Dear Editor,

Current treatment options for patients with advanced hepatocellular carcinoma (HCC) remain limited due to a paucity of drugs that target critical dependencies. Broad-spectrum kinase inhibitors such as sorafenib and lenvatinib, which were approved as first-line treatments in clinic, provide a low response rate and modest overall survival benefit to HCC patients. For the initial responders, the long-term effectiveness is limited by acquired resistance. It is therefore urgent to develop effective second-line treatments for HCC patients who progressed on first-line treatments.

Since the approval of regorafenib in 2017, the list of second-line treatment candidates has expanded continuously in recent years, which currently contains the monotherapies with regorafenib, cabozantinib, ramucirumab, or pembrolizumab as well as the combination with nivolumab plus ipilimumab. Cabozantinib is an orally bioavailable multi-kinase inhibitor targeting c-MET, c-RET, c-KIT, VEGFR, TIE2 and AXL. The phase III CELSESTIAL trial showed that cabozantinib gave a median overall survival/progression-free survival of 10.2/5.2 months compared to 8.0/1.9 months with placebo. Based on the positive data from CELSESTIAL trial, cabozantinib has been approved as a second-line treatment for progressed HCC patients who have previously treated with sorafenib. However, these modest survival benefits highlight the substantial clinical need for identifying combination strategies to improve the clinical efficacy of cabozantinib-based therapies. Shang et al. have recently found that cabozantinib treatment led to a strong anti-tumor effect in HCC mouse models caused by Met overexpression. Since about 44% of HCC samples found that cabozantinib treatment led to a strong anti-tumor effect in HCC mouse models caused by Met overexpression. Since about 44% of HCC samples showed co-activation of AKT/mTOR and c-MET in the TCGA LIHC dataset, the combination of pan-mTOR inhibitor MLN0128 and cabozantinib was also determined in c-Met/β-catenin HCC model. However, it is still not clear at present whether this is the most powerful combination for HCC therapy and therefore large-scale genetic screening and compound screening can be helpful for the identification of the most powerful combination therapies with cabozantinib for HCC.

To address this, we treated a panel of HCC cell lines with increasing concentrations of cabozantinib (Detailed methods shown in Supplementary Data S1). Proliferation was impaired in MHCC97H cells, whereas other HCC cell lines displayed no sensitivity to cabozantinib (Fig. 1a). The activities of MET (p-MET) in HCC cell lines before and after treatment of cabozantinib were detected. It is clear that MHCC97H cells have high MET expression which is consistent with previous finding that this cell line has a MET gene amplification (Fig. 1b). Therefore, we sought to identify compounds, which can enhance the effects of cabozantinib in cabozantinib-insensitive HCC cell lines. A kinome-based CRISPR screening was used to systematically identify the kinase whose inhibition confers sensitivity to cabozantinib in Hep3B cells (Fig. 1c). Hep3B cells were infected with the lentiviral kinome guide RNA (gRNA) library and cultured in the absence or presence of cabozantinib for 14 days. Then, genomic DNA was isolated from both untreated and cabozantinib-treated cells. Several independent gRNAs targeting EGFR were depleted specifically in cabozantinib-treated group, suggesting that EGFR inhibition is synthetic with cabozantinib (Fig.

Ma et al. Cell Discovery (2022) 8:82
https://doi.org/10.1038/s41421-022-00425-y

© The Author(s) 2022

Correspondence: René Bernards (r.bernards@nki.nl) or Wenxin Qin (wxqin@sjtu.edu.cn) or Cun Wang (cwang@shsci.org)
1State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
2Shanghai Immune Therapy Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
Full list of author information is available at the end of the article
These authors contributed equally: Xuhui Ma, Shanshan Wu, Botai Li.
Furthermore, based on a library screen of 2103 compounds in the cabozantinib-insensitive cell line Hep3B (Fig. 1e), a total of three EGFR inhibitors (WZ3146, AST-1306 and poziotinib) were identified, all of which conferred sensitivity to cabozantinib in Hep3B cells (Fig. 1f; Supplementary Table S2).

To validate whether EGFR signaling could be responsible for the limited response to cabozantinib, we...
established EGFR-knockdown cell line. shRNA-mediated EGFR knockdown in Hep3B cells sensitized them to cabozantinib, indicating a causal relationship between EGFR signaling and sensitivity to cabozantinib (Fig. 1g, h). Further validation was performed by treating HCC cell lines (Hep3B and Huh7) with a combination of cabozantinib and the EGFR inhibitor WZ3146, which is identified from compound screening. WZ3146 treatment showed a strong synergy with cabozantinib in HCC cells in both long-term and short-term assays (Fig. 1i, j). We also tested the combined effects of cabozantinib and two FDA-approved EGFR inhibitors including gefitinib and afatinib. WZ3146 and afatinib showed obviously stronger synergistic effects with cabozantinib than that of gefitinib on SNU449 cells (Fig. 1k). It is important in this context to point out that these drugs were designed to inhibit mutant EGFR, while the EGFR gene in most of HCC cell lines is not mutated. Thus, the affinity of these molecules for wild-type EGFR may differ. Indeed, it appears that WZ3146 and afatinib have stronger effects on EGFR activity in the HCC cell line model as compared to gefitinib (data not shown). This most likely explains the relatively weak effect of gefitinib in combination with cabozantinib.

To address the mechanism by which EGFR blockade and cabozantinib synergize to reduce the viability of HCC cell lines, we measured apoptosis induction in the presence of cabozantinib, WZ3146 or the combination of these two drugs. Monotherapy of these two inhibitors showed modest evidence of apoptosis induction in three HCC cell lines. However, strong synergistic induction of apoptosis was observed when cells were treated with the combination of cabozantinib and WZ3146 (Fig. 1l, m). As mentioned above, WZ3146 was developed as a mutant-selective EGFR inhibitor against EGFR T790M, whereas in our study we observed that WZ3146 also obviously inhibited the wild-type EGFR (Fig. 1n). Moreover, western blot analyses indicated that the combination of cabozantinib and WZ3146 resulted in an increased inhibition of p-ERK in Huh7 cells (Fig. 1n). Then we further explored the potential mechanism of the synergy between WZ3146 and cabozantinib. As we mentioned above, cabozantinib is a multi-kinase inhibitor targeting c-MET, c-RET, c-KIT, VEGFR, Tie2 and AXL. We tested the synergy between WZ3146 and c-MET inhibitor (capmatinib), c-RET inhibitor (GSK3179106), c-KIT inhibitor (dasatinib), Tie2 inhibitor (Tie2 kinase inhibitor) or AXL inhibitor (dubermatinib), respectively. Only c-KIT inhibitor (dasatinib) shows some synergy with WZ3146 (Fig. 1o). These data suggest that more than one target have been inhibited by cabozantinib to cause synergy between WZ3146 and cabozantinib.

To test whether our in vitro findings could be recapitulated in pre-clinical models, we established two patient-derived HCC organoids for further analyses. The results indicated that cabozantinib or WZ3146 alone slightly suppressed growth of patient-derived organoids, while their combination significantly inhibited cell viability in both HCC organoids, suggesting a strong synergy between cabozantinib and WZ3146 (Fig. 1p). Then, we generated Huh7 xenografts for in vivo analyses. Compared to monotherapy, treatment with the combination of cabozantinib and WZ3146 showed a more effective inhibition of tumor growth (Fig. 1q). The combination treatment elicited an obvious inhibition of p-ERK accompanied with decreased number of Ki67-positive cells (Fig. 1r). Importantly, no obvious side effects were observed in mice treated with the combination of cabozantinib and WZ3146, which indicates a good tolerability of this drug combination.

Cabozantinib combination therapies can potentially be powerful in the treatment of progressed HCC patients. Shang et al. described the combination therapy of pan-mTOR inhibitor MLN0128 and cabozantinib for oncogene-driven HCC murine models. Based on unbiased approaches, we identified the combination of EGFR blockade and cabozantinib as a potential strategy for the treatment of HCC. In a recent study, EGFR inhibition was also found to enhance the efficacy of lenvatinib in liver cancer. However, EGFR inhibition did not enhance the efficacy of sorafenib in clinical studies, indicating that the synergy of EGFR inhibition is selective for only a subset of multi-kinase inhibitors. Since the two drugs identified here have already been used in the clinic or are in clinical development, our findings could be readily tested in clinic.

Acknowledgements
We thank Mengnuo Chen and Hui Wang for providing advice for the design of the study. The research was funded by the National Natural Science Foundation of China (81920108025, 81874229, 82072633, and 82122047), National Facility for Translational Medicine (TMSK-2020-005 and TMSK-2021-129), National Science Foundation of Shanghai (222R148000), Shanghai Municipal Science and Technology Major Project (20JC141100), Shanghai Cancer Institute (GCYJ-22-03 and ZZ9421-13) and China National Postdoctoral Program for Innovative Talents (BX20211187).

Author details
1State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China. 2Shanghai Immune Therapy Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China. 3National Key Laboratory of Translational Medicine, Shanghai Jiao Tong University School of Medicine, Shanghai, China. 4National Facility for Translational Medicine (Shanghai), State Key Laboratory of Medical Genomics, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China. 5Department of Anesthesiology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China. 6Division of Molecular Carcinogenesis, Oncode Institute. The Netherlands Cancer Institute, Amsterdam, The Netherlands

Author contributions
X.M., S.W., and B.L. designed the experiments, performed the experiments and data analysis; Q.Z. and J.Z. performed compound screening; W.L. and H.Y. performed drug assays in HCC organoids; W.Q., R.B., and C.W. are responsible
for the conception, design, and supervision of the study. All authors commented upon the manuscript.

Data availability
The original data that support the findings are available from the corresponding author (C.W.) on reasonable request.

Ethics approval
This study involves human participants and was approved by the Ethics Committee of Renji Hospital (KY2021-114-B).

Conflict of interest
The authors declare no competing interests.

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary information
The online version contains supplementary material available at https://doi.org/10.1038/s41421-022-00425-y.

Received: 28 September 2021 Accepted: 27 May 2022
Published online: 23 August 2022

References
1. Llovet, J. M. et al. Molecular therapies and precision medicine for hepatocellular carcinoma. Nat. Rev. Clin. Oncol. 15, 599–616 (2018).
2. Wang, C. et al. Exploring liver cancer biology through functional genetic screens. Nat. Rev. Gastroenterol. Hepatol. 18, 690–704 (2021).
3. Llovet, J. M. et al. Sorafenib in advanced hepatocellular carcinoma. N. Engl. J. Med. 359, 378–389 (2008).
4. Kudo, M. et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet 391, 1163–1173 (2018).
5. Abou-Alfa, G. K. et al. Cabozantinib in patients with advanced and progressing hepatocellular carcinoma. N. Engl. J. Med. 379, 54–63 (2018).
6. Shang, R. et al. Cabozantinib-based combination therapy for the treatment of hepatocellular carcinoma. Gut 70, 1746–1757 (2021).
7. Caruso, S. et al. Analysis of liver cancer cell lines identifies agents with likely efficacy against hepatocellular carcinoma and markers of response. Gastroenterology 157, 760–776 (2019).
8. Zhou, W. et al. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. Nature 462, 1070–1074 (2009).
9. Jin, H. et al. EGFR activation limits the response of liver cancer to lenvatinib. Nature 595, 730–734 (2021).
10. Zhu, A. X. et al. SEARCH: a phase III, randomized, double-blind, placebo-controlled trial of sorafenib plus erlotinib in patients with advanced hepatocellular carcinoma. J. Clin. Oncol. 33, 559–566 (2015).