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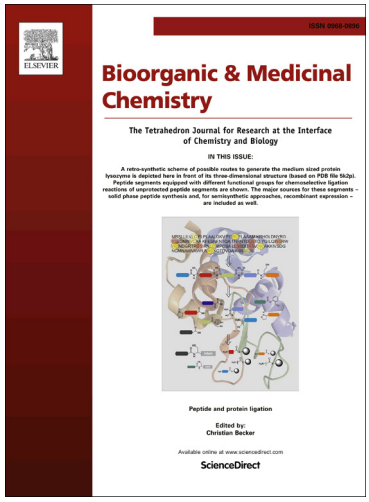

Phosphamide-Containing Diphenylpyrimidine Analogues (PA-DPPYs) as Potent Focal Adhesion Kinase (FAK) Inhibitors with Enhanced Activity against Pancreatic Cancer Cell Lines

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Phosphamide-Containing Diphenylpyrimidine Analogues (PA-DPPYs) as Potent Focal Adhesion Kinase (FAK) Inhibitors with Enhanced Activity against Pancreatic Cancer Cell Lines

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ABSTRCT

A family of phosphamide-containing diphenylpyrimidine analogues (PA-DPPYs) were synthesized as potent focal adhesion kinase (FAK) inhibitors. The PA-DPPY derivatives could significantly inhibit the FAK enzymatic activity within concentrations of lower than 10.69 nM. Among them, compounds **7a** and **7e** were two of the most active FAK inhibitors, possessing IC_{50} values of 4.25 nM and 4.65 nM, respectively. In particular, compound **7e** also displayed strong activity against AsPC cell line, with an IC_{50} of 1.66 μM, but show low activity against the normal HPDE6-C7 cells (IC_{50} > 20 μM), indicating its low cell cytoxicity. Additionally, flow cytometry analysis showed that after treatment of **7e** (8 μM, 72 h), both AsPC and Panc cells were almost totally inhibited, with a cell viability rate of 16.8% and 18.1%, respectively. Overall, compound **7e** may be served as a valuable FAK inhibitor for the treatment of pancreatic cancer.

Keywords: Pancreatic Cancer; FAK; Inhibitor; Pyrimidine.

1. Introduction

Pancreatic cancer (PC), which is characterized by its invasive nature, ability to evade aggressive therapy and frequent late stage diagnosis, leads to high mortality rate, with an average survival of only 10 months after diagnosis.[1] In 2012, PC is the seventh most common cause of cancer deaths, resulting in 330,000 deaths

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globally. PC is also the fifth most common cause of cancer death in the United Kingdom, and the fourth most common in the United States. The disease occurs most often in the developed world, where about 70% of the new cases in 2012 originated.[2–4] Accordingly, there is an urgent need for the development of novel effective and safe agents for the treatment of PC.

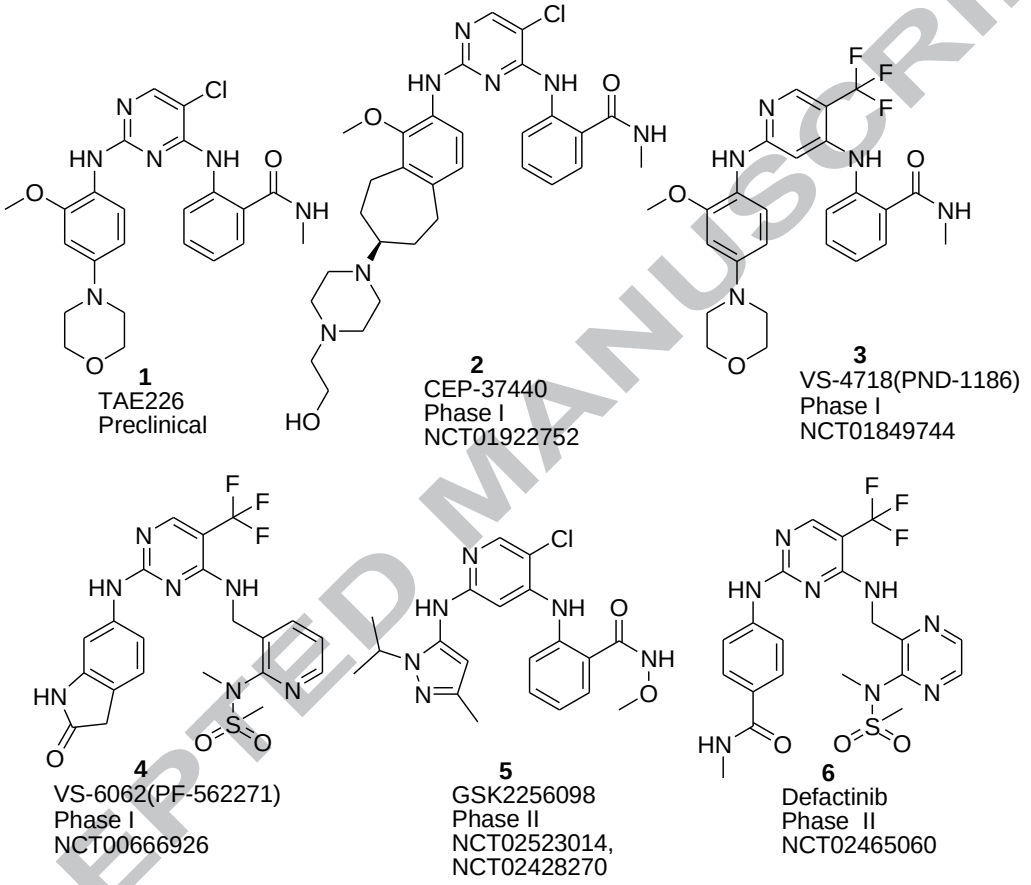

Figure 1. Chemical structures of the typical FAK inhibitors.

Focal adhesion kinase (FAK) is a cytoplasmic protein-tyrosine kinase that modulates cell adhesion, migration, proliferation and survival in response to extracellular signals.[5,6] FAK is aberrantly overexpressed or activated in most solid tumors, including the stubborn PC.[7–9] Phosphorylation of FAK at tyrosine (Y) 397 is required for its kinase activity to trigger down-stream events such as AKT and ERK activation. Therefore, the level of phospho-Y397 FAK has been used as an indicator of FAK activation.[10] Small molecules targeting FAK Y397 are attractive because blocking Y397 phosphorylation is expected to prevent uncontrolled PC growth caused

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by FAK overexpression or hyperactivity.[11]

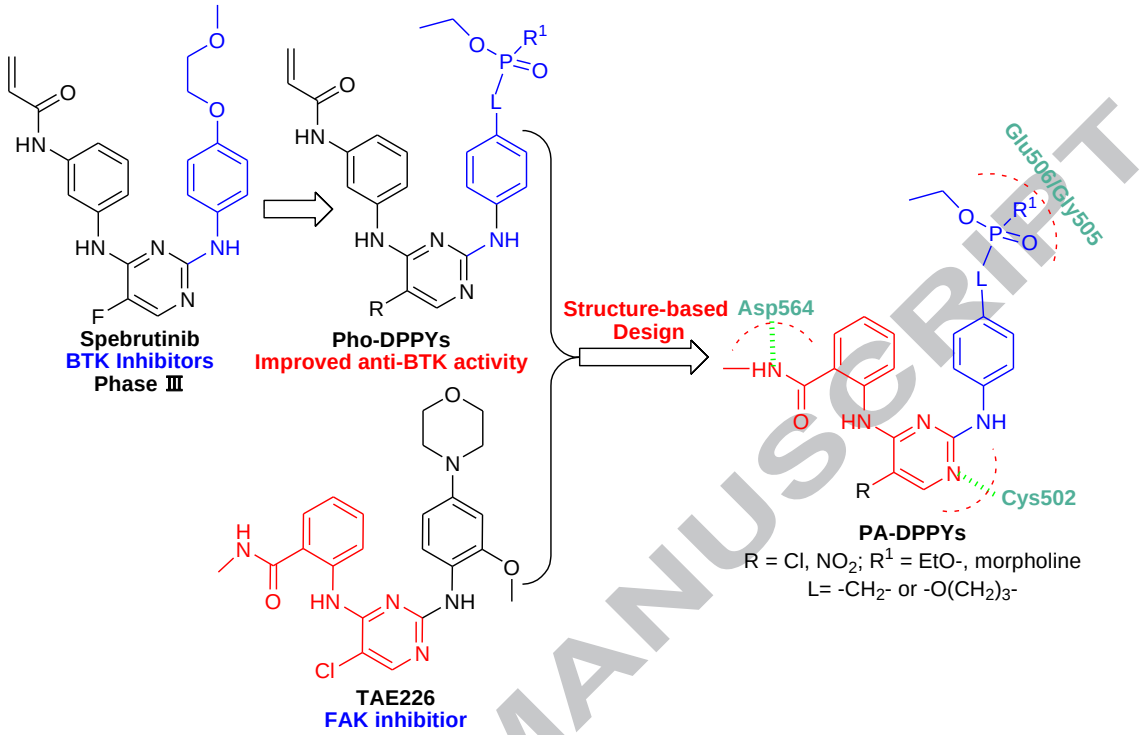

Figure 2. Designed strategy of the title molecules PA-DPPY analogues.

To date, several ATP-competitive reversible FAK inhibitors have been developed to target tumor and endothelial cells, including compounds TAE226 (**1**)[12-14], CEP-37440 (**2**, phase I, NCT01922752)[15,16], VS-4718 (**3**, PND-1186, phase I, NCT01849744)[17], VS-6062 (**4**, PF-562271, phase I, NCT00666926)[18,19], GSK2256098 (**5**, phase II, NCT02523014, NCT02428270)[20,21], and defactinib (**6**, phase II, NCT02465060)[22] (Figure 1). TAE226 is an exemplary FAK inhibitor, showing potent antiproliferative and antitumor effects *in vitro* and *in vivo* in several types of malignancies[12-14]. GSK2256098 was introduced as a novel FAK inhibitor currently in clinical testing for cancer treatment, displaying promising tumor regression effect in a variety of subcutaneously-inoculated xenograft models including LoVo human colon cancer, PC-3M prostate cancer and BxPC human PC[20,21]. Defactinib, a well-known FAK inhibitor, significantly impacts cell viability, decreases anchorage-independent growth and motility in selected PC cells by targeting FAK kinase activity and Y397 phosphorylation[22]. Generally, most of these potent FAK inhibitors possess a pyrimidine template along with a *N*-methylforamide-substituted aniline, which are essential to interact with amino acids Cys502, Asp564 and Leu567 in the FAK protein

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to interfere with FAK activity[23–25]. Phosphoryl functional group is very effective and widely used to improve the aqueous solubility and biological activity of anticancer alkylating agents that require activation *in vivo*[26–28]. Our previous studies also indicated that introduction of a phosphoryl group to the *C*-2 aniline side chain of the pyrimidine scaffold enhanced the activity against the Bruton's tyrosine kinase (BTK) and exhibited excellent anticancer activity against B-cell leukemia cells[29–32]. According to the Fragement-based drug design strategy, a new series of phosphamide-containing diphenylpyrimidine analogues (PA-DPPYs) were synthesized to strengthen the anti-FAK activity for the treatment of PC in this modification work (Figure 2). The synthesis and biological evaluation of these newly-designed PA-DPPYs, in parallel with their putative binding mechanism with the FAK enzyme, are also described in this manuscript.

2. Results and discussion

2.1. Chemistry

According to our reported synthetic method[33–37], these newly designed PA-DPPY analogues were synthesized as shown in Scheme 1. Commercially available 2-nitrobenzoic acid (**8**) was reacted with thionyl chloride, and then directly reacted with methylamine hydrochloride in the $NaHCO_3$ base to produce the 2-nitro-N-methylbenzamide (**9**). Compound **9** was then converted to 2-amino-N-methylbenzamide (**10**) under Fe-NH_4Cl reducing condition. By region selectively coupling with the C-2 chlorine atom in the pyrmidine reagent, compound **10** was converted to the 2-chloropyrimidine intermediate **11a**,**b**. Meanwhile, the chloro-substituted nitrobenzene derivatives **12a,b** were reacted with triethyl phosphate to synthesize the diethoxyphosphinyl nitrobenzenes **13a**,**b**, which were reduced using Fe-NH_4Cl conditions to form the anilines **14a**,**b**. Additionally, compounds **13a,b** were treated with thionyl chloride and then directly reacted with morpholine in the $NaHCO_3$ base to prepare the phosphoramide intermediates **16a,b**. Under the action of the Fe-NH_4Cl reduction reagent, compounds **16a,b** were reduced to the anilines **17a,b**. With these intermediates in hand, the desired compounds **7a-f**

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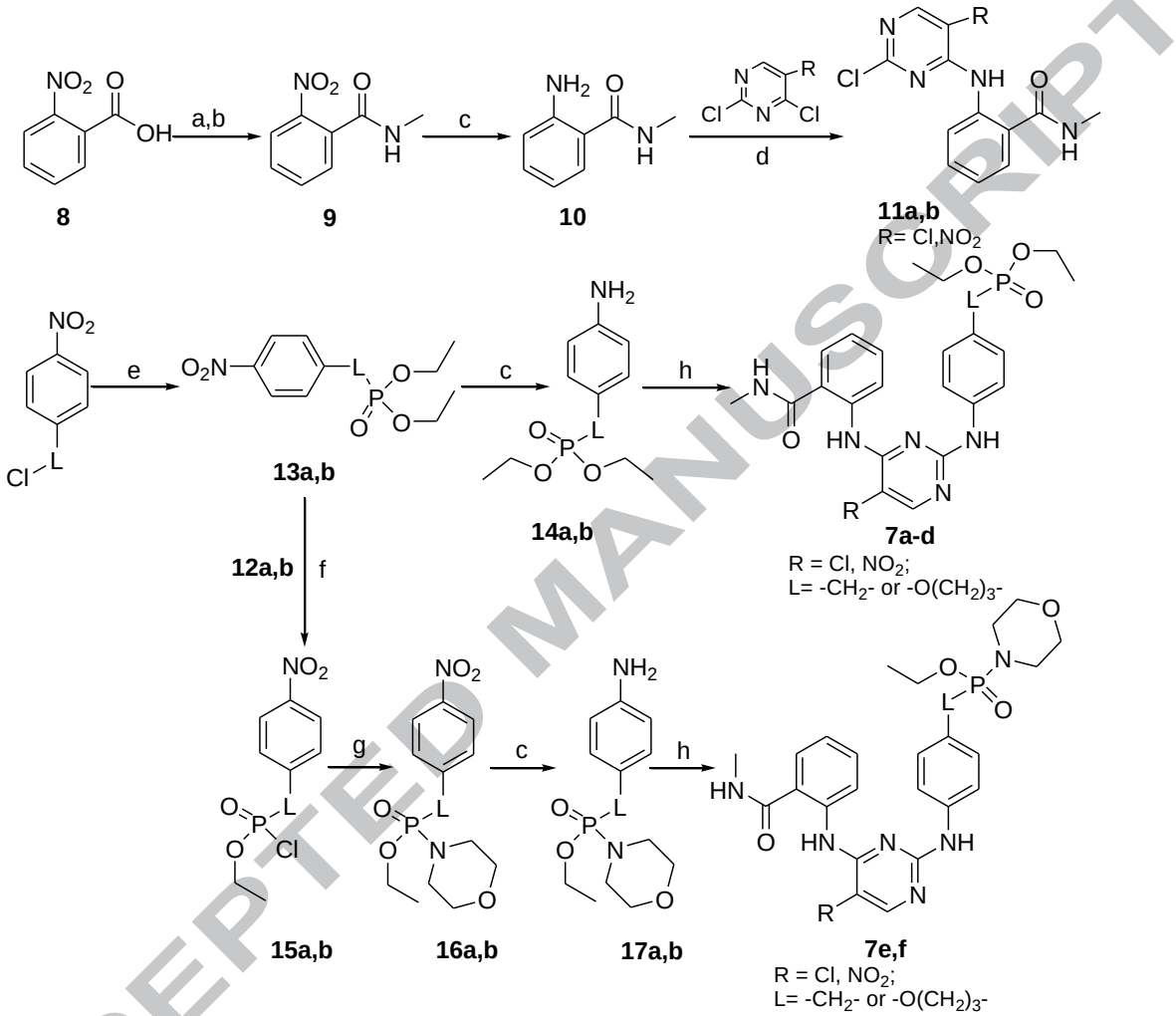
were conventionally synthesized *via* coupling reaction of anilines **14a,b** or **17a,b** with pyrimidines **11a,b** in the presence of trifluoroacetic acid (TFA) under reflux temperature.



Scheme 1. Synthetic route of the title compounds **7a-f**. Reagents and conditions: (a) $SOCl_2$, 1 h, 60 °C; (b) $H_2NMe{\cdot}HCl$, $NaHCO_3$, CH_3CN, 4 h, 80 °C, 95%; (c) Fe-NH_4Cl, MeOH-H_2O, 2 h, 70 °C, 65–78%; (d) *N*,*N*-diisopropylethylamine (DIPEA), isopropanol, 6 h, 85%; (e) $P(OEt)_3$, 5 h, 130 °C, 80-91%; (f) $(COCl)_2$, DMF, 60 °C, 2 h; (g) morpholine, THF, rt., 2 h, 92%; (h) TFA, 2-BuOH, 100 °C, 12 h, 12-30%.

2.2. Biological activity

All the PA-DPPY analogues were evaluated for their activity against the FAK enzyme using the ADP-Glo™ Kinase Assay system[38,39], as well as for the antiprilificative actvity against PC cell lines (AsPC, BXPC and Panc) using the MTT assay[40]. TAE226, the typical FAK inhibitor, was also tested for comparison. The test results are shown in Table 1. Apparently, PA-DPPY analogues displayed strong anti-FAK

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activity, with IC_{50} values ranging from 4.25 to 10.69 nM. Compared with TAE226, most of the PA-DPPY analogues exhibited equivalent or stronger inhibitory activity. In particular, compound **7a** (IC_{50} = 4.25 nM) and **7e** (IC_{50} = 4.65 nM), showed stronger anti-FAK activity than TAE226 (IC_{50} = 6.79 nM). The kinase-based test results indicated that the substitutens installed on the pyrimidine template did produce a little effect on their anti-FAK activity. For example, the *C*-2 chlorine-substituted analogues **7a,b** are as potent as the **7c-f** analogues with a nitro substitutent. Moreover, morpholine subsitutent in analogues **7e** and **7f** is equivalently beneficial to the ethoxyl group in analogues **7a-d**. Additionally, inhibitors **7a**, **7c** and **7f** , bearing a three-carbon linker are as favorable as inhibitors **7b**, **7d** and **7e** with a one-carbon linker. The effects on the viability of PC cell lines showed that compounds **7a** and **7e**, two of the strongest FAK inhibitors among PA-DPPYs, exhibited potent capacitcy to interfer with the proliferaction of PC cells, especially against the BxPC-3 cells, with IC_{50} values between 0.02 and 0.57 μM. Interestingly, compound **7e** also displayed strong activity against the AsPC cell line, with an IC_{50} of 1.66 μM, but was not active against normal HPDE6-C7 cells at concentraion of more than 20 μM, indicating its low cytoxicity towards normal cells. Nevertheless, most of these inhibitors are only moderatively active against the Panc-1 cells (IC_{50} >10.87 μM). Furthermore, the results in Figure 3 revealed that the cell viability was significantly decreased for both AsPC and Panc cells treated with inhibitor **7e,** in a time and concentration-dependent manner. After treatment with 8 μM concentration of **7e** for 72 h, both AsPC and Panc cells were almost totally inhibited, with a cell viability rate of 16.8% and 18.1%, respectively. Taken together, these results indicate that compound **7e** is a potent FAK inhibitor, nontoxic for normal cells, worthy of further research of its effects on PC cells.

Table 1 Biological activity of the newly synthesized compounds **7a-f**. [a]

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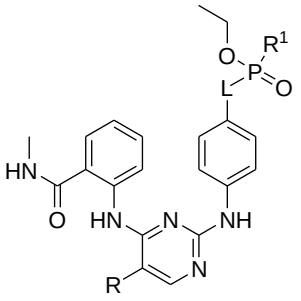

7a-f

Compd.	R	R^1	L	Enzymatic activity (IC_{50}, nM)[b]	Antiproliferative activity (IC_{50}, μM)[c]			
					AsPC-1	BxPC-3	Panc-1	HPDE6-C7
7a	Cl		$-CH_2-$	4.25±0.87	3.31±0.88	0.02±0.0009	14.76±2.12	5.44±1.02
7b	Cl		$-O(CH_2)_3-$	10.69±1.42	4.93±0.49	1.04±0.23	10.87±1.05	4.43±0.89
7c	NO_2		$-CH_2-$	5.01±0.98	15.19±2.31	0.82±0.11	19.19±2.01	4.60±0.91
7d	NO_2		$-O(CH_2)_3-$	6.18±0.89	18.26±2.15	2.04±0.38	25.38±3.12	1.96±0.92
7e	NO_2		$-O(CH_2)_3-$	4.65±0.91	1.66±0.48	0.57±0.089	>40.00	>20.00
7f	NO_2		$-CH_2-$	5.69±0.35	6.42±1.03	1.02±0.11	>40.00	8.11±0.99
TAE226				6.79±1.42	6.73±0.99	1.03±0.35	>20.00	11.38±1.28

[a] Data represent the mean of at least three separate experiments.
[b] Concentration needed to inhibit the autophosphorylation of the cytoplasmic domain of FAK by 50%, as calculated using software GraphPad Prim version 5.0. Mean values of three independent experiments±SE are reported (n=3).
[c] Dose-response curves were determined at five concentrations. The IC_{50} values are the concentrations in micoromolar needed to inhibit cell growth by 50%, as calculated using GraphPad Prism version 5.0.

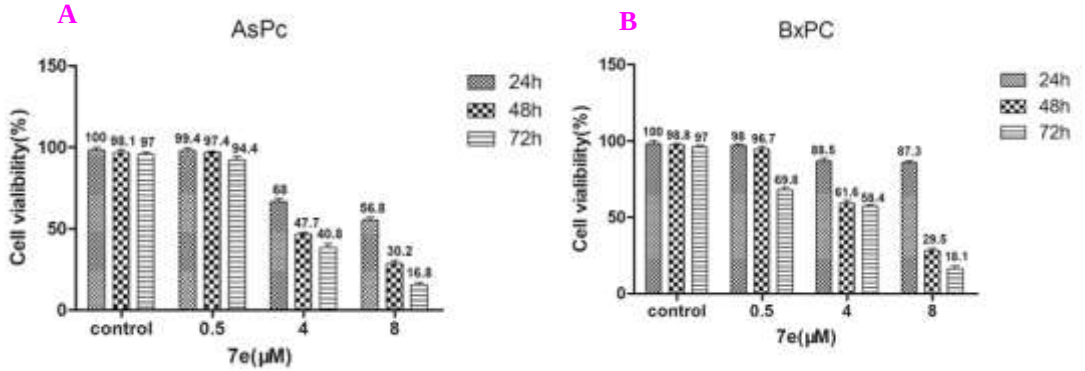

Figure 3. The effects of treating time and concentrations of inhibitor **7e** on cell viability, (A) AsPC cells; (B) BxPC cells.

In addition, flow cytometry performance presented in Figure 4 revealed that the

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active inhibitor **7e** triggered apoptosis in AsPC cells in a dose-and time-dependent manner[31]. The percentages of apoptotic cells increased from 41.7% to 91.1% after treatment with (2.5, 5, and 10 μM) **7e** for 72 h. The effect of **7e** on cell-cycle progression in AsPC cells was also investigated using by flow cytometry analysis (Figure 5). Compared with the control group, the percentages of cells in the G2/M phase increased from 0.89% to 22.1%, and those in the S phase decreased from 59.70% to 37.82% after treatment with **7e** at concentrations of 1, 2.5, and 5 μM for 72 h. While the percentages of cells in the G0/M phase showed only minor changes. Evidently, **7e** significantly blocked AsPC cells in the G2/M phase.

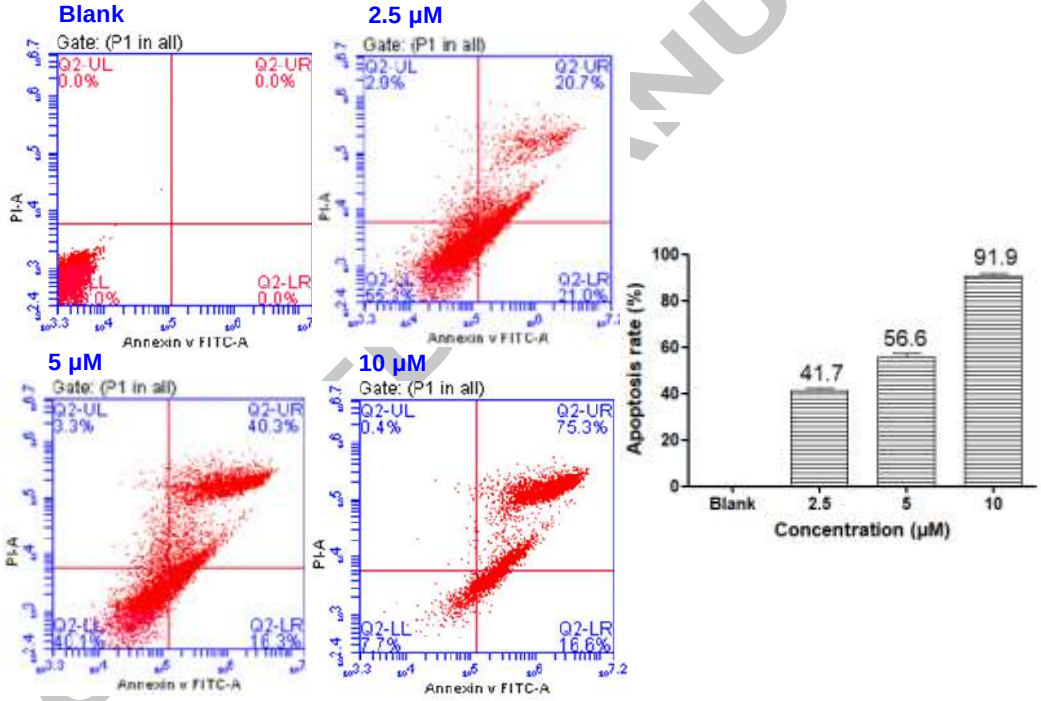

Figure 4. Compound **7e** induced AsPC-1 cell apoptosis *in vitro*. The cells were incubated with the indicated concentrations of **7e** for 72 h, and the cells were stained with annexinV/FTIC, followed by flow cytometry analysis. One representative experiment is shown.

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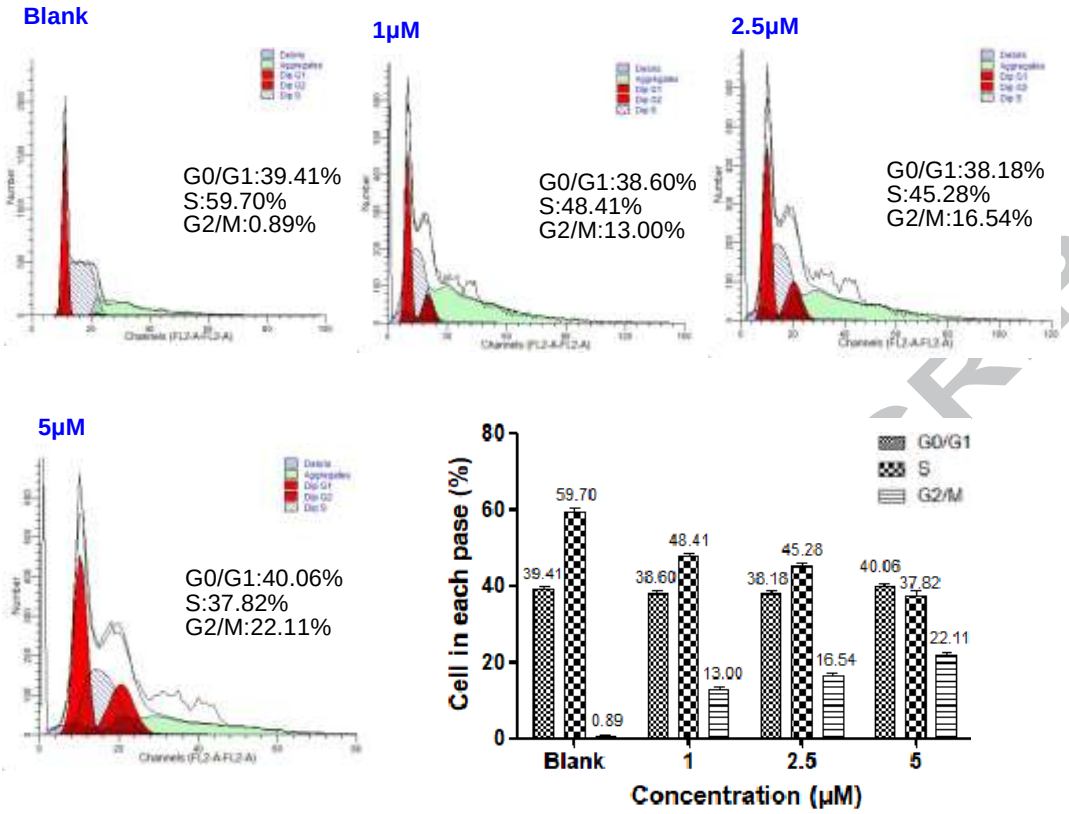

Figure 5. Effects of inhibitor **7e** on AsPC cells cycle arrest detected by flow cytometry assay. Cells were treated with different concentration of **7e** for 72 h, collected and fixed with 70% ethanol at 4 °C overnight. Then, the cells were stained by the mixture containing 5 mL propidium iodide for 10 min at 37 °C, and the cell cycle was analyzed by a flow cytometer.

Table 2. Enzymatic activity of compound **7e** against a small panel of kinases[a].

Kinase	%[b]	Kinase	%
FAK	92	EGFR	22
PYK2	71	EGFRT790M	45
AXL	60	BTK	33
AURK A	48	JAK3	26

[a] This test was conducted by the SelectScreen™ Biochemical Profiling Lab.

[b] Inhibiton rate at a concentration of 100 nM.

Furthermore, the typical FAK inhibitor **7e** was tested against a small panel of kinases at a concentration of 100 nM by using the SelectScreen™ Biochemical Profiling Lab platform (Thermo-Fisher, Waltham, MA, USA). The results presented in Table 2, revealed that, in addition to FAK, compound **7e** also strongly binds to the PYK2 and AXL kinases at a concentration of 100 nM, with inhibition rates of 71 and 60%, respectively. While for other kinase, compound 7e are not active, with inhibition rates lower than 50%.

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2.3. Molecular modeling analysis

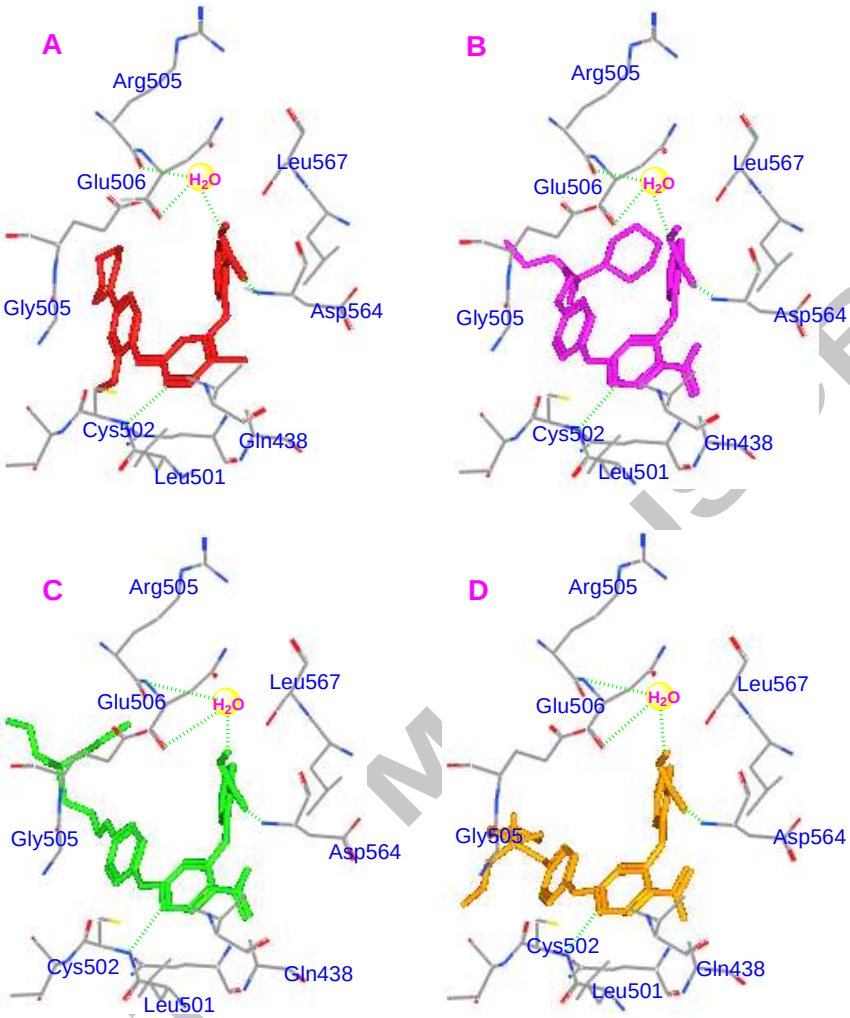

Figure 6. Predicted binding models of the typical PA-DPPY analogues in FAK enzyme (PDB code: 2JKK), A: TAE226; B: inhibitor **7e**; C: inhibitor **7d**; D: inhibitor **7f**.

To investigate the putative interaction mechanism of the PA-DPPY derivatives with the FAK enzyme, three representative molecules **7d**,**7e**, and **7f** were individually docked into the ATP binding pocket of the FAK enzyme (PDB: 2JKK).[41] The lead compound TAE226 was also analyzed for comparison (Figure 6A). The program AutoDock 4.2 with its default parameters was used.[42,43] All the docking results are presented in Figure 6, indicating that PA-DPPY derivatives could tightly contact with the FAK enzyme as to lead compound TAE226. Notably, PA-DPPY analogues formed three strong hydrogen bonds, which are produced by the *N*-1 atom in the pyrimidine core with amino acid Cys502, the carbonyl group in *N*-methylbenzamide with the amino acid Asp564, and the nitrogen atom in *N*-methylbenzamide with Arg505, Gly506 through a water molecule. From the docking results, it can be seen that only

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little differences in the hydrophilic binding sites surrounded by the amino acids Gly505 and Glu506 appeared for PA-DPPYs. Significantly, in this binding sections, the morpholine substituent and the ethoxyl group in PA-DPPYs formed strong contacts with the amino acid Gly505 and Glu506 through the polar induced forces. In addition, the C-5 substituents in the pyrimidine core, including the nitro and chlorine groups produced equivalent interaction forces with the amino acid Gln438. Due to the similar binding interaction forces, all these inhibitors displayed potent inhibitory potency against the FAK kinase activity. Overall, this putative binding models exactly explain their biological activity data.

3. Conclusion

A new class of pyrimidine derivatives featuring a phosphamide functional group were designed and synthesized as potent FAK inhibitors for the treatment of PC.Most of these compounds displayed strong activity against the FAK kinase at concentrations lower than 10.69 nM. Compounds **7a** and **7e,** the two most active inhibitors, could inhibit FAK activity at concentrations of 4.25 nM and 4.65 nM, respectively. The two inhibitors are clearly stronger than the novel FAK inhibitors TAE226 (IC_{50} = 6.79 nM). Compound **7e** also displayed strong activity against AsPC cell line, with an IC_{50} of 1.66 μM, but show low activity against normal HPDE6-C7 cells at a concentration higher than 20 μM, indicating its low cell cytoxicity. In addition, inhibitor **7e** also induced apoptosis in AsPC cells in a dose and time-dependent manner. In summary, this contribution provided an effective FAK inhibitor, namely **7e**, which may serve as a potential lead compound toward pancreatic cancer.

4. Experimental section

4.1. General methods and chemistry

Unless otherwise noted, all solvents and chemicals were used as purchased without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Brucker AV 400 MHz spectrometer and Brucker AV 100 MHz spectrometer, respectively. Coupling constants (J) are expressed in hertz (Hz). Chemical shifts (δ) of NMR are reported in parts per million (ppm) units relative to the residual solvent

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peak (d_6-DMSO: 39.0, TMS: 0.0). ^{1}H NMR spectra are reported in the following order: multiplicity, approximate coupling constant (J value) in hertz, and number of protons; signals are characterized as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet), and br (broad signal). High resolution ESI-MS was performed on an AB Sciex TripleTOF® 4600 LC/MS/MS system. All reactions were monitored by TLC, using silica gel plates with fluorescence F254 (TLC Silica gel 60 F254, Merck) and UV light visualization. Flash chromatography separations were obtained on Silica Gel (300–400 mesh) using dichloromethane/methanol as eluents.

4.2. Preparation of the 2-chloropyrimidine intermediates 11a,b

2-Amino-*N*-methylbenzamide **10** was prepared according to the general procedure in literatures 33 to 35. The prepared 2-amino-*N*-methylbenzamide **10** (1.00 g, 6.67 mmol) was added in one portion to 2,4,5-trichloropyrimidine (1.22 g, 6.67 mmol) or 2,4-dichloro-5-nitropyrimidine (1.29 g, 6.67 mmol) and DIPEA (1.29 g, 10.0 mmol) in isopropanol (100 mL). The resulting mixture was stirred at 85 °C for 6 h. The mixture was evaporated to dryness, and the residue was recrystallised from MeCN/water 20:1 to yield 2-(2-chloro-5-substitudedpyrimidin-4-ylamino)-*N*-methylbenzamide (1.69 g, 85 %).

4.3. Preparation of the title molecules 7a-f

Phosphoryl-substituted anilines **14a,b** and **17a,b** were prepared according to the literature 37. The synthesized anilines **14a,b** or **17a,b** (1.00 mmol), **11a,b** (1.00 mmol), TFA (1.50 mmol), and 2-BuOH (20 mL) was mixed in around-bottom flask. The slurry was heated to 100 °C for 12 h. The reaction mixture was allowed to cool to room temperature and was neutralized with a saturated aqueous sodium bicarbonate solution. The aqueous mixture was then extracted with CH_2Cl_2 (3× 20 mL) three times. The organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo to obtain a crude product. The residue was purified by column chromatography on silica gel (elution with 3% methanol /dichloromethane) to give the title products.

_N_-(3-((5-Chloro-2-((4-((diethoxyphosphoryl)methy))amino)phenylamino-4-pyrimidinyl)amino)phenyl)-_N_-methylbenzamide (7a)

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Orange-yellow solid (164 mg, 12%), Purity (HPLC): 98.9%, ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.61 (s, 1H), 9.46 (s, 1H), 8.73-8.77 (m, 2H), 8.22 (s, 1H), 7.8-7.67 (m, 1H), 7.59 (d, J = 8.4 Hz, 2H), 7.48-7.52 (m, 1H), 7.23-7.08 (m, 3H), 3.90-3.97 (m, 4H), 3.18（s, 1H）, 3.13 (s, 1H), 2.81 (d, J = 4.4 Hz, 3H), 1.17 (t, J = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.16, 157.96, 155.23, 154.90, 139.55, 138.95, 131.70, 129.98 (2C), 128.25, 125.50, 122.19, 121.71, 120.99, 119.85 (2C), 105.28, 61.58 (2C), 32.51, 31.16, 16.53 (2C); HRMS (ESI) m/z calcd for $C_{23}H_{27}ClN_5O_4P$ $[M+H]^+$ 504.1489, found 504.1568.

***N*-(3-((5-Chloro-2-((4-((diethoxyphosphoryl)propoxyl))amino)phenylamino-4-pyrimidinyl)amino)phenyl)-*N*-methylbenzamide (7b)**

Orang-yellow solid (165 mg, 18%), Purity (HPLC): 97.2%, ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.60 (s, 1H), 9.30 (s, 1H), 8.76 (d, J = 4.0 Hz, 2H), 8.18 (s, 1H), 7.75 (d, J = 8.0 Hz, 1H), 7.62-7.40 (m, 3H), 7.13 (t, J = 8.0 Hz, 1H), 6.87 (d, J = 8.0 Hz, 2H), 3.97-4.0 (m, 6H), 2.81 (d, J = 4.0 Hz, 3H), 1.89 (s, 4H), 1.22 (t, J = 8.0 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.40, 158.43, 155.41, 155.12, 154.11, 139.86, 133.83, 131.95, 128.43, 122.31, 122.04 (2C), 121.77, 121.03, 114.78 (2C), 104.96, 67.58, 61.45 (2C), 22.78, 22.31, 20.92, 16.74 (2C); HRMS(ESI) *m/z* calcd for $C_{25}H_{31}ClN_5O_5P$ $[M+H]^+$ 548.1751, found 548.1832.

***N*-(3-((5-Nitro-2-((4-((diethoxyphosphoryl)methyl))amino)phenylamino-4-pyrimidinyl)amino)phenyl)-*N*-methylbenzamide (7c)**

Orang-yellow solid (100 mg, 29%), Purity (HPLC): 98.5%, ^{1}H NMR: (400 MHz, DMSO-d_6) δ 12.05 (s, 1H), 10.50 (s, 1H), 9.12 (s, 1H), 8.65 (d, J = 4.4 Hz, 1H), 8.18 (d, J = 6.8 Hz, 1H), 7.80-7.37 (m, 4H), 7.36-7.01 (m, 3H), 4.27-3.65 (m, 4H), 3.21 (s, 1H), 3.15 (s, 1H), 2.77 (d, J = 4.4 Hz, 3H), 1.17 (t, J = 8.0 Hz, 6H); ^{13}C NMR: (100 MHz, DMSO-d_6) δ 168.30, 159.92, 158.40, 154.08, 137.36, 136.32, 130.69, 130.29, 130.22, 128.35, 127.98, 127.89, 127.11, 126.07, 124.90, 121.60, 121.14, 61.78 (2C), 32.74, 31.40, 16.69 (2C); HRMS (ESI) *m/z* calcd for $C_{23}H_{27}N_6O_6P$ $[M+H]^+$ 515.1730, found 515.1813.

***N*-(3-((5-Nitro-2-((4-((diethoxyphosphoryl)propoxyl))amino)phenylamino-4-pyrimidinyl)amino)phenyl)-*N*-methylbenzamide (7d)**

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Orang-yellow solid (120 mg, 20%), Purity (HPLC): 96.7%, ^{1}H NMR: (400 MHz, DMSO-d_6) δ 12.05 (s, 1H), 10.39 (s, 1H), 9.09 (s, 1H), 8.63 (d, *J* = 4.0 Hz, 1H), 8.15 (d, *J* = 8.0 Hz, 1H), 7.63 (d, *J* = 8.0 Hz, 1H), 7.51 (d, *J* = 12 Hz, 3H), 7.27 (s, 1H), 6.81 (d, *J* = 8.0 Hz, 2H), 3.97-4.02 (m, 6H), 2.77 (d, *J* = 4.0 Hz, 3H), 1.89 (s, 4H), 1.23 (t, *J* = 8.0 Hz, 6H); ^{13}C NMR: (400 MHz, DMSO-d_6) δ 168.30, 159.90, 158.37, 155.43, 154.11, 136.34, 131.90, 130.73, 128.29, 126.93, 125.94, 124.86, 123.13 (2C), 121.30, 114.73 (2C), 67.74, 61.40 (2C), 26.62, 22.29, 20.89, 16.80 (2C); HRMS (ESI) *m/z* calcd for $C_{25}H_{31}N_6O_7P$ $[M+H]^+$ 559.1992, found 559.2080.

***N*-(3-((5-Nitro-2-((4-(((1-morpholino)ethoxyphosphinyl)propoxyl))amino)phenyl amino-4-pyrimidinyl)amino)phenyl)-*N*-methylbenzamide (7e)**

Orang-yellow solid (124 mg, 23%), Purity (HPLC): 98.1%, ^{1}H NMR: (400 MHz, DMSO-d_6) δ 12.05 (s, 1H), 10.39 (s, 1H), 9.09 (s, 1H), 8.63 (d, *J* = 4.6 Hz, 1H), 8.16 (d, *J* = 8.2 Hz, 1H), 7.63 (d, *J* = 8.0 Hz, 1H), 7.56-7.41 (m, 3H), 7.27 (t, *J* = 8.0 Hz, 1H), 6.82 (d, J = 8.0 Hz, 2H), 4.10-3.75 (m, 4H), 3.52 (t, *J* = 4.4 Hz, 4H), 3.01 (d, *J* = 2.6 Hz, 4H), 2.77 (d, *J* = 4.0 Hz, 3H), 1.81-1.88 (m, 4H), 1.21 (t, *J* = 8.0 Hz, 3H); ^{13}C NMR: (100 MHz, DMSO-d_6) δ 168.30, 159.90, 158.38, 155.45, 154.12, 136.35, 131.87, 130.75, 128.30, 126.93, 125.94, 124.87, 123.139 (2C), 121.30, 114.74 (2C), 67.96, 67.02 (2C), 59.26, 43.98 (2C), 26.62, 22.55, 20.97, 16.70; HRMS (ESI) *m/z* calcd for $C_{27}H_{34}N_7O_7P$ $[M+H]^+$ 600.2257, found 600.2340.

***N*-(3-((5-Nitro-2-((4-(((1-morpholino)ethoxyphosphinyl)methy))amino)phenylami no-4-pyrimidinyl)amino)-*N*-methylbenzamide (7f)**

Orang-yellow solid (307 mg ,25%), Purity (HPLC): 98.5%, ^{1}H NMR: (400 MHz, DMSO-d_6) δ 12.04 (s, 1H), 10.51 (s, 1H), 9.12 (s, 1H), 8.65 (s, 1H), 8.18 (d, *J* = 4.0 Hz, 1H), 7.80-7.42 (m, 4H), 7.29 (s, 1H), 7.15 (s, 2H), 4.06-3.68 (m, 2H), 3.42 (d, *J* = 12.4 Hz, 4H), 3.11 (d, *J* = 12.0 Hz, 2H), 3.01-2.66 (m, 7H), 1.21 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR: (100 MHz, DMSO-d_6) δ 168.31, 159.88, 158.37, 154.14, 137.27, 136.32, 130.74, 130.41, 130.35, 128.42, 128.37, 127.18, 126.10, 124.95, 121.58, 120.95 (2C), 66.92 (2C), 59.61, 44.18 (2C), 33.04, 31.80, 16.73; HRMS (ESI) m/z calcd for $C_{25}H_{30}N_7O_6P$ $[M+H]^+$ 556.1995, found 556.2077.

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4.4. In vitro kinase enzymatic assay

FAK enzyme system (Catalog. V9301, Promega) was used for *in vitro* enzymatic assay. Cncentrations consisting of suitable levels from 1 to 200 nM were used for all of the tested compunds. The test was performed in a 384-well plate. Detailed and complete protocols can be founded in the ADP-Glo™ kinase Assay Technical Manual available at: https://cn.promega.com/resources/protocols/product-information-sheets/n/fak-kinase-enzyme-system-protocol/. Briefly, procedures include: (1) perform a 5 μL kinase reaction using 1× kinase buffer (e.g., 1× reactionbuffer A), (2) incubate at room temperature for 60 minutes, (3) add 5 μL of ADP-Glo™ Reagent to stop the kinase reaction and deplete theunconsumed ATP, leaving only ADP and a very low background of ATP, (4) incubate at room temperature for 40 minutes, (5) add 10 μL of Kinase Detection, (6) reagent to convert ADP to ATP andintroduce luciferase and luciferin to detect ATP, (7) incubate at room temperature for 30 minutes, (8) plate was measured on TriStar® LB942 Multimode Microplate Reader (BERTHOLD) to detect the luminescence (Integration time 0.5-1 second). Curve fitting and data presentations were performed using GraphPad Prism version 5.0.

4.5. Cellular activity assay

AsPC-1, Panc-1, BxPC-3, and HPDE6-C7 cells were purchased from Fuheng Biology Company (Shanghai, China). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent was purchased from Biotool Company (Switzerland). The Annexin V-FITC Apoptosis Detection Kit and Cell Cycle Assay were purchased from Beyotime Company (China). All cell lines were grown in DMEM (Gibco®, USA) supplemented with 10% FBS (Gibco®, USA), 1% penicillin-streptomycin (Beyotime Company, China). The cells were maintained and propagated as monolayer cultures at 37 °C in humidified 5% CO_2 incubator.

Cells were seeded in a 96-well plate at a density of 4,000 to 5,000 cells/well and were maintained at 37 °C in a 5% CO_2 incubator in DMEM containing 10% FBS for one day. Cells were then exposed to the synthesiaed inhibitors for 72 h, and the

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number of cells used per experiment for each cell lines was adjusted to obtain an absorbance of 0.5 to 1.2 at 570 nm with a microplate reader (Thermo, USA). Compounds were tested at appropriate concentrations (1.25 to 40 μmol/L), with each concentration duplicated five times. TAE226 was employed as the positive control. The IC_{50} values were calculated using GraphPad Prim version 5.0.

4.6. Cell apoptosis assay

AsPC-1 cells (3 to 5×10^5 cells/well) incubated in 6-well plates were treated with solvent control (DMSO), or compound **7e** (1, 1.2 or 5 μM) in medium containing 5% FBS for 72 h. Then, collected and fixed with 70% ethanol at 4 °C overnight. After being fixed with 75% ethanol at 4 °C for 24 h, the cells were stained with Annexin V-FITC (5 μL)/propidium iodide (5 μL), and analyzed by flow cytometry (Becton-Dickinson, USA).

4.7. Molecular docking study

The AutoDock 4.2 software with its default parameters was used to perform the docking exploration. Detailed tutorials that guide users through basic AutoDock usage, docking with flexible rings, and virtual screening with AutoDock can be founded at: http://autodock.scripps.edu/faqs-help/tutorial/using-autodock-4-with-autodocktools. Generally, the crystal structure (PDB: 2JKK) of the kinase domain of FAK enzyme bound to inhibitor TAE226 was used in the docking studies. Enzyme preparation and the hydrogen atoms adding was performed in the prepared process. The whole EGFR enzyme was defined as a receptor and the site sphere was selected on the basis of the binding location of TAE226. By moving TAE226 and the irrelevant water molecule, the typical inhibitor **7e** was docked into the pocket. The binding interaction energy was calculated to include Van der Waals, electrostatic, and torsional energy terms defined in the tripos force field. The structure optimization was performed using a genetic algorithm, and only the best-scoring ligand protein complexes were kept for analyses.

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Graphical Abstract

Phosphamide-Containing Diphenylpyrimidine Analogues (PA-DPPYs) as Potent Focal Adhesion Kinase (FAK) Inhibitors with Enhanced Activity against Pancreatic Cancer Cell Lines

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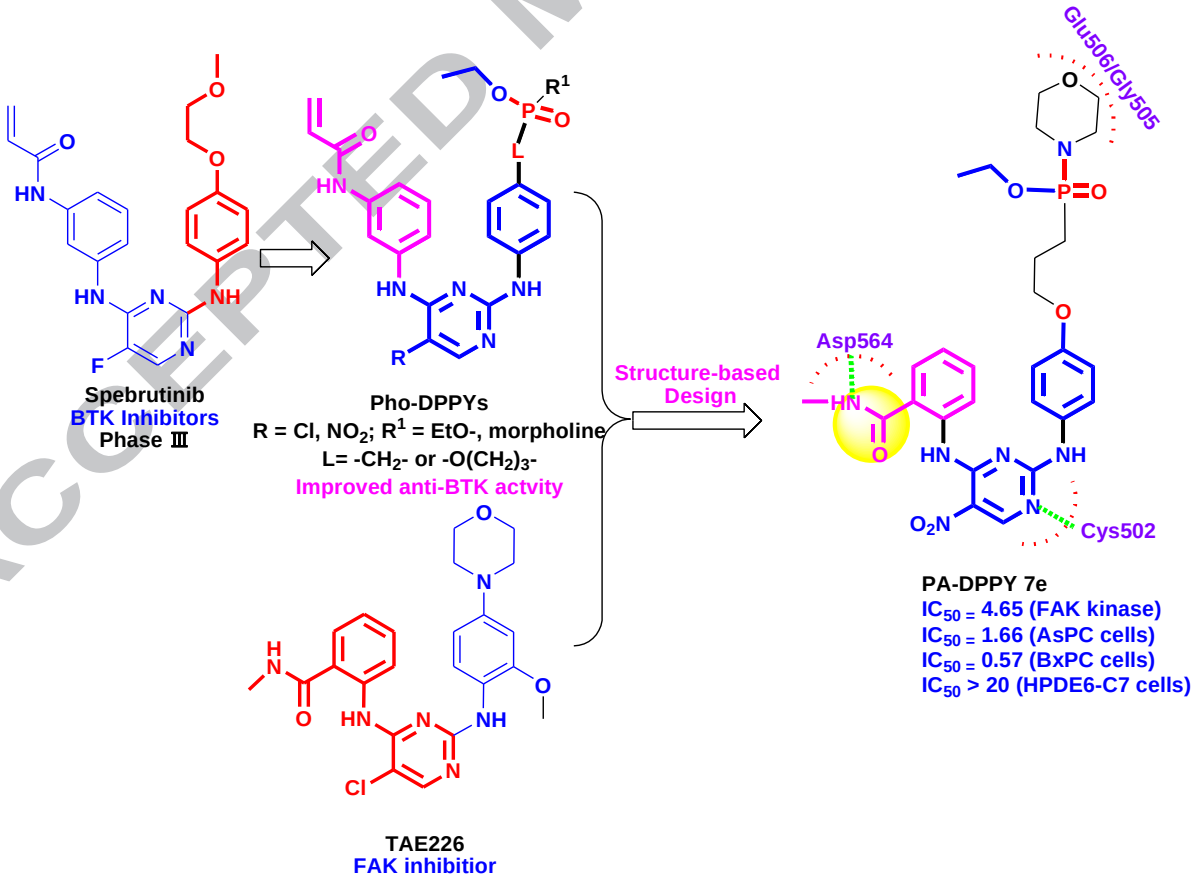

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Highlights

- PA-DPPYs could significantly inhibit FAK activity at concentrations ranged from 4.25 to 4.65 nM.
- PA-DPPYs displayed improved activity against pancreatic cancer cell lines compared with TAE226..
- The typical compound **7e** triggered apoptosis in AsPC cells in a dose and time-dependent manner.

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