Discovery of novel KRAS–PDEδ inhibitors with potent activity in patient-derived human pancreatic tumor xenograft models

Long Chen\textsuperscript{a,\textdagger}, Jing Zhang\textsuperscript{b,\dagger}, Xinjing Wang\textsuperscript{c,d,\textdagger}, Yu Li\textsuperscript{a}, Lu Zhou\textsuperscript{e}, Xiongxiong Lu\textsuperscript{c,d,*}, Guoqiang Dong\textsuperscript{a,*}, Chunquan Sheng\textsuperscript{a,*}

\textsuperscript{a}School of Pharmacy, Second Military Medical University, Shanghai 200433, China
\textsuperscript{b}Department of Pathology, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China
\textsuperscript{c}Department of General Surgery, Pancreatic Disease Center, Ruijin Hospital, Shanghai Jiao Tong University, Shanghai 200025, China
\textsuperscript{d}Research Institute of Pancreatic Diseases, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China
\textsuperscript{e}Department of Medicinal Chemistry, School of Pharmacy, Fudan University, Shanghai 201203, China

Received 19 April 2021; received in revised form 3 July 2021; accepted 5 July 2021

Abstract

KRAS–PDEδ interaction is revealed as a promising target for suppressing the function of mutant KRAS. The bottleneck in clinical development of PDEδ inhibitors is the poor antitumor activity of known chemotypes. Here, we identified novel spiroyl-cyclic PDEδ inhibitors with potent antitumor activity both \textit{in vitro} and \textit{in vivo}. In particular, compound 36l (\(K_D = 127 \pm 16 \) nmol/L) effectively bound to PDEδ and interfered with KRAS–PDEδ interaction. It influenced the distribution of KRAS in Mia PaCa-2 cells, downregulated the phosphorylation of t-ERK and t-AKT and promoted apoptosis of the cells. The novel inhibitor 36l exhibited significant \textit{in vivo} antitumor potency in pancreatic cancer patient-derived xenograft (PDX) models. It represents a promising lead compound for investigating the druggability of KRAS–PDEδ interaction.

© 2022 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

\textsuperscript{*}Corresponding authors. Tel./fax: +86 21 64370045 671003 (Xiongxiong Lu), +86 21 81871242 (Guoqiang Dong), +86 21 81871239 (Chunquan Sheng).

E-mail addresses: simone515night@126.com (Xiongxiong Lu), gdong@smmu.edu.cn (Guoqiang Dong), shengcq@hotmail.com, shengcq@smmu.edu.cn (Chunquan Sheng).

\textsuperscript{\textdagger}These authors made equal contributions to this work.

Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2021.07.009

2211-3835 © 2022 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
1. Introduction

Pancreatic cancer is one of the most fatal solid tumors with a high recurrence rate and poor prognosis. Mutations in KRAS gene are considered to be a key factor of pancreatic cancer progression. However, the development of KRAS-targeting antitumor agents is highly challenging and a number of drug candidates failed in clinical trials. Recently, the development of KRAS\textsuperscript{G12C} inhibitors has gained great attention, which shows promising results in phase I clinical trial. However, KRAS\textsuperscript{G12C} is a comparatively minor part of the KRAS mutation spectrum (approximately 11%)\textsuperscript{13}, inhibitors of other KRAS mutation subtypes, such as KRAS\textsuperscript{G12D} and KRAS\textsuperscript{G12V}, remain to be further investigated\textsuperscript{14,15}.

Chaperone protein PDE\textsuperscript{G} plays an essential role in the function of KRAS protein in the cell\textsuperscript{16,17}. PDE\textsuperscript{G} enhances the diffusion of KRAS in the cytoplasm by binding the farnesyl group, and promotes its distribution over intracellular membrane\textsuperscript{18}. Down-modulation or inhibition of PDE\textsuperscript{G} protein suppresses KRAS signaling, and inhibits the growth and proliferation of cancer cells\textsuperscript{17}.

KRAS–PDE\textsuperscript{G} interaction has been proposed as a potential target for the development of novel antitumor agents. Currently, a series of KRAS–PDE\textsuperscript{G} inhibitors have been reported (Fig. 1)\textsuperscript{19}. Waldmann’s group\textsuperscript{17} discovered the first KRAS–PDE\textsuperscript{G} inhibitor deltarasin (1) by fragment based drug design (FBDD) at 2013. However, the selectivity of deltarasin was poor, which showed apparent cytotoxicity\textsuperscript{20}. Subsequently, more selective inhibitors deltazinone (2) and deltasonamide (3) were designed\textsuperscript{20–22}. Although these inhibitors generally showed high PDE\textsuperscript{G} binding affinity, further development was hampered by poor cellular potency and metabolic stability. More recently, triazole inhibitor (4)\textsuperscript{23}, tetrahydrodibenzofuran inhibitor NHTD (5)\textsuperscript{24} and coumarin inhibitor deltaflexin (6)\textsuperscript{25} were reported. However, the antitumor efficacy of known PDE\textsuperscript{G} inhibitors remains to be significantly improved. Previously, our group reported the discovery and optimization of quinazolinone KRAS–PDE\textsuperscript{G} inhibitors 7 and 8 based on structural biology guided FBDD\textsuperscript{26,27}. Furthermore, we designed fluorescent probes and protein degraders of PDE\textsuperscript{G} that offered new chemical tools to investigate the biological function of KRAS–PDE\textsuperscript{G} interaction. However, known KRAS–PDE\textsuperscript{G} inhibitors are generally limited by weak in vivo efficacy. For example, deltarasin was unspecific to PDE\textsuperscript{G} protein, leading to a “switch-like” response to cell death. Deltazinone was unable to exert in vivo antitumor activity due to poor metabolic stability\textsuperscript{20,21}. In order to identify novel KRAS–PDE\textsuperscript{G} inhibitors with enhanced antitumor potency, herein new spiro-cyclic PDE\textsuperscript{G} inhibitors were discovered from screening of an in-house library.

After structure-based hit optimization, compound 36l was identified to effectively inhibit the KRAS–PDE\textsuperscript{G} interaction and disrupt the distribution of KRAS protein. Interestingly, inhibitor 36l showed potent anti-pancreatic cancer activity both in vitro and in vivo, which represented the first KRAS–PDE\textsuperscript{G} inhibitor with in vivo efficacy in the patient-derived xenograft (PDX) models. Also, compound 36l is a promising lead compound for the treatment of pancreatic cancer.

2. Results and discussion

2.1. Discovery of a spiro-cyclic PDE\textsuperscript{G} inhibitor

Our in-house compound library containing diverse spiro-scaffolds\textsuperscript{30–33} and approved drugs was screened using the fluorescence polarization (FP) assay. First, the inhibitory ratio of all compounds was tested at 5 \( \mu \)mol/L (Supporting Information Table S1). Two compounds (atorvastatin and spiperone) were identified to have an inhibitory rate over 50%. Then, the \( K_D \) value of the hit compounds was further determined. Atorvastatin has been reported to be a PDE\textsuperscript{G} inhibitor and is generally used as the probe in the FP assay\textsuperscript{17}. Spiperone (9), which shared a spiro scaffold, exhibited moderate activity in a low micro-molar range to PDE\textsuperscript{G} (\( K_D \) = 1471 ± 246 nmol/L, Fig. 2A and B). Subsequently, it was chosen for further structural optimization.

2.2. Molecular docking and drug design

In order to determine the binding mode of compound 9 towards PDE\textsuperscript{G} protein, Glide docking with extra precision (XP) was performed. According to the predicted docking pose (Fig. 2C), the amide group within the spiro scaffold of compound 9 formed a hydrogen bond to Tyr149, while the \( p \)-fluorobenzoyl moiety binds to the Arg61 pocket through hydrogen bonding interactions between the ketone group and Arg61 and Gln78, respectively. The docking pose exhibited an obvious hydrophobic cavity in the Arg61 pocket, which suggested that the introduction of hydrophobic groups could improve the binding affinity. Guided by the binding conformation, structure-based drug design (SBDD) was applied to design a series of new inhibitors (Fig. 2D). First,
various amides were introduced to replace the p-fluorobenzoyl moiety. Then N-monosubstitution or N-di-substitution was introduced by aromatic or aliphatic ring. The spiro moiety was reserved to maintain the binding conformation.

2.3. Chemistry

The synthetic route for the synthesis of target compounds 23a–23e is shown in Scheme 1. The substituted primary amines reacted with 4-chlorobutyl chloride to give intermediates 22, subsequently 22 reacted with intermediate 18 to afford target compounds 23a–23e.

The synthetic route for compounds 36a–36o is shown in Scheme 2. Reductive amination was conducted to prepare intermediates 33 with substituted aldehydes 31 and aniline 32. Then, compounds 33 were substituted by γ-butyrolactone to obtain primary alcohols 34, which were oxidized to aldehydes 35. Finally, reductive amination was conducted between intermediates 35 and 18 to afford target compounds 36a–36o.

2.4. Biological evaluations and structure—activity relationships

Initially, compounds 23a–23e with N-monosubstitutions were assayed. As shown in Table 1, these compounds generally showed modest inhibitory activities (Kd range: 1538–2186 nmol/L), which were comparable to hit compound 9 (Kd = 1471 ± 246 nmol/L). However, it was worth noting that introducing a large size group led to decreased PDEδ binding affinities. For example, naphthalen-1-yl derivative 23c showed the weakest activity (Kd = 2186 ± 385 nmol/L).

When an additional phenyl group was added to the amine, most di-substituted compounds 36 showed comparable or superior activity to the lead compound. In particular, cyclohexyl (compound 36l, Kd = 127 ± 16 nmol/L) and cyclopentyl (compound 36m, Kd = 159 ± 29 nmol/L) derivatives showed excellent PDEδ binding affinity, which was about 7–9 fold more active than lead compound 9 (Kd = 1471 ± 246 nmol/L). In contrast, less steric cyclobutyl (compound 36n) or cyclopropyl (compound 36o) derivative showed decreased activity. Replacement of the cycloalkyl-methyl substitions by benzyl (36a–36d, Kd range: 328–1311 nmol/L) or heterocyclic methyl group (36e–36k, Kd range: 1240–1657 nmol/L) generally led to reduced PDEδ binding affinity. The docking result (Fig. 3A) revealed that the amide substitutions of 36l could engange the hydrophobic pocket of PDEδ protein in which the cyclohexyl moiety formed hydrophobic interactions with residues Trp32, Val145 and Leu147. Moreover, the key hydrogen interactions with Arg61, Gln78 and Tyr149 were retained. The hydrophobic interaction between phenyl moiety and Leu38, Thr131 and Phe133 could also be observed, which was absent in the docking pose of compound 9 (Fig. 3B).

Furthermore, in vitro antitumor activity of the target compounds was assayed against human pancreatic cancer cell line Mia PaCa-2 (Table 1). PDEδ inhibitors 9 and 2 failed to exert cellular potency (IC50 > 100 μmol/L). Interestingly, most target compounds showed moderate to good antitumor activity. Among them, compound 36l (Kd = 127 ± 16 nmol/L, IC50 = 6.3 ± 1.7 μmol/L) possessed balanced inhibitory activity both at the molecular and cellular level. Furthermore, patient-driven primary cells 098 and 099 were applied to evaluate the potential clinical application of compound 36l (Fig. 4). The 098 and 099 were both KRASG12D mutant pancreatic cancer cells. The result showed that 36l (IC50 range: 6.4–7.6 μmol/L) exhibited potent activity against primary pancreatic cancer cell line, while compound 2 (IC50 > 100 μmol/L) was totally inactive. Moreover, water solubility of inhibitor 36l (0.63 mmol/L) was about 20 fold higher than compound 2 (0.030 mmol/L). Considering the activity and physicochemical properties, compound 36l was chosen for further biological evaluations.

2.5. The cellular thermal shift assay (CETSA)

The CETSA assay is a useful method for the discovery of the intracellular target of inhibitors. The method evaluates the change in the stability when protein binds with the small molecules, which is fast and convenient without purifying the protein34,35. Mia Paca-2 cells were treated by compound 36l at 20 and 50 μmol/L, respectively, using compound 2 as a positive drug (Fig. 5A). Increased Tmagg (aggregation temperature) values were observed in the group treated by compounds 2 and 36l. In addition, compound 36l introduced a higher Tmagg value change than that of compound 2 at 50 μmol/L. The experiment verified PDEδ protein was the target of compound 36l in Mia Paca-2 cells.

2.6. Co-immunoprecipitation (Co-IP) experiment

Co-IP is a powerful technique for the identification of protein–protein interaction36,37. Co-IP experiment was further conducted
to evaluate the inhibition of KRAS–PDEδ by compound 36l (Fig. 3B). The cells were treated with compound 36l at 20 μmol/L for 24 h. Western blotting experiment was used to identify KRAS and PDEδ protein, respectively. The level of PDEδ/KRAS in the 36l treating group was lower than the control group. The results confirmed that the compound 36l effectively disrupted the interaction of KRAS–PDEδ.

### 2.7. Compound 36l inhibited the phosphorylation of AKT in Mia PaCa-2 cells

The mutant KRAS protein activates downstream signal pathways, such as RAS-RAF-MAPK and PI3K-AKT-mTOR pathway, and then stimulates the growth, proliferation, and differentiation of cancer cells. Thus, the effects of compound 36l on the phosphorylation of total extracellular signal-regulated kinase (t-ERK) and protein kinase B (t-AKT) were evaluated. Based on our previous work, the epidermal growth factor (EGF) was applied to induce the expression and phosphorylation of t-ERK and t-AKT. Phosphorylation levels were determined by treating Mia PaCa-2 cells with compounds 36l for 4 h followed by stimulating for another 10 min with EGF. Compound 2 was used as positive control. As shown in Fig. 6, compounds 36l interfered with phosphorylation of t-ERK and t-AKT in a dose-dependent manner in Mia PaCa-2 cells, and significantly reduced their phosphorylation at 100 μmol/L, which was more potent than that of positive compound 2.

### 2.8. Compound 36l increased KRAS-dependent apoptosis and influenced the distribution of KRAS protein in Mia PaCa-2 cells

The results of apoptosis assay demonstrated that positive control 2 induced cell apoptosis with an apoptotic rate of 28.55% at 50 μmol/L and 38.32% at 100 μmol/L (respectively, Fig. 7). Similarly, compound 36l effectively promoted apoptosis of Mia PaCa-2 cells, and induced apoptosis in 19.42%, 31.22% and 54.28% at 5, 10 and 20 μmol/L, respectively. The results suggested that both compounds 2 and 36l induced apoptosis in a dose-dependent manner. Spiro-cyclic inhibitor 36l was more potent even at a lower concentration. Immunofluorescence staining was further applied to evaluate the KRAS distribution in Mia PaCa-2 cells (Fig. 8). Compound 36l was observed to accumulate PDEδ around the cell nucleus and disrupt the distribution of KRAS to endomembrane at 20 μmol/L, while compound 2 showed similar effects at the higher concentration (100 μmol/L).

### 2.9. Compound 36l inhibited proliferation of the pancreatic tumor in patient-derived xenograft (PDX) model

In order to evaluate the in vivo anti-pancreatic tumor activity, patient-derived primary cell lines were applied to detect the anti-proliferative effects of compound 36l (Fig. 9). Four KRAS mutant clinical primary pancreatic cancer cell lines [0001 (KRAS<sup>G12A</sup>), 0034 (KRAS<sup>G12D</sup>), 0037 (KRAS<sup>G12V</sup>) and 0043 (KRAS<sup>G12D</sup>)] were treated with 36l at different time. The results revealed that compound 36l dose-dependently inhibited the growth of primary cell lines. Moreover, 0001, 0037 and 0034 cell lines were more sensitive to inhibitor 36l than the 0043 cell line. Pharmacokinetic study and cellular permeability of 36l were further conducted in ICR mice. The results indicated that the spiro-cyclic inhibitor 36l exhibited acceptable plasma exposures (C<sub>max</sub> = 15.50 μg/mL, AUC<sub>0- infinity</sub> = 59.23 h·μg/mL) and moderate cellular permeability (Supporting Information Fig. S1 and Table S3), which enabled further in vivo studies.

Based on the cellular potency of inhibitor 36l, PDX models were further applied to evaluate the in vivo antitumor potency. Guided by the antiproliferative assay to primary cells, the excised tumor tissue of patients produced 0034 primary cells was implanted into nude female mice. Consideration of the metabolic instability of compound 2, gemcitabine, a cytotoxic antitumor agent, was chosen as the positive drug. As depicted in Fig. 9, Compound 36l significantly inhibited the tumor growth with the tumor growth inhibition (TGI) rate of 57.3% (Fig. 10A and C). Moreover, the significant difference of tumor weight was also observed between compound 36l and the control group (Fig. 10C). Despite that the in vivo antitumor activity of compound 36l was lower than cytotoxic antitumor agent gemcitabine, it was the first KRAS–PDEδ inhibitor with potent antitumor efficacy in PDX models. To further validate the in vivo antitumor potency, compound 36l (25, 50 and 75 mg/kg, QD) and positive control 2 (50 mg/kg, QD) were evaluated in Mia PaCa-2 pancreatic cancer mouse xenograft models. In consistent with the results from PDX models, compound 36l dose dependently inhibited the tumor growth without obvious adverse effects (Supporting Information Fig. S2). These results highlighted the therapeutic potential of this new class of PDEδ inhibitors in treating pancreatic cancer.

Furthermore, hematoxylin-eosin (H&E) staining and immunohistochemistry (IHC) for Ki-67 was performed for the investigation of the action mechanism of inhibitor 36l against the pancreatic tumor in vivo. As shown in Fig. 10D and E, hyalination and reduction of cellularity was observed in the tumor treated with compound 36l (marked by dark arrow respectively) in the images of H&E staining and magnification image, while decreased proliferation was exhibited as shown by Ki-67 immunostaining. The same variation was visible in the gemcitabine group. The results supported the in vivo efficacy of 36l.

### 3. Conclusions

In summary, new spiro-cyclic KRAS–PDEδ inhibitors were discovered by screening an in-house compound library and structure-based hit optimization. As compared with the hit compound, inhibitor 36l showed improved PDEδ binding affinity and antitumor activity. In KRAS-dependent Mia PaCa-2 cells, compound 36l promoted the apoptosis, induced down-regulation of t-AKT phosphorylation and disrupted the diffusion of KRAS in the cytoplasm. The spiro-cyclic KRAS–PDEδ inhibitors revealed remarkable advantages over known ones due to the potent anti-tumor activity both in vitro and in vivo. In particular, compound 36l showed good therapeutic efficacy in the pancreatic cancer PDX models. Taken together, this study provides a promising lead compound for investigating the druggability of KRAS–PDEδ protein–protein interaction. Further structural optimization of the spiro-cyclic inhibitors is in progress.

### 4. Experimental

#### 4.1. Chemistry

All reagents were commercially available and used without further purification. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded on
Bruker AVANCE300 or AVANCE600 spectrometer (Bruker Company, Germany). CDCl₃ or DMSO-d₆ were solvents with TMS as an internal standard. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and Hz, respectively. HRMS spectra were detected on an Esquire 3000 LC−MS mass spectrometer. Precoated plates GF-254 (Qingdao Haiyang Chemical, China) and silica gel 60G (Qindao Haiyang Chemical) were adopted for monitoring reaction and column chromatography respectively. The purities of final compounds were analyzed by HPLC (Agilent 1260), and were greater than 95%.

4.1.1. 1-Phenyl-1,3,8-triazaspiro[4.5]decan-4-one (18)

The synthesis of intermediate 18 was according to the literature. White solid. 1H NMR (600 MHz, DMSO-d₆) δ 8.91 (s, 1H), 7.21 (dd, 2H, J = 7.5, 8.7 Hz), 6.98 (d, 2H, J = 8.2 Hz), 6.76 (t, 1H, J = 7.5 Hz), 4.60 (s, 2H), 3.43−3.48 (m, 2H), 3.18−3.21 (m, 2H), 3.69−2.75 (m, 2H), 1.73 (d, 2H, J = 14.2 Hz).

4.1.2. 4-Chloro-N-phenylbutanamide (22a)

A mixture of aniline 21a (470 mg, 1 eq, 5 mmol), 4-chlorobutyryl chloride (850 mg, 1.2 eq, 6 mmol), TEA (1.01 g, 2 eq, 10 mmol) in DCM (20 mL) was reacted at room temperature for 2 h. The mixture was washed with saturated Na₂CO₃ solution. The organic layer was combined and washed with brine, then it was evaporated to afford the crude product. Intermediate 22a was purified by flash column chromatography (dichloromethane/ethyl acetate 75:25) as white solid (yield 51.4%). 1H NMR (300 MHz, DMSO-d₆) δ 9.98 (s, 1H), 7.59 (d, 2H, J = 7.7 Hz), 7.28 (t, 2H, J = 7.7 Hz), 7.02 (t, 1H, J = 7.7 Hz), 3.70 (t, 2H, J = 6.8 Hz), 2.48 (t, 2H, J = 6.8 Hz), 1.98−2.07 (m, 2H).

4.1.3. 4-(4-Oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-phenylbutanamide (23a)

Intermediate 22a (400 mg, 2 eq, 2 mmol), 18 (230 mg, 1 eq, 1 mmol) and KI (17 mg, 0.1 eq, 0.1 mmol) were added into acetonitrile (10 mL) and reacted under reflux. Additional 22a (1 eq, 1 mmol) was added to the reaction 2 h later. The mixture was reacted for another 24 h under reflux, and then concentrated under reduced pressure and purified by flash column chromatography (dichloromethane/methanol 100:7) to give target molecules 23a (yield 36.4%). 1H NMR (600 MHz, DMSO-d₆) δ 10.02 (s, 1H), 8.69 (s, 1H), 7.52 (d, 2H, J = 8.2 Hz), 7.27 (t, 2H, J = 8.2 Hz), 7.20 (t, 2H, J = 8.2 Hz), 7.11 (t, 1H, J = 7.2 Hz), 6.87 (d, 2H, J = 8.2 Hz), 6.78 (t, 1H, J = 7.6 Hz), 4.57 (s, 2H), 3.18 (br, 4H), 2.78 (t, 2H, J = 7.6 Hz), 2.61−2.66 (m, 2H), 2.37 (t, 2H, J = 6.9 Hz), 1.85−1.90 (m, 2H), 1.73 (d, 2H, J = 14.4 Hz), 13C NMR (150 MHz, DMSO-d₆) δ 176.46, 171.46, 143.75, 139.81, 129.47, 129.10, 123.37, 119.50, 118.16, 114.79, 59.14, 58.51, 57.49, 49.59, 34.69, 28.48, 22.53. HRMS: m/z calcd. for C₂₃H₂₈N₄O₂ 392.2212, found 393.2270 [M+H]+. HPLC purity: 95.3%, tₚ = 8.942 min.

4.1.4. N-(2,3-Dihydro-1H-inden-5-yl)-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)butanamide (23b)

Pale solid, 135 mg, yield 31.3%. Synthesized by the protocol of compound 23a with 22b (475 mg, 2 mmol), 18 (230 mg, 1 mmol) and KI (17 mg, 0.1 mmol) and purified by flash column chromatography (dichloromethane/methanol = 100:7). 1H NMR (600 MHz, DMSO-d₆) δ 10.02 (s, 1H), 8.69 (s, 1H), 7.52 (d, 2H, J = 8.2 Hz), 7.27 (t, 2H, J = 8.2 Hz), 7.20 (t, 2H, J = 8.2 Hz), 7.11 (t, 1H, J = 7.2 Hz), 6.87 (d, 2H, J = 8.2 Hz), 6.78 (t, 1H, J = 7.6 Hz), 4.57 (s, 2H), 3.18 (br, 4H), 2.78 (t, 2H, J = 7.6 Hz), 2.61−2.66 (m, 2H), 2.37 (t, 2H, J = 6.9 Hz), 1.85−1.90 (m, 2H), 1.73 (d, 2H, J = 14.4 Hz), 13C NMR (150 MHz, DMSO-d₆) δ 176.46, 171.46, 143.75, 139.81, 129.47, 129.10, 123.37, 119.50, 118.16, 114.79, 59.14, 58.51, 57.49, 49.59, 34.69, 28.48, 22.53. HRMS: m/z calcd. for C₂₃H₂₈N₄O₂ 392.2212, found 393.2270 [M+H]+. HPLC purity: 95.3%, tₚ = 8.942 min.
Novel spiro-cyclic PDE\textsubscript{1}\textalpha inhibitors with potent activity in PDX models.

Scheme 2  Reagents and conditions: (h) MgSO\textsubscript{4}, NaBH\textsubscript{4}, MeOH, rt, overnight; (i) \(\gamma\)-butyro-lactone, trimethylaluminum, toluene, 80 °C, 12 h; (j) DMP, DCM, rt, 2 h; (j) 18, MgSO\textsubscript{4}, NaBH\textsubscript{4}, MeOH, rt, overnight.

4.58 (s, 2H), 3.02 (br, 4H), 2.71 (t, 2H, \(J = 9.8 \text{ Hz}\)), 7.29 (m, 1H), 7.08 (t, 2H, \(J = 7.6 \text{ Hz}\)), 6.90 (d, 2H, \(J = 8.7 \text{ Hz}\)), 6.78 (t, 1H, \(J = 7.6 \text{ Hz}\)), 6.47 (s, 2H), 2.00 (t, 2H, \(J = 7.6 \text{ Hz}\)), 1.55 (m, 2H), 1.17 (m, 3H). 13C NMR (150 MHz, DMSO-\(d_6\)) \(\delta 175.70, 171.54, 143.16, 134.71, 133.32, 130.91, 129.02, 128.50, 127.58, 126.21, 125.79, 125.61, 123.52, 117.86, 114.34, 58.74, 57.63, 56.49, 48.79, 40.23, 32.83, 27.39, 21.58. HRMS: \(m/z\) calcd. for C\textsubscript{20}H\textsubscript{19}N\textsubscript{2}O\textsubscript{2} 456.2525, found 457.2604 [M+H]\textsuperscript{+}. HPLC purity: 99.3%, \(t_k = 11.154 \text{ min.}\)

4.1.6. N-Cyclohexyl-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl]butanamide (23d)

White solid, 142 mg, yield 35.7%. Synthesized by the protocol of compound 23a with 22d (410 mg, 2 mmol), 18 (230 mg, 1 mmol) and Ki (17 mg, 0.1 mmol) and purified by flash column chromatography (dichloromethane/methanol = 100:7). 1H NMR (600 MHz, DMSO-\(d_6\)) \(\delta 8.71 (s, 1H), 7.78 (d, 1H, J = 7.6 \text{ Hz}), 7.25 (t, 2H, J = 7.6 \text{ Hz}), 6.90 (d, 2H, J = 7.6 \text{ Hz}), 6.78 (t, 1H, J = 7.6 \text{ Hz}), 4.60 (s, 2H), 3.98-4.01 (m, 1H), 2.86-2.90 (m, 4H), 2.58-2.63 (m, 2H), 2.47-2.50 (m, 2H), 2.10 (t, 2H, J = 7.6 \text{ Hz}), 1.77-1.81 (m, 2H), 1.70-1.75 (m, 2H), 1.60-1.65 (m, 4H), 1.49-1.51 (m, 2H), 1.33-1.39 (m, 2H). 13C NMR (150 MHz, DMSO-\(d_6\)) \(\delta 176.39, 171.70, 143.74, 129.49, 118.26, 114.85, 59.17, 57.402, 57.402, 50.624, 49.491, 33.625, 32.773, 28.338, 23.905, 22.686. HRMS: \(m/z\) calcd. for C\textsubscript{29}H\textsubscript{23}N\textsubscript{2}O\textsubscript{2} 384.2525, found 385.2602 [M+H]\textsuperscript{+}. HPLC purity: 98.9%, \(t_k = 9.887 \text{ min.}\)

4.1.8. N-(2-Fluorobenzyl)aniline (33a)

The synthesis of secondary amines 33a was applied the reductive amination procedure according to the literature procedure [22]. In brief, a solution of 2-fluorobenzaldehyde 31a (465 mg, 1 eq, 5 mmol) and aniline 32 (0.47 g, 1 eq, 5 mmol) in methanol (25 mL) was treated with MgSO\textsubscript{4} (1.2 g, 10 mmol) at room temperature overnight. Sodium borohydride (95 mg, 0.5 eq, 2.5 mmol) was added into the reaction under ice bath and reacted for another 2 h. The mixture was filtered. The methanol kept and removed in vacuo to give conditions for another 2 h. The mixture was filtered. The methanol kept and removed in vacuo to give the secondary amines. The product 33a (825 mg) was used directly in the next step without purification.

1H NMR (300 MHz, DMSO-\(d_6\)) \(\delta 7.35 (t, 1H, J = 7.5 \text{ Hz}), 7.22-7.29 (m, 1H), 7.08-7.17 (m, 2H), 7.01 (t, 2H, J = 7.8 \text{ Hz}), 2.62-2.64 (m, 2H), 2.11 (t, 2H, \(J = 8.7 \text{ Hz}\)), 1.55-1.75 (m, 9H), 1.23-1.29 (m, 2H), 1.09-1.17 (m, 3H). 13C NMR (150 MHz, CDCl\textsubscript{3}) \(\delta 173.00, 143.77, 129.55, 118.31, 114.87, 59.24, 58.38, 57.32, 49.48, 47.90, 33.70, 33.05, 28.26, 25.79, 25.14, 22.61. HRMS: \(m/z\) calcd. for C\textsubscript{23}H\textsubscript{25}N\textsubscript{2}O\textsubscript{2}. 398.2782, found 399.2763 [M+H]\textsuperscript{+}. HPLC purity: 99.2%, \(t_k = 9.850 \text{ min.}\)
6.46–6.55 (m, 2H), 6.16 (t, 2H, J = 5.9 Hz), 4.26 (d, 2H, J = 6.2 Hz).

4.1.9. N-(2-Fluorobenzyl)-4-hydroxy-N-phenylbutanamide (34a)
The secondary amines 33a (600 mg, 1 eq, 3 mmol) was dissolved into dry toluene (10 mL) and protected by nitrogen. The reaction was cooled down to 0 °C, and trimethylaluminum (2 mol/L in toluene, 2 eq, 3 mL) was added to the solution dropwise. The mixture was reacted for 2 h and γ-butyrolactone (510 mg, 2 eq, 6 mmol) was added. The solution was reacted at 65 °C for 24 h. The reaction mixture was moved to ice bath. Then, methanol (5 mL) was added dropwise followed by saturated sodium tartrate solution. Ethyl acetate (30 mL) was used to wash the solution and combined. The crude product was afford by removing the ethyl acetate and purified by flash column chromatography (hexane/ethylacetate = 40:60–0:100) to give intermediate 34a (105 mg, 12.2% yield). 1H NMR (300 MHz, DMSO-d6) δ 7.05–7.39 (m, 9H), 4.90 (s, 2H), 4.36 (t, 1H, J = 5.1 Hz), 3.28 (t, 2H, J = 6.3 Hz), 2.08 (t, 2H, J = 7.2 Hz), 1.57–1.66 (m, 2H).

4.1.10. N-(2-Fluorobenzyl)-4-oxo-N-phenylbutanamide (35a)
A solution of 34a (145 mg, 1 eq, 0.5 mmol) in dry DCM (15 mL) were stirred at 0 °C added Dess–Martin reagent (425 mg, 2 eq, 1 mmol). The resulting suspension was reacted for 1 h at room temperature. After completion of the reaction, the solution was added saturated sodium thiosulfate solution followed by saturated Na2CO3 solution. The water phase was washed with DCM twice. The organic layer was combined, washed with brine and evaporated under reduced pressure to afford crude product. The crude product was purified by column chromatography (hexane/ethyl acetate = 75:25) to give target molecules 35a (86 mg, 60.3% yield). 1H NMR (300 MHz, DCDCl3) δ 9.80 (s, 1H), 7.31–7.37 (m, 4H), 7.17–7.22 (m, 1H), 7.05–7.10 (m, 3H), 6.92 (t, 1H, J = 8.8 Hz), 4.96 (s, 2H), 2.78 (t, 2H, J = 6.4 Hz), 2.36 (t, 2H, J = 6.4 Hz).

4.1.11. N-(2-Fluorobenzyl)-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-phenylbutanamide (36a)
Target compounds 36a were prepared by the reductive amination by using 35a (142 mg, 1 eq, 0.5 mmol), 18 (115 mg, 1 eq, 0.5 mmol) and MgSO4 (300 mg, 2.5 mmol) in dry methanol (10 mL) as the procedure of preparation of 33a. The product was purified by flash C18 column chromatography [H2O (0.1% TFA)/MeOH = 40:60–80:20] to give target molecules 36a (pale solid, 76 mg, yield 30.4%). 1H NMR (600 MHz, DMSO-d6) δ 8.60 (s, 1H), 7.32–7.35 (m, 3H), 7.24–7.27 (m, 2H), 7.18–7.21 (m, 4H), 7.11–7.13 (m, 1H), 7.06–7.09 (m, 1H), 6.75–6.78 (m, 3H), 4.91 (s, 2H), 4.55 (s, 2H), 2.58 (br, 4H), 2.35–2.40 (m, 2H), 2.21 (t, 2H, J = 6.9 Hz), 2.11 (t, 2H, J = 6.7 Hz), 1.65–1.67 (m, 2H), 1.59 (d, 2H, J = 13.5 Hz). 13C NMR (150 MHz, DMSO-d6) δ 163.53, 161.33, 159.69, 157.18, 153.61, 147.75, 135.13, 135.01, 134.64, 132.52, 131.97, 131.92, 131.29, 130.27, 129.67, 129.44.

### Table 1 Structures, binding affinity and anti-pancreatic cancer activities of PDEδ inhibitors.

| Compd. | R1 | R2          | PDEδ (K_{IC50}, nmol/L) | Mia PaCa-2* (IC_{50}, μmol/L) |
|--------|----|-------------|-------------------------|-------------------------------|
| 23a    | H  | Ph          | 1670 ± 260              | 46 ± 14                       |
| 23b    | H  | 2,3-Dihydro-1H-inden-5-yl | 1538 ± 166              | 3.6 ± 2.1                    |
| 23c    | H  | Naphthalen-1-ylmethyl     | 2186 ± 385              | 4.8 ± 1.2                     |
| 23d    | H  | Cyclobexyl              | 1699 ± 178              | 64 ± 8.1                     |
| 23e    | H  | Cyclopentyl             | 1543 ± 244              | >100                          |
| 36a    | Ph | 2-F-Benzyl              | 1311 ± 219              | 16 ± 0.1                      |
| 36b    | Ph | 4-Cl-Benzyl             | 328 ± 59                | 4.3 ± 2.1                     |
| 36c    | Ph | 2-Br-Benzyl             | 485 ± 35                | 12 ± 0.60                     |
| 36d    | Ph | 3-Br-Benzyl             | 690 ± 198               | 4.9 ± 1.4                     |
| 36e    | Ph | 1H-Imidazole-4-ylmethyl | 1657 ± 436              | >100                          |
| 36f    | Ph | Thiophen-2-ylmethyl     | 1475 ± 197              | 15 ± 5.4                      |
| 36g    | Ph | Thiophen-3-ylmethyl     | 1240 ± 106              | 17 ± 8.3                      |
| 36h    | Ph | Pyridin-2-ylmethyl      | 1373 ± 317              | 46 ± 21                       |
| 36i    | Ph | Pyridin-4-ylmethyl      | 1482 ± 18               | 55 ± 2.8                      |
| 36j    | Ph | Quinolin-6-ylmethyl     | 1343 ± 36               | 12 ± 4.0                      |
| 36k    | Ph | Quinolin-3-ylmethyl     | 1328 ± 32               | 13 ± 2.8                      |
| 36l    | Ph | Cyclohexylmethyl        | 127 ± 16                | 6.7 ± 1.7                     |
| 36m    | Ph | Cyclopropylmethyl       | 159 ± 29                | 18 ± 1.5                      |
| 36n    | Ph | Cyclobutylmethyl        | 393 ± 27                | 19 ± 4.0                      |
| 36o    | Ph | Cyclopropylmethyl       | 1125 ± 140              | 26 ± 1.6                      |
| 9      | /  | /                       | 1471 ± 246              | >100                          |
| 2      | /  | /                       | 34 ± 4                  | >100                          |

*Tested by fluorescent anisotropy assay.
*Tested by the CCK8 method.
129.10, 128.57, 128.48, 125.77, 117.96, 117.10, 116.96, 115.86, 115.82, 115.53, 114.00, 69.18, 66.93, 45.82. MS (ESI): \( m/z \) calcd. for C\(_{30}\)H\(_{33}\)FN\(_4\)O\(_2\) 500.2588, found 501.2658 [M+H].

HPLC purity: 98.7%, \( t_R = 13.036 \) min.

4.1.12. N-(4-Chlorobenzyl)-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-phenylbutanamide (36b)

Pale solid, 86 mg, yield 33.2%. Synthesized by the protocol of compound 36a with 35b (150 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography [H\(_2\)O (0.1% TFA)/methanol = 40:60—80:20]. \(^1\)H NMR (600 MHz, DMSO-d\(_6\)) \( \delta \) 8.65 (s, 1H), 7.34—7.39 (m, 4H), 7.30 (t, 1H, \( J = 7.1 \) Hz), 7.18—7.24 (m, 6H), 6.78—6.82 (m, 3H), 4.86 (s, 2H), 4.58 (s, 2H), 2.71 (d, 4H, \( J = 7.8 \) Hz), 2.41—2.46 (m, 2H), 2.23 (t, 2H, \( J = 6.5 \) Hz), 2.14 (t, 2H, \( J = 8.4 \) Hz), 1.69—1.74 (m, 2H), 1.56 (d, 2H, \( J = 13.6 \) Hz). \(^{13}\)C NMR (150 MHz, DMSO-d\(_6\)) \( \delta \) 176.00, 171.88, 143.28, 142.14, 136.79, 131.62, 129.82, 129.48, 128.98, 128.27, 128.16, 117.83, 114.48, 58.64, 58.08, 56.64, 51.30, 48.97, 31.23, 28.08, 22.12. HRMS (ESI): \( m/z \) calcd. for C\(_{30}\)H\(_{33}\)ClN\(_4\)O\(_2\) 516.2292, found 517.2381 [M+H].

HPLC purity: 96.9%, \( t_R = 14.829 \) min.

4.1.13. N-(2-Bromobenzyl)-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-phenylbutanamide (36c)

Yellow solid, 99 mg, yield 35.3%. Synthesized by the protocol of compound 36a with 35c (172 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography [H\(_2\)O (0.1% TFA)/methanol = 50:50—80:20]. \(^1\)H NMR (600 MHz, DMSO-d\(_6\)) \( \delta \) 8.62 (s, 1H), 7.55 (d, 1H, \( J = 8.1 \) Hz), 7.27—7.39 (m, 7H), 7.16—7.22 (m, 3H), 6.76—6.79 (m, 3H), 4.93 (s, 2H), 4.56 (s, 2H), 2.56—2.59 (m, 4H), 2.36—2.41 (m, 2H), 2.24 (t, 2H, \( J = 7.1 \) Hz), 2.18 (t, 2H, \( J = 7.7 \) Hz), 1.66—1.71 (m, 2H), 1.56 (d, 2H, \( J = 14.1 \) Hz). \(^{13}\)C NMR (150 MHz, DMSO-d\(_6\)) \( \delta \) 176.15, 172.47, 143.34, 142.34, 136.60, 132.47, 129.71, 129.41, 129.09, 128.96, 128.04, 127.73, 122.58, 117.71, 114.40, 58.61, 58.29, 56.82, 52.20, 49.91, 48.58, 31.22, 28.42, 22.47. HRMS (ESI): \( m/z \) calcd. for C\(_{30}\)H\(_{33}\)BrN\(_4\)O\(_2\) 560.1787, found 561.1846 [M+H].

HPLC purity: 99.3%, \( t_R = 14.700 \) min.

4.1.14. N-(3-Bromobenzyl)-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-phenylbutanamide (36d)

Yellow solid, 101 mg, yield 36.3%. Synthesized by the protocol of compound 36a with 35d (172 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography [H\(_2\)O (0.1% TFA)/methanol = 50:50—80:20]. \(^1\)H NMR (600 MHz, DMSO-d\(_6\)) \( \delta \) 8.65 (s, 1H), 7.55 (d, 1H, \( J = 8.1 \) Hz), 7.27—7.39 (m, 7H), 7.16—7.22 (m, 3H), 6.76—6.79 (m, 3H), 4.93 (s, 2H), 4.56 (s, 2H), 2.56—2.59 (m, 4H), 2.36—2.41 (m, 2H), 2.24 (t, 2H, \( J = 7.1 \) Hz), 2.18 (t, 2H, \( J = 7.7 \) Hz), 1.66—1.71 (m, 2H), 1.56 (d, 2H, \( J = 14.1 \) Hz). \(^{13}\)C NMR (150 MHz, DMSO-d\(_6\)) \( \delta \) 176.15, 172.05, 143.34, 142.17, 136.13, 132.47, 129.71, 129.41, 129.09, 128.96, 128.04, 127.73, 122.58, 117.71, 114.40, 58.61, 58.29, 56.82, 52.20, 49.91, 48.58, 31.22, 28.42, 22.47. HRMS (ESI): \( m/z \) calcd. for C\(_{30}\)H\(_{33}\)BrN\(_4\)O\(_2\) 560.1787, found 561.1846 [M+H].

HPLC purity: 99.9%, \( t_R = 14.829 \) min.

Figure 3  Molecular docking of 36l with PDE\(_d\) protein. (A) Proposed-bonding mode of 36l with PDE\(_d\) protein. The red dash line represented hydrogen interaction with residues Arg61, Gln78 and Tyr149. (B) The overlay image binding mode of compounds 36l (yellow) and 9 (green) with PDE\(_d\) protein.

Figure 4  \textit{In vitro} inhibitory activity of compound 36l against two clinical pancreatic cancer cell lines. The primary cell lines 098 and 099 (KRAS\(_{G12D}\) mutation) were treated with 36l at various concentrations for 72 h. The percentages of viability were measured by CCK8 method. The studies with primary cell lines were approved by the Changhai Hospital Ethics Committee. Data were represented as means ± SEM, \( n = 3 \).
0.5 mmol) and purified by flash C18 column chromatography [H2O (0.1% TFA)/methanol = 50:50–80:20]. 1H NMR (600 MHz, DMSO-d6) δ 8.59 (s, 1H), 7.42 (t, 1H, J = 8.0 Hz), 7.35–7.37 (m, 3H), 7.28 (t, 1H, J = 7.4 Hz), 7.25 (t, 1H, J = 7.9 Hz), 7.18–7.21 (m, 5H), 6.75–6.78 (m, 3H), 4.84 (s, 2H), 4.54 (s, 2H), 2.55–2.60 (m, 4H), 2.34–2.39 (m, 2H), 2.22 (t, 2H, J = 7.8 Hz), 2.13 (t, 2H, J = 6.8 Hz), 1.64–1.69 (m, 2H), 1.50 (d, 2H, J = 13.2 Hz). 13C NMR (150 MHz, DMSO-d6) δ 176.15, 172.05, 143.34, 142.17, 141.12, 130.96, 130.40, 129.95, 129.43, 128.55, 128.21, 127.37, 122.02, 118.26, 114.99, 59.08, 58.75, 57.26, 51.87, 49.58, 31.68, 28.88, 22.96. HRMS (ESI): m/z calcd. for C30H33BrN2O2 560.1787, found 561.1865 [M+H]⁺. HPLC purity: 97.9%, tR = 14.361 min.

4.1.15.  (1H-Imidazole-4-yl)methyl)-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-phenyl-N-(thio-phen-2-ylmethyl)butanamide (36e)

Yellow solid, 61 mg, yield 25.8%. Synthesized by the protocol of compound 36a with 35f (129 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography [H2O (0.1% TFA)/methanol = 50:50–80:20]. 1H NMR (600 MHz, DMSO-d6) δ 8.97 (s, 1H), 7.20–7.40 (m, 7H), 6.94–6.99 (m, 4H), 6.78 (t, 1H, J = 7.5 Hz), 4.89 (s, 2H), 4.62 (s, 2H), 3.31–3.43 (m, 3H), 2.79–2.95 (m, 4H), 2.16 (br, 2H), 1.90 (br, 2H), 1.79 (d, 2H, J = 14.2 Hz). 13C NMR (150 MHz, DMSO-d6) δ 175.58, 171.52, 144.01, 143.34, 142.44, 129.95, 129.53, 128.64, 128.38, 122.23, 118.43, 114.71, 59.29, 57.42, 56.01, 48.79, 46.59, 31.46, 26.72, 20.18. HRMS (ESI): m/z calcd. for C27H32N6O2 472.2587, found 473.2679 [M+H]⁺. HPLC purity: 98.0%, tR = 7.85 min.

4.1.16.  4-(4-Oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-phenyl-N-(thio-phen-2-ylmethyl)butanamide (36f)

Brown solid, 71 mg, yield 29.1%. Synthesized by the protocol of compound 36a with 35f (136 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography [H2O (0.1% TFA)/methanol = 50:50–80:20]. 1H NMR (600 MHz, DMSO-d6) δ 8.62 (s, 1H), 7.37–7.41 (m, 3H), 7.31 (t, 1H, J = 7.0 Hz), 7.21 (t, 1H, J = 7.8 Hz), 7.15 (d, 1H, J = 7.4 Hz), 6.88–6.90 (m, 1H), 6.76–6.80 (m, 4H), 4.97 (s, 2H), 4.55 (s, 2H), 2.57 (br, 4H), 2.34–2.39 (m, 2H), 2.20 (t, 2H, J = 6.7 Hz), 2.07 (t, 2H, J = 7.4 Hz), 1.63–1.66 (m, 2H), 1.90 (br, 2H), 1.49 (d, 2H, J = 13.3 Hz). 13C NMR (150 MHz, DMSO-d6) δ 178.01, 173.47, 145.17, 143.78, 141.96, 131.28, 130.97, 130.81, 129.99, 129.68, 128.68, 128.24, 127.82, 119.58, 116.29, 60.46, 60.13, 58.67, 50.94, 48.81, 33.10, 30.25, 24.36. HRMS (ESI): m/z calcd. for C28H32N4O2S 488.2251, found 489.2323 [M+H]⁺. HPLC purity: 98.9%, tR = 12.334 min.

Brown solid, 77 mg, yield 31.5%. Synthesized by the protocol of compound 36a with 35g (136 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography [H2O (0.1% TFA)/methanol = 50:50–80:20]. 1H NMR (600 MHz, DMSO-d6) δ 8.71 (s, 1H), 7.46–7.47 (m, 1H), 7.38 (t, 2H, J = 7.4 Hz), 7.31 (t, 1H, J = 6.5 Hz), 7.18–7.24 (m, 5H), 6.98 (d, 1H, J = 5.1 Hz), 6.84 (d, 2H, J = 7.8 Hz), 6.79 (d, 1H, J = 7.4 Hz), 4.84 (s, 2H), 4.58 (s, 2H), 2.83–2.86 (m, 4H), 2.49–2.54 (m, 2H), 2.42 (t, 2H, J = 6.9 Hz), 2.12 (t, 2H, J = 13.6 Hz).

Figure 5  Mechanism study of compound 36l. (A) Cellular thermal shift assays of PDEδ. 2 (50 µmol/L) and 36l (20 and 50 µmol/L) were incubated with Mia PaCa-2 cells for 2 h. The results were shown with melt curves. (B) Co-IP assay of compound 36l. Immunoprecipitation antibody: KRAS and control rabbit IgG; Western blotting antibody: PDEδ. Statistical difference was indicated by gray intensity analysis. Data were represented as means ± SEM, n = 3. ***P < 0.001.
$\delta 176.31, 171.82, 143.69, 142.71, 138.94, 129.88, 129.46, 128.63, 128.18, 126.78, 123.33, 118.32, 114.88, 59.16, 58.33, 56.89, 49.29, 47.95, 37.10, 28.21, 22.15$. HRMS (ESI): $m/z$ calc. for $C_{28}H_{32}N_{4}O_{2}S$ 488.2251, found 489.2322 [M+H]$^+$. HPLC purity: 99.7%, $t_R = 12.682$ min.

Figure 6  Western blotting analysis of KRAS downstream signal pathways. (A) Phosphorylation levels of t-ERK and t-AKT by stimulated KRAS-dependent Mia PaCa-2 cells with EGF (125 ng/mL, 10 min). From top to bottom: phosphorylated ERK on Thr202 and Tyr204 (p-ERK), total level of ERK (t-ERK), phosphorylated AKT on S473 (p-AKT), total level of AKT (t-AKT), and loading control (GAPDH). (B and C) Gray intensity analysis of the blots in quantification of p-ERK/t-ERK $\pm$ SEM (top) and p-AKT/t-AKT $\pm$ SEM (bottom) was standardized to the EGF-stimulated control (1% DMSO). Data were represented as means $\pm$ SEM, $n = 3$, ***$P < 0.001$.

Figure 7  Apoptosis assay staining with Annexin V-FITC/PI in the Mia PaCa-2 cells. (A) Representative scatter plots of samples treated with 2 (50 and 100 $\mu$mol/L) and compound 36l (5, 10 and 20 $\mu$mol/L) for 48 h. (B) and (C) Bar charts show quantitative data of apoptosis assay in Mia PaCa-2 cells.
7.71–7.74 (m, 1H), 7.32–7.36 (m, 5H), 7.27 (t, 1H, J = 6.9 Hz),
7.19–7.23 (m, 3H), 6.82 (d, 2H, J = 7.5 Hz), 6.79 (t, 1H,
J = 6.9 Hz), 4.93 (s, 2H), 4.56 (s, 2H), 2.71–2.79 (m, 4H),
2.34–2.46 (m, 4H), 2.18 (br, 2H), 1.71 (br, 2H), 1.56 (d, 2H, 
J = 12.9 Hz). 13C NMR (150 MHz, DMSO-d6) δ 176.41, 174.31,
149.36, 143.31, 137.08, 129.88, 129.45, 128.53, 128.09,
122.64, 122.26, 118.27, 114.89, 59.13, 58.50, 57.11,
54.77, 49.47, 31.64, 28.49, 22.54. HRMS (ESI): m/z calcd. for
C29H33N2O2 483.2634, found 484.2707 [M+H]+. HPLC purity:
97.7%, tR = 9.197 min.

4.1.19. 4-(4-Oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-
phenyl-N-(pyridin-4-ylmethyl)butanamide (36l)
Brown solid, 68 mg, yield 28.1%. Synthesized by the protocol of
Figure 8 Immunostaining of Mia PaCa-2 cells with anti-PDEδ (green) and anti-KRAS (red) by treated with tested compounds (2, 100 μmol/L;
36l, 20 μmol/L) for 4 h. 1% DMSO was used as the vehicle control. Leica confocal microscope was applied for the image collection. Scale bar: 10 μm.

4.1.20. 4-(4-Oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-
phenyl-N-(quinolin-3-ylmethyl)butanamide (36j)
Brown solid, 88 mg, yield 33.0%. Synthesized by the protocol of compound 36a with 35j (159 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography
H2O (0.1% TFA)/methanol = 50:50–80:20). 1H NMR (600 MHz, DMSO-d6) δ 8.72 (s, 1H), 8.50 (br, 1H), 7.17–7.38 (m, 10H), 6.82 (d, 2H, J = 8.7 Hz), 6.75 (t, 1H, J = 7.5 Hz), 4.85 (s, 2H), 4.55 (s, 2H), 2.86–2.90 (m, 4H), 2.50–2.54 (m, 2H), 2.17–2.19 (m, 2H), 1.74–1.76 (m, 2H), 1.60 (d, 2H, J = 13.7 Hz). 13C NMR (150 MHz, DMSO-d6) δ 176.73, 172.45, 150.01, 143.65, 142.69, 130.66, 129.46, 128.48, 128.31, 118.36, 114.91, 59.16, 58.26, 56.78, 49.26, 31.51, 28.09, 21.95. HRMS (ESI): m/z calcd. for C30H32N2O2 483.2634, found 484.2708 [M+H]+. HPLC purity: 99.4%, tR = 8.833 min.

4.1.21. 4-(4-Oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-
phenyl-N-(quinolin-3-ylmethyl)butanamide (36k)
Brown solid, 70 mg, yield 26.2%. Synthesized by the protocol of compound 36a with 35k (159 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography
H2O (0.1% TFA)/methanol = 50:50–80:20). 1H NMR (600 MHz, DMSO-d6) δ 8.74 (d, 1H, J = 1.7 Hz), 8.67 (s, 1H), 8.10 (s, 1H), 7.98 (d, 1H, J = 8.7 Hz), 7.93 (d, 2H, J = 7.7 Hz), 7.71–7.74 (m, 1H), 7.57–7.58 (m, 1H), 7.33 (t, 2H, J = 7.7 Hz), 7.18–7.31 (m, 5H), 6.75–6.82 (m, 3H), 5.08 (s, 2H), 4.57 (s, 2H), 2.75–2.80 (m, 4H), 2.42–2.46 (m, 4H), 2.17 (t, 2H, J = 6.3 Hz), 1.73–1.78 (m, 2H), 1.59 (d, 2H, J = 13.5 Hz). 13C NMR (150 MHz, DMSO-d6) δ 176.36, 172.44, 151.47, 147.26, 143.70, 142.43, 135.02, 131.19, 130.05, 129.76, 129.45, 129.12, 128.76, 128.40, 128.31, 127.83, 127.25, 118.36, 114.99, 59.14, 58.41, 57.00, 50.37, 49.40, 31.73, 28.35, 22.30. HRMS (ESI): m/z calcd. for C31H34N2O2 533.2791, found 534.2875 [M+H]+. HPLC purity: 98.9%, tR = 10.733 min.

4.1.22. N-(Cyclohexylmethyl)-4-(4-oxo-1-phenyl-1,3,8-
triazaspiro[4.5]decan-8-yl)-N-butanamide (36l)
Pale solid, 85 mg, yield 34.8%. Synthesized by the protocol of compound 36a with 35l (136 mg, 0.5 mmol) and 18 (115 mg,
0.5 mmol) and purified by flash C18 column chromatography [H₂O (0.1% TFA)/methanol = 50:50–80:20]. ¹H NMR (600 MHz, DMSO-d₆) δ 8.59 (s, 1H), 7.43 (t, 2H, J = 7.5 Hz), 7.29–7.33 (m, 3H), 7.21–7.24 (m, 2H), 6.77–6.79 (m, 3H), 4.93 (s, 2H), 3.51 (d, 2H, J = 7.5 Hz), 2.53–2.55 (m, 4H), 2.33–2.39 (m, 2H), 2.17 (t, 2H, J = 6.2 Hz), 2.02 (t, 2H, J = 7.5 Hz), 1.56–1.64 (m, 7H), 1.50 (d, 2H, J = 13.0 Hz), 1.33–1.35 (m, 1H), 1.06–1.11 (m, 3H), 0.87–0.92 (m, 2H). ¹³C NMR (150 MHz, DMSO-d₆) δ 176.23, 171.75, 143.39, 142.96, 129.58, 129.02, 128.24, 127.55, 117.80, 114.49, 58.66, 58.36, 56.98, 54.03, 49.14, 35.73, 31.50, 30.27, 28.45, 26.06, 25.37, 22.62. HRMS (ESI): m/z calcd. for C₃₀H₄₀N₄O₂ 488.3151, found 489.3231 [M + H]⁺. HPLC purity: 99.0%, tᵣ = 16.157 min.

4.1.23. N-(Cyclopentylmethyl)-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-phenylbutanamide (36m)

White solid, 86 mg, yield 36.3%. Synthesized by the protocol of compound 36a with 35m (130 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography [H₂O (0.1% TFA)/methanol = 50:50–80:20]. ¹H NMR (600 MHz, DMSO-d₆) δ 8.70 (s, 1H), 7.49 (t, 2H, J = 8.1 Hz), 7.30–7.35 (m, 3H), 7.22 (t, 2H, J = 7.9 Hz), 6.77–6.82 (m, 3H), 4.57 (s, 2H), 3.61 (d, 2H, J = 7.9 Hz), 2.76 (br, 4H), 2.48–2.50 (m, 2H), 2.35 (t, 2H, J = 6.0 Hz), 2.02 (t, 2H, J = 6.9 Hz), 1.87–1.92 (m, 1H), 1.66–1.68 (m, 2H), 1.54–1.60 (m, 6H), 1.43–1.45 (m, 2H), 1.15–1.19 (m, 2H). ¹³C NMR (150 MHz, DMSO-d₆) δ 176.23, 171.75, 143.25, 142.47, 129.53, 128.99, 128.32, 127.59, 117.80, 114.38, 58.67, 57.94, 56.59, 52.48, 48.87, 37.75, 31.42, 29.72, 27.86, 24.73, 21.89. HRMS (ESI): m/z calcd. for C₂₉H₃₈N₄O₂ 474.2995, found 475.3070 [M + H]⁺. HPLC purity: 99.2%, tᵣ = 15.359 min.

4.1.24. N-(Cyclobutylmethyl)-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-phenylbutanamide (36n)

White solid, 75 mg, yield 32.6%. Synthesized by the protocol of compound 36a with 35n (122 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography [H₂O (0.1% TFA)/methanol = 50:50–80:20]. ¹H NMR (600 MHz, DMSO-d₆) δ 8.67 (s, 1H), 7.43 (t, 2H, J = 7.6 Hz), 7.34 (t, 2H, J = 7.3 Hz), 7.21–7.26 (m, 4H), 6.77–6.81 (m, 3H), 4.56 (s, 2H), 3.70 (d, 1H, J = 7.6 Hz), 2.71–2.72 (m, 4H), 2.42–2.47 (m, 2H), 2.29–2.37 (m, 3H), 2.00 (t, 2H, J = 7.1 Hz), 1.85–1.91 (m, 2H), 1.73–1.79 (m, 2H), 1.63–1.65 (m, 2H), 1.54–1.61 (m, 4H). ¹³C NMR (150 MHz, DMSO-d₆) δ 175.95, 171.31, 143.26, 142.43, 129.47, 128.98, 128.33, 127.63, 117.79, 114.40, 58.65, 57.99, 56.68, 53.04, 48.94, 33.61, 31.35, 27.99, 25.67, 22.05, 17.80. HRMS (ESI): m/z calcd. for C₂₈H₃₆N₄O₂ 460.2838, found 461.2914 [M + H]⁺. HPLC purity: 99.2%, tᵣ = 14.265 min.

4.1.25. N-(Cyclopropylmethyl)-4-(4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-N-phenylbutanamide (36o)

White solid, 74 mg, yield 33.2%. Synthesized by the protocol of compound 36a with 35o (116 mg, 0.5 mmol) and 18 (115 mg, 0.5 mmol) and purified by flash C18 column chromatography.
**Figure 10** Therapeutic efficacy of compound 36l in the pancreatic tumor PDX model. When the tumor reached about 100 mm³ (set to day 0), the mice were divided into three groups randomly. Then, the groups were respectively treated with the compound 36l (50 mg/kg, QD) intraperitoneally, gemcitabine (30 mg/kg, once every 3 days) and saline intravenously until the endpoint. (A) Effect of 36l on tumor volume growth in PDX models. (B) Effect of 36l on body weight in PDX models. (C) Weight of the PDX tumor treated with vehicle, gemcitabine and 36l. (D) Quantification of Ki-67 positive cells. (E) Representative images of H&E staining and IHC staining for Ki-67 of tumor tissues treated by vehicle, gemcitabine and 36l. Scale bar: 200 μm. Data were represented as the mean ± standard deviation, n = 5. *P < 0.05, **P < 0.01, ***P < 0.001.

[H₂O (0.1% TFA)/methanol = 50:50–80:20]. ¹H NMR (600 MHz, DMSO-d₆) δ 8.65 (s, 1H), 7.45 (t, 2H, J = 6.9 Hz), 7.31–7.37 (m, 3H), 7.21–7.24 (m, 3H), 6.77–6.83 (m, 3H), 4.57 (s, 2H), 3.51 (d, 1H, J = 6.9 Hz), 2.69 (br, 4H), 2.45–2.48 (m, 2H), 2.29 (t, 2H, J = 8.1 Hz), 2.00 (t, 2H, J = 6.9 Hz), 1.64–1.69 (m, 2H), 1.55 (d, 2H, J = 13.0 Hz), 0.85–0.91 (m, 1H), 0.36–0.39 (m, 2H), 0.04–0.05 (m, 2H). ¹³C NMR (150 MHz, DMSO-d₆) δ 176.48, 171.71, 143.75, 143.17, 129.91, 129.45, 128.99, 128.12, 118.25, 114.89, 59.13, 58.57, 57.26, 52.99, 49.47, 31.90, 28.57, 22.65, 10.34, 3.90. HRMS (ESI): m/z calc'd. for C₂₂H₂₄N₄O₂ 446.2682, found 447.2757 [M+H]⁺. HPLC purity: 97.2%, tᵣ = 12.547 min.

## 4.2. Fluorescence anisotropy assay

The assay was carried out using our established protocol previously. In Brief, 80 nmol/L PDEδ protein and 25 nmol/L Atorvastatin-FITC probe in PBS buffer were mix in the plate (Corning 3650). The compounds with various concentrations were added to each well. PBS buffer was supplied until the volume...
reached 200 µL. The plate was incubated at 30 °C for 10 h. The fluorescence anisotropy and fluorescence intensity were detected by Bioteck Synergy H2 with 485 nm excitation and 528 nm emission. The results were calculated by Mathematic 9.0 (Wolfram Research).

4.3. Molecular docking

The crystal complex 5X7426 was chosen for the docking study. Firstly, the reliability of the docking method was established with the following procedure: 1) the protocol of protein preparation was according to previous works. The water molecules were removed, hydrogen atoms were complemented and energy minimized with OPLS2005 to the RMSD reaching 0.30 Å; 2) the program LigPrep was used for ligand preparation with OPLS2005 force-field. Other settings follow the default parameters. 20 low energy ring conformations were generated for each ligand; 3) the protein grid was generated by Receptor Grid Generation using H-bond constraints with Agr61, Gin78, Glu88 and Try149. Extra Precision (XP) and H-bond constraints were applied in the docking study. The figures were generated using PyMol academic version (http://pymol.sourceforge.net/).

4.4. Determination of the solubility of compounds in water

The solubility assay was conducted according to the literature. In brief, 5 mg compound was dissolved in 1 mL PBS (pH = 7.4) for 24 h in 37 °C. Then the suspension was filtered to afford the saturated solution. Standards in acetonitrile (0.2 mL) with concentration at 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL and saturated solution were added into a quartz 96-well plate. Acetonitrile and PBS solution was added as blank respectively. UV absorbance value at 254 nm was measured with Biotek Synergy H2. The solubility was calculated using the absorbance value and concentration of standards according to Lambert–Beer law.

4.5. Cell culture

Mia PaCa-2 cells were cultured in DMEM (Hyclone) containing 10% fetal bovine serum (Gibco), 2.5% horse serum (BioSharp), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). The primary cell lines were cultured in 1640 (Hyclone) containing 10% fetal bovine serum (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). Cells were maintained in a humidified incubator with 95% air/5% CO₂ at 37 °C.

4.6. In vitro antiproliferative assay

Mia PaCa-2 cell line and primary cell lines (0001, 0034, 0037 and 0043) were chosen to determine the inhibitory activity towards pancreatic cancer. The density was 5000 per well in 96-well plates. After cultured for 24 h, each compound was added to triplicate wells at different concentrations and 0.5% DMSO for control. After 72 h of incubation, the medium was removed and 10% CCK8 in DMEM was added to each well following incubating further for the suitable time at 37 °C. The absorbance (OD) at 450 nm was quantitated with Biotek Synergy H2. Wells with no cells and drugs were used as blanks. The concentration inhibited cell growth by 50% (IC₅₀) was calculated by GraphPad Prism 5.0.

4.7. Cell apoptosis assay

Mia PaCa-2 cells were seeded at a density of 3 × 10⁵ cells per well in 6-well plates. After 24 h, the cells were treated with compounds of different concentrations for the proper time. Cells were collected and treated with Annexin V-FITC and PI according to the manufacture’s protocol. The stained cells were analyzed by a flow cytometer (BD Accuri C6).

4.8. Western blotting

Mia PaCa-2 cells were seeded at a density of 6 × 10⁴ cells per well in 6-well plates. Then cells were starved and treated with EGF followed by compounds for 2 h as the procedure reported. Then, the protein was extracted, denatured and run on SDS-PAGE gels. Each sample was loaded with 30 µg total protein. The gels were electroblotted onto polyvinylidene fluoride membrane (0.22 µm) and blocked for 2 h at room temperature. The primary antibodies used for western blotting were: p44/42 MAPK (ERK1/2) (Cell Signaling Technology # 9107, 1:1000), anti-Phospho AKT (Cell Signaling Technology # 4606, 1:800), anti-AKT1 (Cell Signaling Technology, 1:1000), GAPDH (Abcam, 1:1000), Phospho p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling Technology # 4370, 1:1000). And secondary FITC-labeled antibodies including goat anti rabbit IgG (Abcam, 1:5000) and goat anti mouse IgG (Abcam, 1:8000) were applied. The blots were scanned on the LI-COR Odyssey imaging system. The gray values of the bands were applied for quantification of the protein level while the control group as the standard.

4.9. CETSA

Prepared the cell in 4 separate 10 cm flasks. Complete medium containing the different concentrations of compounds were added, and the control group containing 1% DMSO. Then cultured the cells for another 2 h. Harvested and re-suspended the cells with 1 mL PBS buffer with protease inhibitors, then divided into 10 tubes. Heated the tubes successively at different temperatures for 3 min, followed by incubation at room temperature for 3 min. Snap-freeze the tube in liquid nitrogen for 3 circles. The cell lysates were reserved on ice after the third snap-freeze. Centrifuge the tubes at 4 °C, 12,000 rpm, 15 min. The supernatant was kept and the loading buffer was added. Then heated the mixture at 70 °C for 10 min. Western blotting experiments and gray-scale statistics were conducted to quantify the protein.

4.10. Co-IP

Six dishes of Mia PaCa-2 cells were prepared. Renewed the medium with 20 µmol/L compound 36l and 1% DMSO to every 3 dishes respectively and incubated for another 2 h. Harvested and combined the cells, then added 500 µL of IP lysate (Beyotime, containing 1% protease inhibitor) and lysed the cells on ice for 30 min. The prepared Protein A/G beads (Santa Cruz) were added into the cell lysates. Shacked the tubes at 4 °C for 1 h followed by a centrifuge at 4 °C, 12,000 rpm, 15 min. Quantify the supernatant by the BCA method and adjust the total protein concentration to 5 µg/mL. Respectively added KRAS primary antibody (Abcam #ab275876) and IgG (Abcam #ab125938) antibody to 100 µL the above lysates followed by rotating at 4 °C for 1 h. Add the Protein...
The PDX model was constructed as the literature⁴⁵. All the studies ability was calculated by GraphPad Prism 5.0.

Peak areas of each sample were detected and converted to concentration according to the standard curve. The bioavailability of TFA/methanol Z/L/min. 10 min at 4000 rpm, which were further precipitated protein with plasma were afforded by centrifuging the blood samples for 1, 2, 4, and 8 h in micro-tubes containing 0.1% heparin. The mice were divided into three groups randomly. The mice were respectively treated with the compound gemcitabine (30 mg/kg, intraperitoneally until the endpoint. The body weight were evaluated. The tumor size was calculated using formula *L*/2 *W*/2 *π*/6, where *L* is the long axis and *W* is the width axis. Tumor size and body weight were recorded every 3 days over the course of study. Mice were sacrificed on Day 14 and all tumors were harvested and analyzed.

4.14. H&E staining and immunohistochemical staining

All tumor samples were fixed in 10% formaldehyde overnight, then dehydrated in graded concentrations of ethanol. For hematoxylin and eosin (H&E) staining, slides were stained with Mayer’s hematoxylin for 2 min, blued in 0.1% sodium bicarbonate for 1 min, washed in water and counterstained with Eosin Y solution for 1 min. For immunohistochemistry, sections were fixed onto poly-l-lysine coated slides. The slides were incubated with rabbit anti-Ki67 antibody (1:500, GB111141, Servicebio, China) in a humid incubator at 4° C overnight. The secondary antibody system (PV9000, Golden Bridge International, Beijing, China) was followed according to the manufacturer’s instructions. A positive staining result was recorded when the nucleus of cells was stained yellow or brown. Per slide were randomly chosen to measure the proportion of immunopositive cells. Quantification of Ki-67 positive drug was calculated by ImageJ 1.51.

4.15. Statistical analysis

The Student’s *t*-test and one-way analysis of variance were used for comparison among all different groups represented with the mean values ± standard errors. A probability of 0.05 or less (*P* < 0.05, **P** < 0.01, and ***P*** < 0.001) was considered statistically significant.

Acknowledgments

This work was supported by the National Key R&D Program of China (Grant No. 2020YFA0509100), the National Natural Science Foundation of China (Grants 21738002, 82030105, 81725020 and 81903436).

Author contributions

Long Chen synthesized the target compounds and completed most biological and cell-based assays. Jing Zhang and Xinxing Wang constructed the (PDX) models and completed the in vivo anti-tumor assays. Yu Li and Lu Zhou participated in research design and conducted experiments. Xiongxiang Lu, Guoqiang Dong and Chunquan Sheng proposed the project, performed data analysis and contributed to the writing—review&editing of the manuscript. All authors have given approval to the final version of the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2021.07.009.
Novel spiro-cyclic PDEδ inhibitors with potent activity in PDX models.

References

1. Kamerkar S, LeBleu VS, Sugimoto H, Yang S, Ruivo CF, Melo SA, et al. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* 2017;546:498–503.

2. Zhu Z, Xiao S, Hao H, Hou Q, Fu X. Kirsten rat sarcoma viral oncogene homologue (kra) mutations in the occurrence and treatment of pancreatic cancer. *Curr Top Med Chem* 2019;19:2176–86.

3. Gillson J, Ramsawamy Y, Singh G, Gorfe AA, Pavlakis N, Samra J, et al. Small molecule KRAS inhibitors: the future for targeted pancreatic cancer therapy?. *Cancers (Basel)* 2020;12:1341–9.

4. Bryant KL, Mancias JD, Kimmelman AC, Der CJ. KRAS: feeding pancreatic cancer proliferation. *Trends Biochem Sci* 2014;39:91–100.

5. Nagasaka M, Li Y, Sukari A, Ou SI, Al-Hallak MN, Azmi AS. KRAS G12C game of thrones, which direct KRAS inhibitor will claim the iron throne?. *Cancer Treat Rev* 2020;84:101974.

6. Misale S, Fatheree JP, Cortez E, Li C, Bilton S, Timonina D, et al. KRAS G12C NSCLC models are sensitive to direct targeting of PDEδ in combination with PI3K inhibition. *Clin Cancer Res* 2019;25:796–807.

7. Nollmann FI, Ruess DA. Targeting mutant KRAS in pancreatic cancer: futile or promising?. *Biomedicines* 2020;8:281–94.

8. Seton-Rogers S. KRAS-G12C in the crosshairs. *Nat Rev Cancer* 2020;20:3.

9. Canon J, Rex K, Saiki AY, Mohr C, Cooke K, Bagal D, et al. The clinical KRAS(G12C) inhibitor AMG 510 drives anti-tumour immunity. *Nature* 2019;578:217–23.

10. Hata AN, Shaw AT. Resistance looms for KRAS(G12C) inhibitors. *Nat Med* 2020;26:169–70.

11. Veluswamy R, Mack PC, Houldsworth J, Ekhlohy E, Hirsh FR. KRAS G12C-mutant non-small cell lung cancer: biology, developmental therapeutics, and molecular testing. *J Mol Diagn* 2021;23:507–20.

12. Kim D, Xue JY, Lito P. Targeting KRAS(G12C): from inhibitory mechanism to modulation of antitumor effects in patients. *Cell 2020;183:850–9.*

13. Grapas D, Syrigos K. Direct KRAS inhibition: progress, challenges, and a glimpse into the future. *Expert Rev Anticancer Ther* 2020;20:437–40.

14. Khan I, Rhett JM, O’Bryan JP. Therapeutic targeting of RAS: new hope for drugging the ‘undruggable’. *Biochim Biophys Acta Mol Cell Res* 2020;1867:118570–85.

15. Jones GD, Caso R, Tan KS, Mastrogiacomo B, Sanchez-Vega F, Liu Y, et al. KRAS (G12C) mutation is associated with increased risk of KRAS mutant nonsmall cell lung cancer. *Int J Cancer* 2019;145:1334–45.

16. Siddiqui FA, Alam C, Rosenqvist P, Ora M, Saht M, Manoharan GB, et al. PDEδ inhibitors with a new design principle selectively block K-Ras activity. *ACS Omega* 2020;5:8322–42.

17. Tian J, Zhuang C, Chen L, Jiang Y, Sheng C. Discovery of novel KRAS-PDEdelta inhibitors by fragment-based drug design. *J Med Chem* 2018;61:2604–10.

18. Dong G, Chen L, Zhang J, Liu T, Du L, Sheng C, et al. Discovery of turn-on fluorescent probes for detecting PDEdelta in living cells and tumor slices. *Anal Chem* 2020;92:9516–22.

19. Cheng J, Li Y, Wang X, Dong G, Sheng C. Discovery of novel PDEdelta degraders for the treatment of KRAS mutant colorectal cancer. *J Med Chem* 2020;63:7892–905.

20. Wang S, Zhang Y, Dong G, Wu S, Zhu S, Miao Z, et al. Asymmetric synthesis of chiral dihydrothiopyrans via an organocatalytic enantioselective formal thio [3 + 3] cycloaddition reaction with binucleophilic bisketone thioetheres. *Org Lett* 2013;15:5570–3.

21. Wang S, Jiang Y, Wu S, Dong G, Miao Z, Zhang W, et al. Meeting oncogenetics with drug discovery: asymmetric synthesis of 3,3’-spirooxindoles fused with tetrahydrothiopyrans as novel P53-MDM2 inhibitors. *Org Lett* 2016;18:1028–31.

22. Wu S, Li Y, Xu G, Chen S, Zhang Y, Liu N, et al. Novel spiro- pyrazoline antitumor scaffold with potent activity: design, synthesis and structure–activity relationship. *Eur J Med Chem* 2016;115:141–7.

23. Wang S, Chen S, Guo Z, He S, Zhang F, Liu X, et al. Synthesis of spiro-tetrahydrothiopyranoxides by Michael-aldol cascade reactions: discovery of potential P53-MDM2 inhibitors with good antitumor activity. *Org Biomol Chem* 2018;16:625–34.

24. Chambers M, Delport A, Hewer R. The use of the cellular thermal shift assay for the detection of intracellular beta-site amyloid precursor protein cleaving enzyme-1 ligand binding. *Mol Biol Rep* 2021;48:2957–62.

25. Chernobrovkin AL, Cazares-Korner C, Friman T, Caballero JM, Amadio D, Martinez Molina D. A tale of two tails: efficient profiling of protein degraders by specific functional and target engagement readouts. *SAS Discov* 2021;26:534–46.

26. Husain A, Begum NA, Kobayashi M, Honjo T. Native co-immunoprecipitation assay to identify interacting partners of chromatin-associated proteins in mammalian cells. *Bio Protoc* 2020;10:e3837.

27. Zhang J, He S. Co-immunoprecipitation assay for blue light-dependent protein interactions in plants. *Methods Mol Biol* 2021;2297:141–6.

28. Del Rosario Taco Sanchez M, Soler-Monzo T, Petit A, Azcarate J, Lasheras A, Artul C, et al. Digital quantification of Ki-67 in breast cancer. *Virochows Arch* 2019;474:169–76.

29. Morciano G, Preti D, Pedriali G, Aquila G, Missiroli S, Fantinati A, et al. Discovery of novel 1,3,8-triazaspiro[4.5]decane derivatives that...
target the $c$ subunit of $f_1/f_0$-adenosine triphosphate (ATP) synthase for the treatment of reperfusion damage in myocardial infarction. J Med Chem 2018;61:7131–43.

40. Roy D, Ducher F, Laumain A, Legendre JY. Determination of the aqueous solubility of drugs using a convenient 96-well plate-based assay. Drug Dev Ind Pharm 2001;27:107–9.

41. Jafari R, Almqvist H, Axelsson H, Ignatushchenko M, Lundback T, Nordlund P, et al. The cellular thermal shift assay for evaluating drug target interactions in cells. Nat Protoc 2014;9:2100–22.

42. Seashore-Ludlow B, Axelsson H, Lundback T. Perspective on CETSA literature: toward more quantitative data interpretation. SLAS Discov 2020;25:118–26.

43. Tung Z, Takahashi Y. Analysis of protein–protein interaction by co-ip in human cells. Methods Mol Biol 2018;1794:289–96.

44. Lin JS, Lai EM. Protein–protein interactions: co-immunoprecipitation. Methods Mol Biol 2017;1615:211–9.

45. Wen CL, Huang K, Jiang LL, Lu XX, Dai YT, Shi MM, et al. An allosteric PGAM1 inhibitor effectively suppresses pancreatic ductal adenocarcinoma. Proc Natl Acad Sci U S A 2019;116:23264–73.