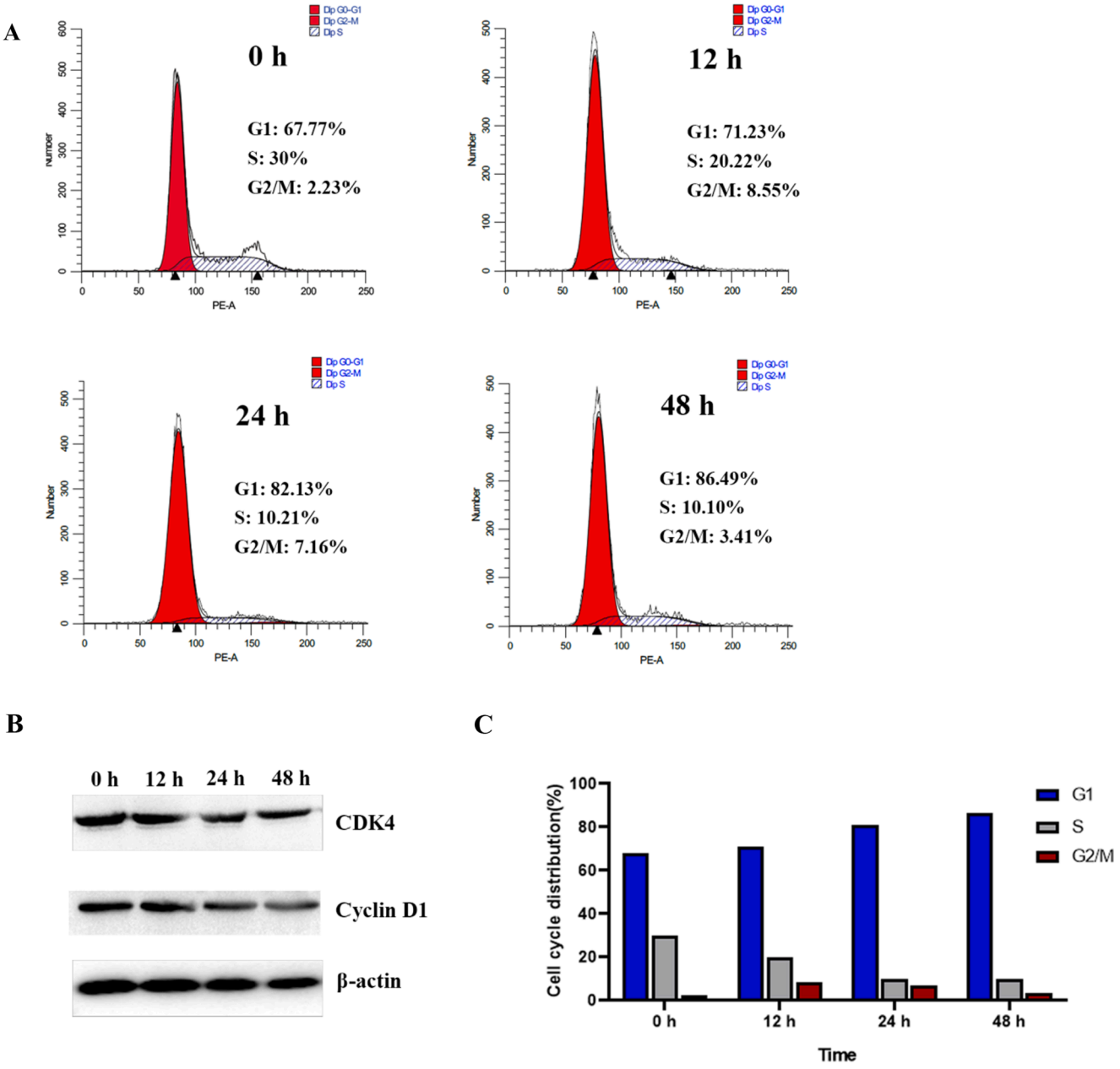


Fig. 3. The cell cycle distribution of NCI-H2228 cells treated with **9e** (20 nM) for the indicated time (0, 12, 24 or 48 h) was measured by flow cytometry with PI staining. (A) The expression levels of G0/G1 phase-related proteins in the NCI-H2228 cells treated with **9e** (20 nM) for 0–48 h. (B) NCI-H2228 cells were treated for 0, 12, 24 or 48 h with **9e** (20 nM), cell cycle regulatory protein were analyzed by western blotting. (C) Quantitative analysis of the percentage of cells in each cell cycle phase was analyzed by EXPO32 ADC analysis software. The experiments were performed three times, and the results of representative experiments are shown.

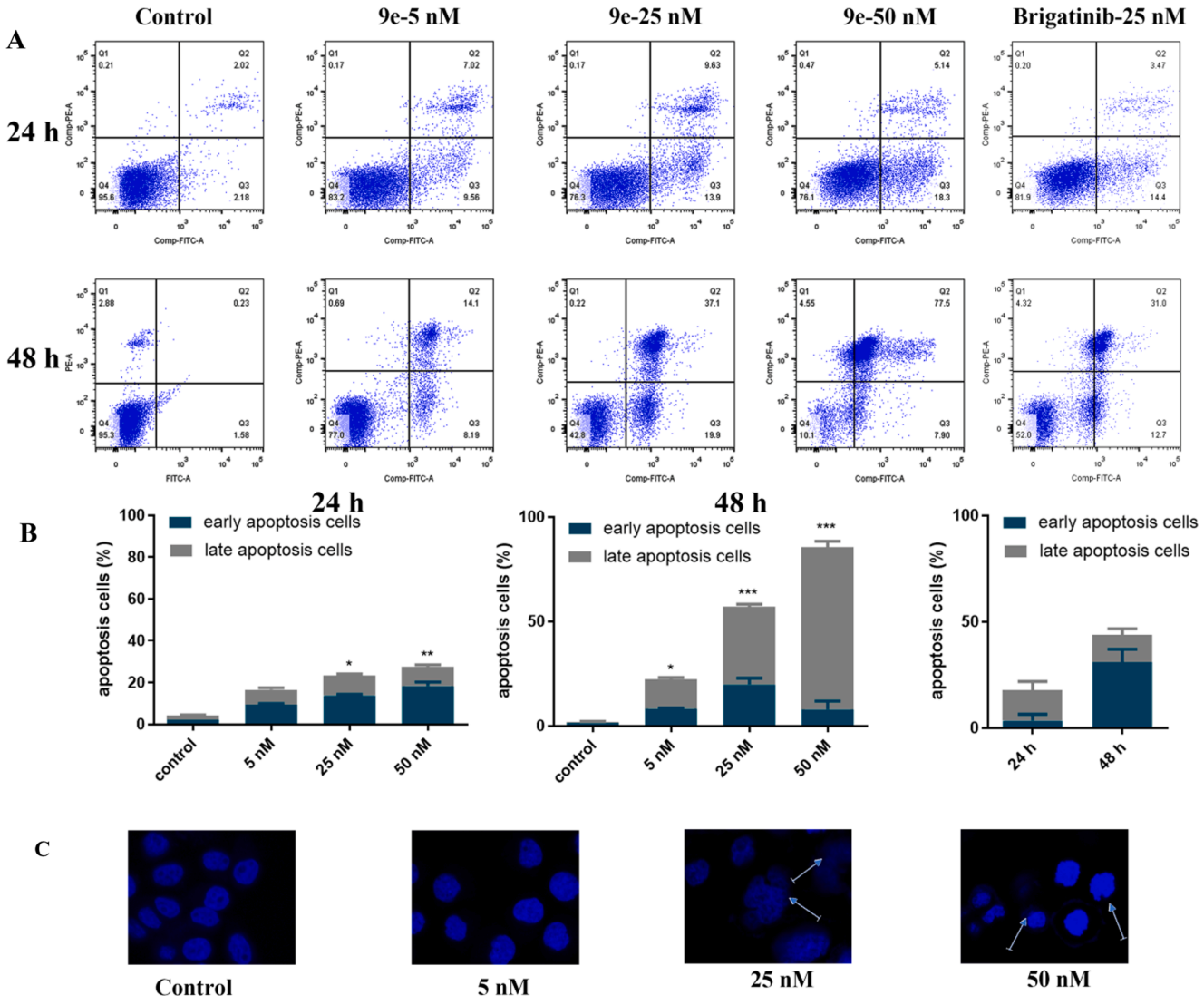


Fig. 4. Apoptosis of NCI-H2228 cells with or without **9e** treatment at the indicated concentrations. (A) Treated with **9e** (5, 25 and 50 nM) or Brigatininb (25 nM) for 24 h, or 48 h, x-axis is Annexin-V-FITC-fluorescence vs y-axis is PI-fluorescence. (B) The percentages of cells in each stage of apoptosis were quantitated by EXPO32 ADC analysis software. (C) NCI-H2228 cells were treated with **9e** (5, 25 and 50 nM) for 48 h and then stained with Hoechst 33,342 and photographed with an Olympus inverted fluorescence microscope. The experiments were performed three times, and the results of representative experiments are shown.

cell migration. The wound closure in the migration assay was significantly suppressed when the time was extended to 24 h. In contrast, the tumor cells spanned the wound closure and nearly covered the whole area in the control. These results suggested that compound **9e** effectively inhibited the migration of NCI-H2228 cells and might be a promising candidate for chemotherapy of metastatic cancer.

In this study, we have developed a series of 2,4-diaminopyrimidines derivatives bearing a sulfoxide moiety as novel potent ALK inhibitors. The optimal compound, **9e** exhibited nearly the same potent antiproliferative activity as Ceritinib and better than Brigatinib which approved by FDA in 2017. The mechanism study indicated that compound **9e** induced cell cycle arrest at the G0/G1 phase, down regulated the related proteins CDK4 and Cyclin D1, thereby inducing cell apoptosis. It is noteworthy that compound **9e** effectively inhibited the migration of NCI-H2228 cells as tumor-cell spreading is one of the major causes of death in tumor patients. Overall, these results demonstrated the promising value of **9e** as a potential anticancer leading compound. Further progression concerning the chiral properties of these sulfoxide analogs, including the asymmetric preparation and anti-tumor activity assays, will be reported in due course.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

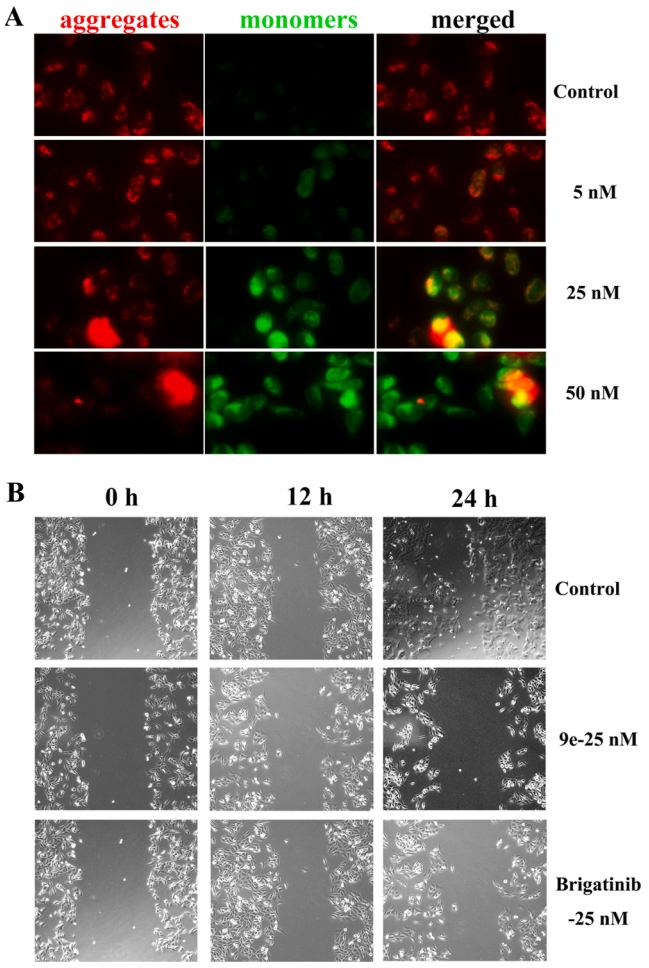


Fig. 5. Compound **9e** decreased the mitochondrial membrane potential and inhibited cell migration of NCI-H2228 cells. (A) The NCI-H2228 cells were treated with **9e** at different concentrations (5, 25, and 50 nM) or DMSO (0.01%) for 24 h, followed by incubation with the fluorescence probe JC-1 for 30 min. Then, the cells were analyzed by fluorescence microscopy. (B) NCI-H2228 cells were suspended in 1640 medium containing **9e** or Brigatininb for 12 h or 24 h and photographed under a phase contrast microscope (magnification: 4 × objective). The experiments were performed three times, and the results of representative experiments are shown.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.128253.

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