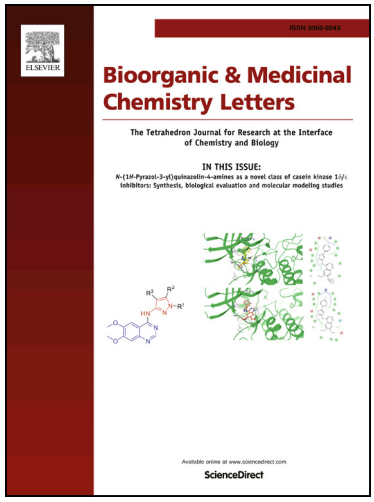

Discovery of a highly potent, selective and novel CDK9 inhibitor as an anti-cancer drug candidate

Yongtao Li, Qingxiang Guo, Chao Zhang, Zhi Huang, Tianqi Wang, Xin Wang, Xiang Wang, Guangwei Xu, Yanhua Liu, Shengyong Yang, Yan Fan, Rong Xiang

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Discovery of a highly potent, selective and novel CDK9 inhibitor as an anticancer drug candidate

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Author Contributions: [⊥] Yongtao Li and Qingxiang Guo are contributed equally to this work.

Abstract

A series of novel hybrid structure derivatives, containing both LEE011 and Cabozantinib pharmacophore, were designed, synthesized and evaluated. Surprisingly, a compound **4d** was discovered that highly exhibited effective and selective activity of CDK9 inhibition with IC_{50} = 12 nM. It effectively induced apoptosis in breast and lung cancer cell lines at nanomolar level. Molecular docking of **4d** to ATP binding site of CDK9 kinase demonstrated a new hydrogen bonding between F atom of 4-(3-fluorobenzyloxy) group and ASN116 residue, compared with the positive control, LEE011. The compound **4d** could block the cell cycle both in G0/G1 and G2/M phase to prevent the proliferation and differentiation of cancer cells. Mice bared-breast cancer treated with compound **4d** showed significant suppression of cancer with low toxicity. Taken together, this novel compound **4d** could be a promising drug candidate for clinical application.

Keywords: CDK9, LEE011, Inhibitor, Cancer

Leland H. Hartwell, R. Timothy Hunt, and Paul M. Nurse received the 2001 Nobel Prize in Physiology or Medicine for their complete description of how cells regulate proliferation and the central role of Cyclin-dependent kinases (CDKs).[1] The CDKs have two major functional groups based on their roles that mediate cell cycle (subtypes 1–4 and 6) and regulate transcription control (subtypes 5, 7-9).[2] Cdk4–CyclinD complexes regulate the G0/G1 transition and the early phases of G1 through phosphorylation of the Rb. CDK9 is a component of the multiprotein complex TAK/P-TEFb and a key regulatory kinase of mRNA polymerase II (PII) and a well-validated target for treating cancers.[3] Inhibition of CDK9 associated with blocking RNA synthesis by inhibitors can lead to a reduction in the levels of short-lived pro-survival proteins and the induction of apoptosis, which provides an effective approach to the control of tumor growth.[4-5]

Multiple generations of drugs targeting CDK have been designed and synthesized through binding in the ATP-binding cleft of CDK enzymes. The first generation of CDK-directed drugs entered clinical trials in the 1990s and were found to be nonspecific, pan-CDK, cytotoxic and ineffective. Second-generation CDK-directed drugs have toxicities which limit their clinical utility. Third-generation CDK-directed drugs have low toxicity, potent and selective CDK4 inhibition[6-8]. LEE011 (Novartis/Astex) is a bioavailable, selective inhibitor targeting both CDK4 and CDK6 that prevents Rb phosphorylation, resulting in cell cycle G0/G1 phase arrest.[9-10] It received FDA breakthrough therapy designation as first-line treatment for HR+/HER2- advanced breast cancer in 2016.[7] But in clinical trials, LEE011 needed to combine with letrozole for the treatment of $ER^{+}/HER2^{-}$ metastatic breast cancer,[11-13] which indicated a lack of sensitivity to CDK4 monotherapy. Therefore, we reasoned that development of single drug with multiple biological targets based on CDK4 to improve efficacy in cancer treatment.

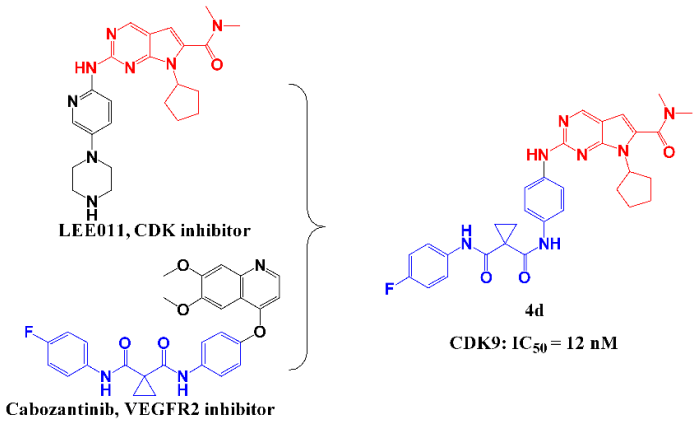
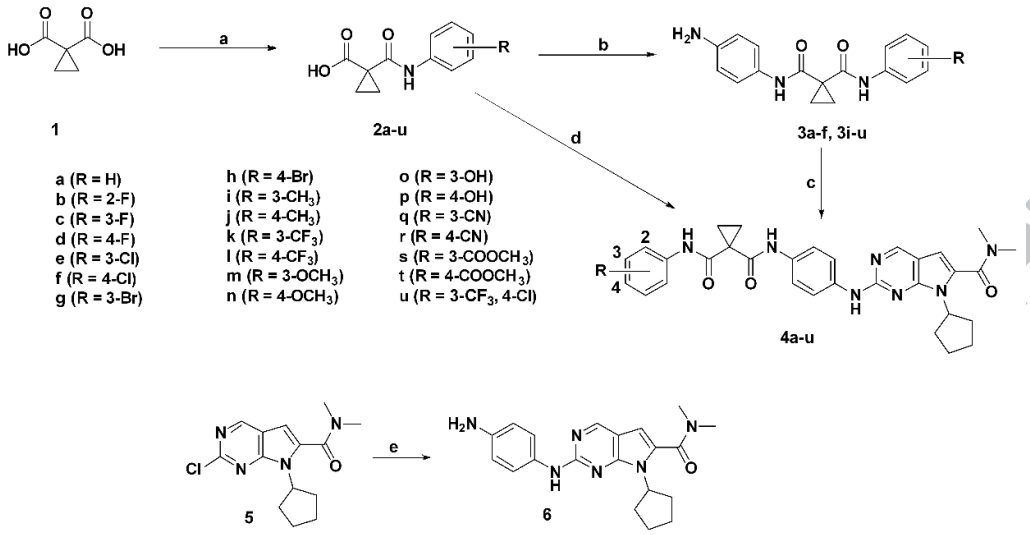


Figure 1. Schematic showing design for merged CDK-VEGFR pharmacophore.

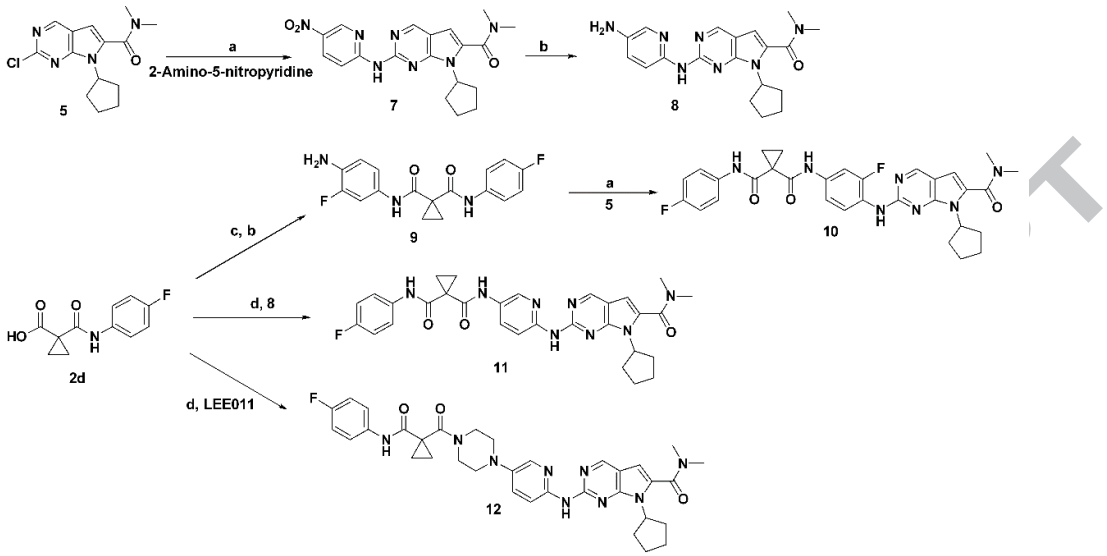
The vascular endothelial growth factor (VEGF) is a primary regulator of physiological new vessel formation. Disruption of VEGF signaling has been demonstrated to retard angiogenesis and inhibit tumor growth[14]. It has been reported that VEGFR-2 inhibitors possess synergistic antitumor effects in rational combinations with anticancer drugs[15-16]. Cabozantinib is a highly potent small molecule inhibitor against VEGFR-2 (IC_{50} = 0.035 nM).[17-18] In our study, a series of novel dual-action compounds was designed and synthesized to target CDK4 and VEGFR2. In vitro kinase profiling and cell-based screening together with in vivo assays and structure–activity relationship (SAR) studies finally led to the discovery of compound **4d**, which was a hybrid structure containing both pyrrolo[2,3-d] pyrimidines-2-amine of LEE011 and N-(4-fluorophenyl)-N-phenylcyclopropane-1,1-dicarboxamide moiety of cabozantinib. Fortuitously, it was found that the compound **4d** has a high potent inhibition of CDK9 (IC_{50} = 12 nM). This compound showed potent anti-tumor activities both in vitro and in vivo, and low toxicity. In this paper, we report the synthesis and biological evaluation of the resulting novel CDK9 compounds (Figure 1).



Scheme 1. Synthesis of Compounds **4a-u.**

Reagents and conditions: (a) $SOCl_2$, Et_3N, THF, then aniline or substituted aniline; (b) *p*-phenylenediamine, EDCI, HOBt, DIEA, rt; (c) 2-chloro-7-cyclopentyl-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide(**5**), $Pd(AcO)_2$, BINAP, Cs_2CO_3, 100 °C; (d) **6**, EDCI, HOBt, DIEA; (e) *p*-Phenylenediamine, $Pd(AcO)_2$, BINAP, Cs_2CO_3, 100 °C.

Scheme 1 outlines the routes used to synthesize compounds **4a**–**u**. Briefly, compound **2a-u** was synthesized by cyclopropane-1,1-dicarboxylic acid condensating with aniline or substituted aniline.[19] The condensation of *p*-phenylenediamine with carboxylic acids **2a-u** afforded **3a–f** and **3i-u** under condensing agent 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDCI) in the presence of 1-hydroxybenzotriazole (HOBt), N,N-diisopropylethylamine (DIEA).[20] The condensation of carboxylic acids **2g-h** with compound **6**, which was obtained from compound **5** that was coupled to the *p*-Phenylenediamine using the Buchwald–Hartwig reaction, afforded **4g-h.** A palladium catalyzed cross-coupling reaction of compounds **3a–f** and **3i-u** with compound **5** provided compounds **4a–f** and **4i-u**, respectively.[21]

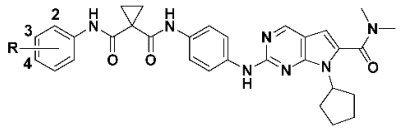


Scheme 2. Synthesis of Compounds **10-12**.

Reagents and conditions: (a) $Pd(AcO)_2$, BINAP, Cs_2CO_3, 100 °C; (b) Fe, NH_4Cl, $EtOH/H_2O$, reflux; (c) $SOCl_2$, then 3-fluoro-4-nitroaniline; (d) EDCI, HOBt, DIEA.

Synthetic routes for compounds **10-12** are outlined in Scheme 2. A palladium catalyzed cross-coupling reaction of 2-Amino-5-nitropyridine or compounds **9** with compound **5** provided compounds **7** or **10**, respectively. Compound **7** was treated with ammonium chloride and iron powder for reduction of nitro to obtain compound **8.** The intermediate aniline **9** was obtained from the nitro, which was prepared by condensation reaction of 3-fluoro-4-nitroaniline with **2d**.[19] The condensation of carboxylic acids **2d** with **8** or **LEE011** afforded **11** or **12** under condensing agent EDCI in the presence of HOBt and DIEA**,** respectively.

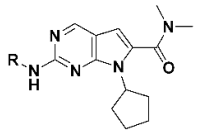
Table 1. Structure and inhibitory activity of **4a-u** against CDK4/@ 1 μM and VEGFER2/@ 1 μM[a]

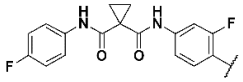
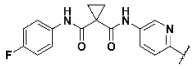
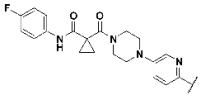


Compound	R	CDK4/@ 1 μM (Inhibition%)	VEGFR2/@ 1 μM (Inhibition%)
4a	H	82	8
4b	2-F	75	-2
4c	3-F	61	7
4d	4-F	102	IC_{50} >10 μM
4e	3-Cl	44	15
4f	4-Cl	65	5
4g	3-Br	72	6
4h	4-Br	48	26
4i	3-CH_3	66	5
4j	4-CH_3	62	9
4k	3-CF3	26	-10
4l	4-CF3	32	1
4m	3-OCH_3	66	-2
4n	4-OCH_3	77	19
4o	3-OH	94	24
4p	4-OH	95	36
4q	3-CN	53	29
4r	4-CN	84	21
4s	3-$COOCH_3$	76	-7
4t	4-$COOCH_3$	77	2
4u	3-CF_3, 4-Cl	24	4

[a]IC_{50} values and inhibition values were determined using KinaseProfiler by Eurofins. The data represent the mean values of two independent experiments.

Table 2. Structure and inhibitory activity of compound **10-12** against CDK4/@1μM and VEGFER2 /@1μM[a]



Compound	R	CDK4/@ 1 μM (Inhibition%)	VEGFR2/@ 1 μM (Inhibition%)
10		87	33
11		85	-3
12		97	-8

[a]IC_{50} values and inhibition values were determined using KinaseProfiler by Eurofins. The data represent the mean values of two independent experiments.

The enzyme inhibitory activities are summarized in Table 1 and Table 2. Analogue **4d** displayed a better enzymatic potency than the 2-fluoro analogue **4b** and the 3-fluoro analogue **4c**, suggesting that 4-fluoro substitution is superior to 2-fluoro and 3-fluoro substitution. Analogue **4a**, **10** and **11** displayed similar enzymatic potency, suggesting that pyridine ring could be replaced by benzene ring and fluoro-substitued benzene. Compound **4o** and **4p** have stronger inhibition targeting CDK4 than other analogs except compound **12**, most likely because R groups of compound **4o** and **4p** are hydrophilic groups. Compound **12** shows stronger inhibition targeting CDK4 than other analogs, suggesting that piperazine ring is important for CDK4 enzymatic activity. Compound **4k, 4l** and **4u,** which possess a trifluoromethyl group, showed very low binding affinity. Collectively, among all the compounds synthesized, **4d, 4o, 4p** and **12** are the promising compounds targeting CDK4. Unfortunately, these compounds show low inhibition effect targeting VEGFR2.

Table 3. In vitro growth inhibitory activities of compound **4d** against human cancer cell lines.

	IC_{50} (nM)				
Cell lines	T47D	MCF7	H460	H1299	A549
LEE011	6227	>10000	>10000	7637	>10000
Cabozantinib	4448	2889	5669	1919	4205
4d	358	338	359	221	456

In the meanwhile, we detected the antiviability activity of these compounds against a panel of human cancer cell lines using standard CCK8 assay. Cancer cell lines inhibition profiling assays with a fixed concentration of 1 μM and 10 μM were first carried out. The data is presented in Table S1. The compounds **4d** and **4p** that showed a high inhibitory rate at 1 μM and 10 μM, were consistent with the kinase result of CDK4. Compared with **4p**, compound **4d** demonstrated strong inhibition in T47D (breast cancer cell line) and A549 and H1299 (lung cancer cell lines). Negligible activity was observed for other selected cell lines, HeLa and SiHa (cervical cancer), OVCAR5(ovarian cancer), which indicated that **4d** has a considerable selectivity. Then we confirmed the inhibition on tumor cells of compound **4d** as shown in Table 3. **4d** potently inhibited the proliferation of the breast cancer cell lines T47D and MCF7, and lung cancer cell lines, H460, H1299 and A549, with half-maximal inhibitory concentration (IC_{50}) values of 358 nM, 338 nM, 359 nM, 221 nM and 456 nM, respectively. The CDK4 selective inhibitor LEE011 was not potent in these cells, indicating a lack of sensitivity to CDK4 monotherapy. This result demonstrated that compound **4d** and **4p** maybe have other target kinase.

Table 4. Inhibition of compound **4d** and **4p** against CDK kinases at 1 μM. [a]

	Inhibition%/@ 1 μM										
Compound	CDK1 /cyclinB(h)	CDK2 /cyclinA(h)	CDK2 /cyclinE(h)	CDK3 /cyclinE(h)	CDK5 /p25(h)	CDK5 /p35(h)	CDK6 /cyclinD3(h)	CDK7 /cyclinH/MAT1(h)	CDK9 /cyclin T1(h)	CDK12 /cyclinK(h)	CDK18 /cyclinY(h)
4d	73	29	85	65	86	81	71	7	98	24	6
4p	90	90	88	89	94	88	86	-9	26	23	0

[a] IC_{50} values and inhibition values were determined using KinaseProfiler by Eurofins. The data represent the mean values of two independent experiments.

Table 5. Inhibition of compound **4d** and **LEE011** against CDK9 and CDK4[a]

Compound	CDK4 IC_{50} (nM)	CDK9 IC_{50} (nM)
LEE011	13	197
4d	142	12

[a]IC50 values were determined using KinaseProfiler by Eurofins. The data represent the mean values of two independent experiments.

We next evaluated the kinase inhibitory activities against CDK kinases of compound **4d** and **4p** with a fixed concentration of 1μM through the Eurofins kinase profiling. The results are provided in Table 4 indicated **4d** have comparable potency and selectivity with **4p**. Both the preliminary study on cell and kinase inhibition declared **4d** is the most promising candidate for the treatment of cancer. In-depth studies including in vitro and in vivo anti-tumor studies were then carried out on **4d**. CDK4 and CDK9 further IC_{50} were examined and the results are shown in Table 5. As indicated in the data, we found that compound **4d** has exhibited highly potent inhibition of CDK9 and selectivity to other CDKs. **4d** showed high inhibitory activity against CDK9 (IC_{50} = 12 nM) and low inhibitory activity against CDK4 (IC_{50} = 142 nM). While, LEE011 showed high inhibitory activity against CDK4 with IC_{50} value of 13 nM rather than CDK9 with IC_{50} value of 197 nM. Through a complicated process of structural synthesis and optimization, we finally discover a new compound, **4d**, has potent effect on CDK9 and selectivity for other CDKs and tumor cells in vitro.

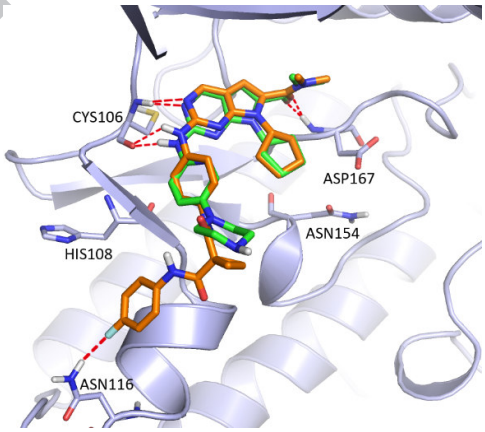


Figure 2. The docking of compound **4d** to the active site of CDK9 (PDB:4bcg), using Discovery Studio 3.1(Accelrys Inc., San Diego, CA, USA): LEE011 (green), and compound **4d** (brown). The H-bonds are shown by dashed red lines.

Molecular docking of **4d** to ATP binding site of CDK9 kinase was performed by

using Discovery Studio 3.1(Accelrys Inc., San Diego, CA, USA). Here CDK9 in complex with cyclin T and a 2-amino-4-heteroaryl-pyrimidine inhibitor crystal structure (PDB:4bcg)[22] was selected as the binding model. The docking results showed compound **4d** and LEE011 were in similar binding mode as shown in Figure 2. LEE011 (green) and **4d** (brown), both bound in the ATP binding pocket of the kinases, form two hydrogen bonds with conserved CYS106 and one hydrogen bond with conserved ASP167. While the F atom of 4-(3-fluorobenzyloxy) group of compound **4d** was directly involved in hydrogen bonding with ASN116 residue. These changes should be the reason of their different inhibitory activity on CDK9. In an attempt to find the CDK4 inhibitory difference between the compound **4d** and LEE011, we docked compound **4d** and LEE011 into the CDK4 protein in Figure 3. Compared with the LEE011, the hydrogen bonding interaction between dimethylcarbamoyl group and ASP-158 in **4d** was too weak, which lead to the significant selectivity of compound **4d** for CDK9 over CDK4.

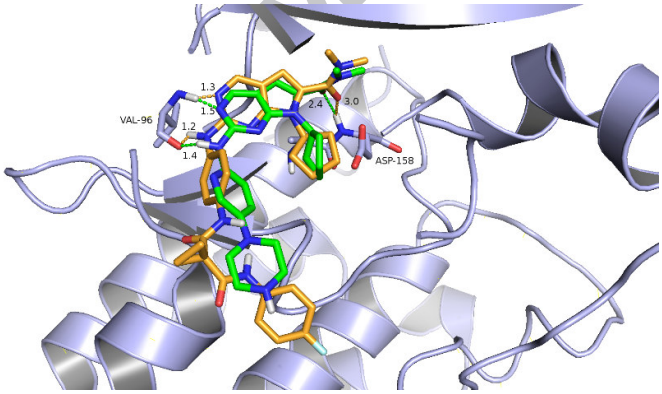


Figure 3. The docking of compound **4d** to the active site of CDK4 (PDB: 2W96), using Discovery Studio 3.1(Accelrys Inc., San Diego, CA, USA): LEE011 (green), and compound **4d** (brown). The H-bonds are shown by dashed lines.

We investigated the effect of compound **4d** treatment on breast cancer cell line (T47D) cell cycle progression (Figure 3). Cells were treated with compound **4d** with varying concentrations (0.1 μM, 0.5 μM and 1 μM), LEE011 and vehicle (DMSO) for 24h, and subjected to flow cytometry analysis to determine the distribution of cells in various phases of the cell cycle. LEE011 treatment for 24h led to a majority of cells in G0/G1 phase, which is associated with CDK4 inhibition. Compared to the

vehicle-treated control, cells treated with compound **4d** have been demonstrated an increase of G2 and G0/G1 phase, with smaller percentages in S phase. Compound **4d** blocked the cells in G2 and G0/G1 phase, which resulted in decreased S-phase populations. The increase of the G2 phase by compound **4d** was indicative of cells undergoing apoptosis, which may be related to the excellent cellular CDK9 inhibitory. The result in the appearance of G0/G1 population confirmed that compound **4d** had multi-intracellular targets activity on CDK4.

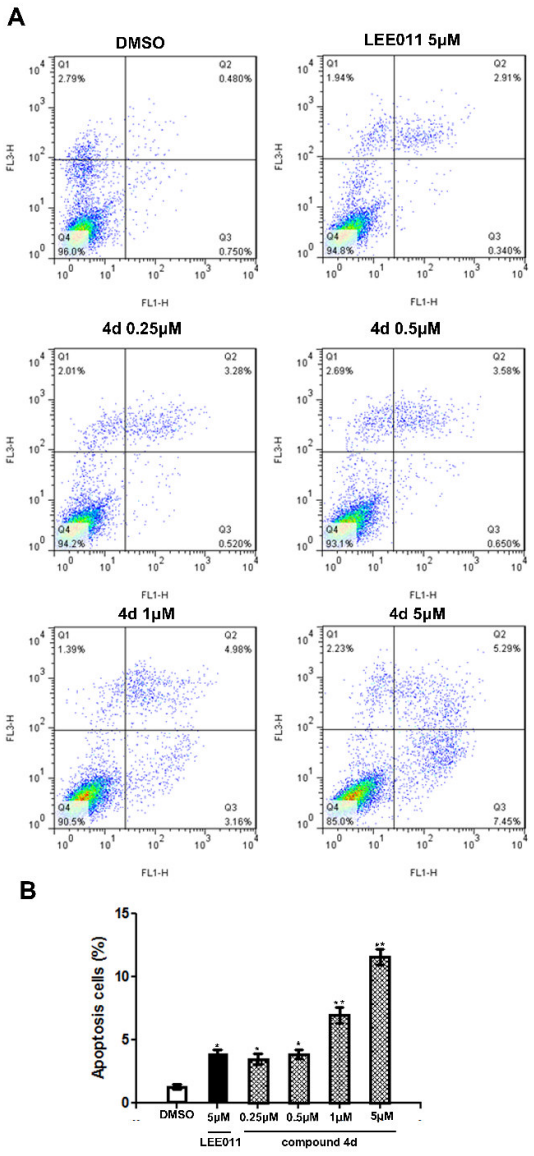


Figure 4. Compound **4d** induces cell apoptosis of T47D cell line in vitro. (A) Flow cytometry and (B) quantitative analysis of apoptotic cells. Cells were incubated with the indicated concentrations of compound 4d or LEE011 for 48h and were stained with FITC-Annexin V/PI, followed by flow

cytometry analysis. Data are expressed as means ± SD of the percentages of apoptotic cells from three independent experiments, $P < 0.05$.

Breast cancer cells (T47D) were treated with compound **4d**, vehicle (DMSO) and LEE011 as positive control for 48h and analyzed by annexin V/PI staining. As shown in Figure 4, compound **4d** induced cell apoptosis in a dose-dependent manner. An increase in dose led to increase in annexin V and PI positive cells, indicating apoptotic events. Compared with the LEE011 and vehicle condition, compound **4d** was significantly more active in apoptosis induction as the concentration increasing. This study illustrated that compound **4d** could induce cell death via apoptosis. In addition, migration of **4d**-treated T47D cells, suggested by transwell migration assay (Fig. 5a and b), was significantly decreased compared to the untreated group.

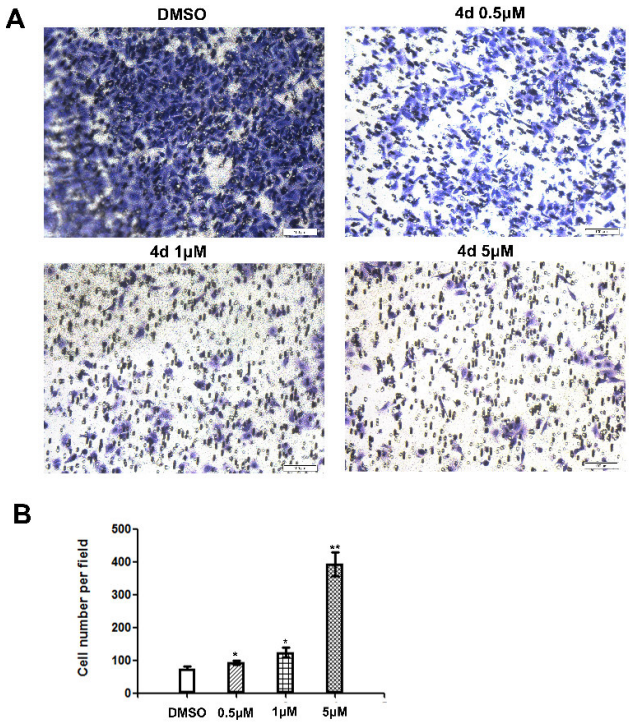


Figure 5. (A) Representative images of transwell migration assay for compound **4d**-treated T47D cells and (B) statistically analyzed. Cells were incubated in the upper transwell chambers and the number of migrated cells was counted after 16 h incubation using microscope in five random fields per chamber.

The in vivo antitumor efficacy of **4d** was assessed using 4T1 xenograft models. 4T1

cells were orthotopically implanted into the mammary fat pads of 6−8 weeks old female BALB/c mice. Once the tumors reached an average volume of 100 mm^3, **4d** was administered daily via IP injection. They received different doses of compound **4d** for 21 consecutive days. As shown in Figure 6A, IP injection dosing of **4d** at 50, 100 and 150 mg/kg/day inhibited tumor growth in a dose-dependent manner in models. During the treatment period, no weight loss (Figure 6B) was observed in all the treatment groups. Our data clearly demonstrated that **4d** had potent antitumor activity against the growth of tumors in vivo.

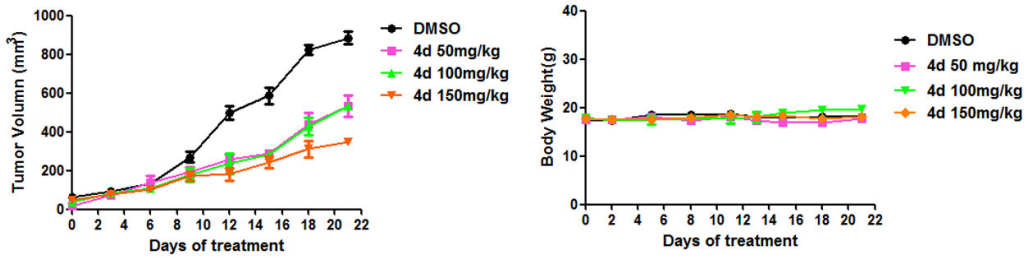


Figure 6. In vivo antitumor efficacy of **4d** against 4T1 (A) tumor xenograft models and (B) average body weights for treated mice. Animals were treated with solvent control, compound 4d at doses of 150, 100 and 50 mg/kg/day through IP injection. Points indicate mean tumor volumes (mm^3) or mean body weights (g); bars indicate SD.

In summary, through a hybrid-design approach based upon two privileged pharmacophores, namely pyrrolo[2,3-d] pyrimidines-2-amine and N-(4-fluorophenyl)-N-phenylcyclopropane-1,1-dicarboxamide, we finally successfully discovered a novel inhibitor, compound **4d**. Compound **4d** is a novel CDK9 inhibitor, which potently inhibited CDK9 with IC_{50} values of 12 nM. **4d** showed good selectivity in CDKs kinase profiling assay against CDK kinases and cell proliferation inhibition. In vitro cellar assays, **4d** showed potent activities against human breast cancer cell lines, T47D and MCF7, human lung cancer cell lines, A549, H1299 and H460. In vivo, compound **4d** exhibited a considerable ability to inhibit tumor and a low body weight loss of mice. These results confirmed that **4d** showed good antitumor efficacy and could be used as a novel CDK9 inhibitor to be further

researched on the therapy of solid tumors. More works will be done to characterize the therapeutic relevance of compound **4d**.

Acknowledgments

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A series of novel hybrid structure derivatives, containing both LEE011 and Cabozantinib pharmacophore, were designed, synthesized and evaluated. Surprisingly, a compound **4d** was discovered that highly exhibited effective and selective activity of CDK9 inhibition with IC_{50} = 12nM. It effectively induced apoptosis in breast and lung cancer cell lines at nanomolar level. The compound **4d** could block the cell cycle both in G0/G1 and G2/M phase to prevent the proliferation and differentiation of cancer cells. Mice bared-breast cancer treated with compound **4d** showed significant suppression of cancer with low toxicity.