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LETTER TO THE EDITOR

Discovery of a potent and selective cell division cycle 7 inhibitor from 6-(3-fluoropyridin-4-yl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one derivatives as an orally active antitumor agent

KEY WORDS

Cell division cycle 7;
DNA replication;
CDC7 inhibitor;
Anti-tumor

To the Editor:

Kinase cell division cycle 7 (CDC7), a cell division cycle protein, takes a vital role in mediating DNA replication[1]. CDC7 complexes in the nucleus can phosphorylate the mini-chromosome maintenance complex (MCM) family members that bind to chromosomes. In addition, CDC7 kinase, as a molecular switch regulating DNA replication, can mediate DNA damage signaling pathways to stimulate cell cycle termination as well as DNA replication[2]. Studies have shown that CDC7 is overexpressed in many types of cancer cells, and its overexpression was related to poor patient survival, tumor grade, genetic instability, aneuploidy and so on[3]. Therefore, CDC7 is a promising target for antitumor therapy.

Owing to the important role of CDC7, a large number of commercial drugs and newly discovered compounds were found to have the effect on inhibiting CDC7, and some of which have entered clinical trials (Supporting Information Fig. S1). Among them, TAK931 was once a globally leading CDC7 kinase inhibitor which had been carried out for clinical studies in a number of aspects simultaneously[4]. It had also been touted as the most promising CDC7 inhibitor to be approved for clinical application. However, phase II clinical trials of TAK931 showed some unsatisfactory results in terms of high clearance *in vivo*, short half-life and limited efficacy as a single agent. Therefore, the development of CDC7 inhibitors with high potency, high selectivity and low toxicity remains an urgent and challenging task.

By analyzing the discovery process and structure−activity relationship (SAR) of TAK931, it is believed that there is still a large room for improving the structure and activity of TAK931 (Fig. 1A). Therefore, TAK931 was chosen as a lead compound to develop its derivatives as new potential CDC7 inhibitors. Finally, among a number of the resulting compounds, EP-05 was found to be a CDC7 inhibitor with high efficiency, low toxicity, acceptable pharmacokinetic parameters and strong anti-tumor activity *in vivo*, which has a potential promise as a drug candidate.

1. Synthesis, characterization and SAR of the target compounds

The synthetic steps of target compounds EP-01−10 and EP-11−22 are shown in Supporting Information Schemes S1 and S2, respectively. All the resulting compounds were identified *via* NMR and HR-MS techniques. As potential CDC7 inhibitors, the inhibitory effects of all the target compounds on CDC7 enzyme and COLO 205 cell line were firstly detected with TAK931 as a positive control, and then their SAR was analyzed. As shown in Supporting Information Tables S1 and S2, among all the target compounds, EP-03 and EP-05 exhibited stronger inhibitory activity on both CDC7 enzymatic activity and COLO 205 cell proliferation than TAK931. The SAR analysis showed that a substituent F atom at the 3-position of the pyridine ring is

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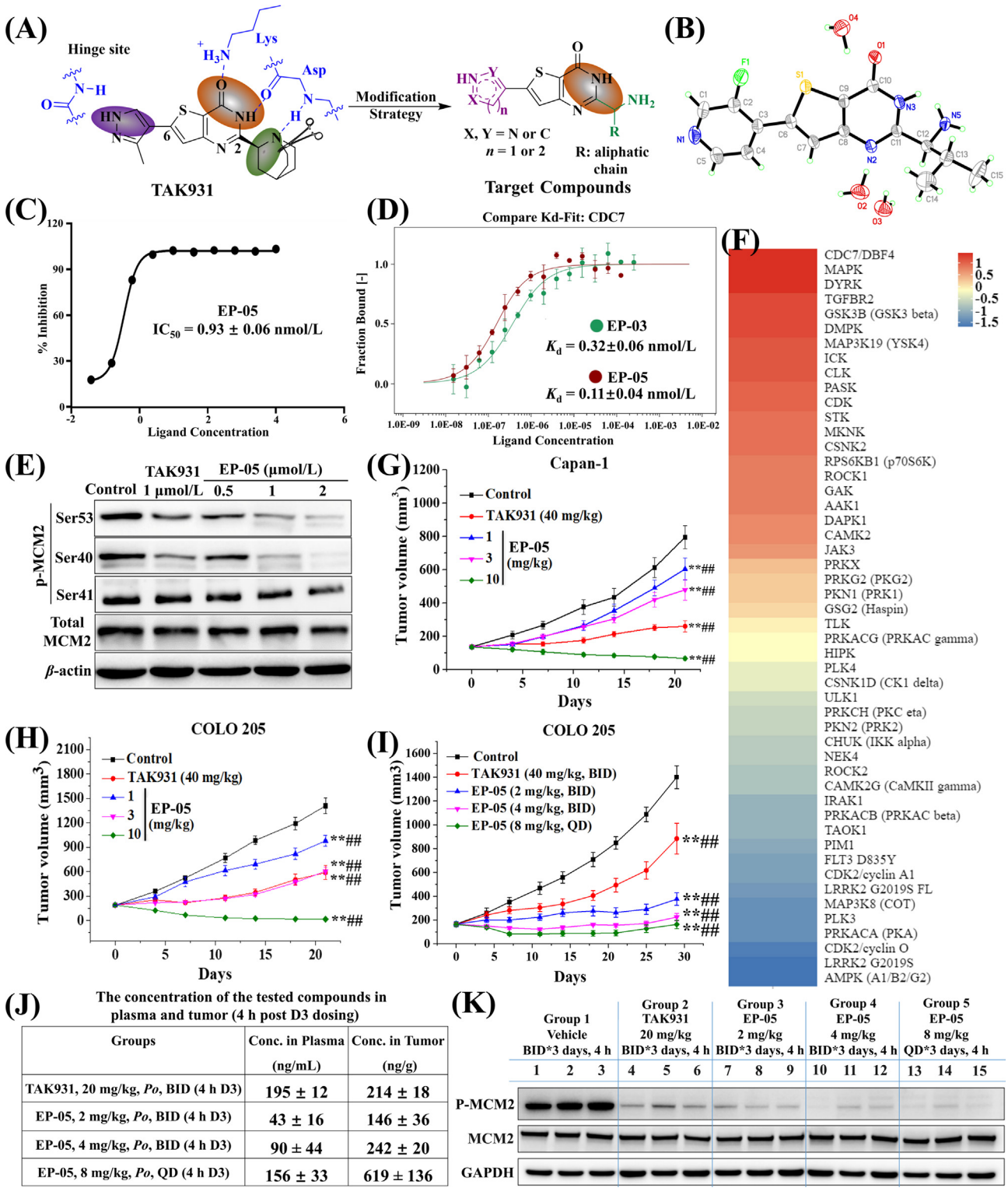

Groups	Conc. in Plasma (ng/mL)	Conc. in Tumor (ng/g)
TAK931, 20 mg/kg, *Po*, BID (4 h D3)	195 ± 12	214 ± 18
EP-05, 2 mg/kg, *Po*, BID (4 h D3)	43 ± 16	146 ± 36
EP-05, 4 mg/kg, *Po*, BID (4 h D3)	90 ± 44	242 ± 20
EP-05, 8 mg/kg, *Po*, QD (4 h D3)	156 ± 33	619 ± 136

Figure 1 (A) Structural modification strategy for the target compounds with TAK931 as a lead compound. (B) Crystal and molecular structures of EP-05. (C) Inhibition curve of compound EP-05 against CDC7 enzyme activity. (D) MST results of EP-03 and EP-05 binding to CDC7 enzyme. (E) The kinase inhibition ratio of EP-05 on the top 50 kinases. (F) Western blotting assessments on the expression levels of MCM2 phosphorylated proteins in COLO 205 cells treated with TAK931 and EP-05 at the indicated concentration for 24 h, respectively. (G–I) The change curves of tumor volume in mice treated with different doses of EP-05 in different xenograft tumor mice models. (J) The drug concentration in mice plasma and tumor tissue detected at the fourth hour after 3 days of continuous administration. (K) The expression levels of P-MCM2 and MCM2 proteins in tumor tissues of mice in each group at the fourth hour after 3 days of continuous administration. Error bars = SD. $^{**}P < 0.01$, $^{\#\#}P < 0.01$.

favorable, and *S* configuration of the asymmetric C12 atom with R_1 as a branched alkyl group is much helpful to promote the biological activity of the target compound. An unsubstituted $-NH_2$ moiety as designed is necessary to maintain the activity of the target compound. Moreover, a 2-fluoropyridinyl group at the left end of the pharmacophore was found much favorable to improve the *in vitro* activity of the target compound.

2. *In vitro* activity of representative compounds

Since EP-03 and EP-05 exhibited excellent inhibitory effects on CDC7 enzyme activity and COLO 205 cells proliferation among all the target compounds, they were selected for subsequent studies. First, the molecular structures of EP-03 (Supporting Information Fig. S2) and EP-05 (Fig. 1B) including the asymmetric C12 atom as an *S*-isomer in two compounds were determined by X-ray diffraction techniques. Secondly, these two compounds not only exhibited strong inhibition on CDC7 enzyme activity in a dose-dependent manner (Supporting Information Fig. S3, and Fig. 1C), but also showed potent affinity with CDC7 protein, and their equilibrium dissociation constant (K_d) values reached 0.32 and 0.11 nmol/L, respectively (Fig. 1D). Moreover, EP-03 and EP-05 were also found to exhibit strong anti-proliferation activity against Capan-1 cells, with IC_{50} values of 0.049 and 0.028 μmol/L, respectively (Supporting Information Table S3 and Fig. S4). Notably, they expressed a lower cardiotoxicity, with the hERG inhibitory activity greater than 30 μmol/L (Supporting Information Table S4).

As we know, CDC7 takes part in the phosphorylation of its downstream MCMs complexes, especially for MCM2 proteins. As illustrated in Supporting Information Fig. S5, and Fig. 1E, EP-03 and EP-05 can inhibit the phosphorylation at Ser53 and Ser40 of MCM2 in a dose-dependent manner as well, and at the same concentration, their inhibitory effects are superior to that of TAK931. In addition, they can also promote the expression of Cleaved-PARP, Cleaved-caspase-3 and DNA damage marker γ-H2AX proteins (Supporting Information Fig. S6), indicating that the observed cells death was caused by the selective inhibition of the compound on endogenous CDC7 activity.

Upon its good performance, EP-05 was selected for further evaluation on its cytotoxicity against other 29 cancer cell lines and a normal endothelial cell line. As listed in Supporting Information Table S5, EP-05 showed strong anti-proliferation activity against these cancer cells with IC_{50} values below 0.54 μmol/L. Among these cancer cells, EP-05 showed the strongest cytotoxicity to SW620 and DLD-1 cell lines, with IC_{50} values of 0.068 and 0.070 μmol/L, respectively. Meanwhile, EP-05 displayed lower toxicity to normal cells, and its IC_{50} value for HUVEC cells was 33.41 μmol/L. In addition, the selectivity of EP-05 to CDC7 was also evaluated by measuring its inhibitory activity against a panel of 439 human kinases. As shown in Fig. 1F, Supporting Information Table S6 and Fig. S7, EP-05 can only completely inhibit the activity of CDC7, MAPK and DYPK at 1 μmol/L. However, in further evaluation, it was found that the IC_{50} values of EP-05 were higher than those of CDC7 for kinases with greater than 95% inhibition, including MAPK and DYPK3. Among them, EP-05 showed the highest inhibitory activity against GSK3α, but its IC_{50} value was only 4.02 nmol/L (Supporting Information Table S7). Therefore, EP-05 not only possesses potent cytotoxicity against cancer cells, but also has considerably high inhibitory activity and selectivity for CDC7.

3. Toxicokinetic and pharmacokinetic study

First, the metabolic stability of EP-05 was detected in the liver microsomes of mouse, rat, beagle, monkey and human. As shown in Supporting Information Table S8, EP-05 was stable in different liver microsomes, with a half-life ($t_{1/2}$) value over 48 min and a relatively low clearance rate (CL_{int}).

Second, the maximum tolerated dose, acute toxicity and toxicokinetic of EP-05 were investigated by intragastric administration at different doses (1.5, 5 and 10 mg/kg) in rats of different genders every day for 14 days. As shown in Supporting Information Table S9, on the first day of administration and the 14th day of continuous administration in both male and female rats, the T_{max} of EP-05 in all dose groups occurred between 0.1 and 1.0 h after administration. The $AUC_{0-24\ h}$ and C_{max} values of EP-05 in both male and female rats increased proportionally with dose as increased. Unfortunately, after repeating oral administration of EP-05 at 10 mg/kg/day for one week, both male and female rats experienced severe systemic toxicity symptoms such as vomiting, convulsions, and death. But no significant systemic toxicity symptoms were observed in the other groups of rats.

And finally, EP-05 showed favorable pharmacokinetic (PK) parameters in mouse and rat (Supporting Information Table S10). After a single oral administration at a dose of 10 mg/kg, the bioavailability (*F*%) of EP-05 in two types of animals reached 45.29% and 51.26%, respectively. EP-05 has a lower $t_{1/2}$ value and higher clearance in mice than in rats, possibly due to its lower microsomal stability in mouse. Overall, EP-05 has acceptable PK parameters, which is worthy of further study.

4. Anti-tumor activity *in vivo*

The anti-tumor activity of EP-05 was evaluated in Capan-1 and COLO 205 xenograft mice models. In two models, the groups with drug were administrated orally once a day (QD) for 21 days. Results indicated that EP-05 could obviously inhibit tumor growth. In the Capan-1 xenograft mice model, the maximum tumor growth inhibition (TGI) of EP-05 reached 91.61% at 10 mg/kg. In contrast, the anti-tumor effect of TAK931 was much weak, with TGI of 67.33% at 40 mg/kg (Fig. 1G and Supporting Information Fig. S8A). In the COLO 205 xenograft mice model, its TGI was 56.93% at 3 mg/kg almost comparable to TAK931 (58.24%) at 40 mg/kg. When at 10 mg/kg, the TGI of EP-05 reached 98.85% (Fig. 1H and Fig. S8C). In addition, the body weight and health status of all mice hardly showed noticeable change during the treatments in two models (Fig. S8B and S8D).

In order to explore the optimal administration mode, the anti-tumor activity of EP-05 in COLO 205 xenograft mice model, drug concentration in mouse plasma and tumor tissue, p-MCM2 and MCM2 protein levels in tumor tissues were investigated by changing the dose and frequency. As illustrated in Fig. 1I, Supporting Information Fig. S8E and Table S11, the TGI of EP-05 at 2 mg/kg was even higher than that of TAK931 at 20 mg/kg (85.6% *versus* 51.5%) after 21 days of continuous administration. When EP-05 was dosed bis in die (BID) at 4 or QD at 8 mg/kg, the TGI reached 101.3% and 110.6%, respectively, after 21 days. Significantly, the TGI in these two groups were still 95.1% and 100%, respectively, even after ending administration for 7 days.

In addition, at the fourth hour after three consecutive days of administration, the enrichment concentration of EP-05 in tumor tissue was 2.6−4.0 times as much as that in plasma under different

administration concentrations and modes, which was much better than that of TAK931 (Fig. 1J). Moreover, EP-05 administered QD at 8 mg/kg had more advantages than that administered BID at 4 mg/kg, because the drug concentration in tumor tissue *via* the former administration mode was 2.6 times as much as that *via* the latter one at the same time point. Western blot analysis showed that both EP-05 and TAK931 could strongly inhibit the expression of p-MCM2 protein in tumor tissues, but the inhibitory activity of the former was significantly higher than that of the latter (Fig. 1K). Therefore, the optimal dosage and administration mode of EP-05 at 8 mg/kg *po* QD can effectively inhibit tumor growth by increasing the concentration of the drug in tumor tissue and the expression of p-MCM2 protein without obvious systemic toxicity.

In summary, a series of thieno[3,2-*d*]pyrimidin-4(3*H*)-one derivatives were designed and prepared to serve as potential CDC7 inhibitors by using TAK931 as a lead compound. Among the resulting target compounds, EP-05 presented high selectivity and strong inhibitory activity against both endogenous and exogenous CDC7 enzyme, and displayed strong anti-proliferation activity against a variety of cancer cell lines, especially against Capan-1 and COLO 205 cells with IC_{50} values less than 0.03 μmol/L. Moreover, EP-05 had favorable PK parameters and exhibited potent anti-tumor activity *in vivo*. Particularly, in the COLO 205 xenograft mice model, its TGI reached over 98% when administered orally at 8−10 mg/kg per day. Consequently, EP-05 is proved to be a novel CDC7 inhibitor with potential promise as a drug candidate.

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Author contributions

Shaohua Gou and Min Ge conceived the project. Yuanjiang Wang and Mingwei Fu designed and performed the majority of experiments. Chunchen Hu and Xiaowei Li contributed to the Western blot. Xiaowei Li and Wanxiang Yang contributed to the *in vitro* experiments. Mingwei Fu and Yuanjiang Wang compiled the figures and wrote the paper with significant inputs from Shaohua Gou. All authors discussed the results and commented on the manuscript.

Conflicts of interest

The authors proclaim no potential conflict of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2023.11.026.

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