Anticarin-β shows a promising anti-osteosarcoma effect by specifically inhibiting CCT4 to impair proteostasis

Gan Wang¹, Min Zhang², Ping Meng³, Chengbo Long⁴, Xiaodong Luo⁵, Xingwei Yang⁶, Yunfei Wang⁷, Zhiye Zhang⁸, James Mwangi⁹, Peter Muiruri Kamau¹⁰, Zhi Dai¹¹, Zunfu Ke¹², Yi Zhang¹³, Wenlin Chen¹⁴, Xudong Zhao¹⁵, Fei Ge¹⁶, Qiumin Lv¹⁷, Mingqiang Rong¹⁸, Dongsheng Li¹⁹, Yang Jin²⁰, Xia Sheng²¹, Ren Lai¹,²,³,⁴,⁵

¹Key Laboratory of Animal Models and Human Disease Mechanisms, Chinese Academy of Sciences/Key Laboratory of Bioactive Peptides of Yunnan Province, KIZ-CUHK Joint Laboratory of Bioresources and Molecular Research in Common Diseases, National Resource Center for Non-Human Primates, Kunming Primate Research Center, and National Research Facility for Phenotypic & Genetic Analysis of Model Animals (Primate Facility), Kunming Institute of Zoology, Kunming 650107, China
²University of Chinese Academy of Sciences, Beijing 100049, China
³State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China
⁴Institutes for Drug Discovery and Development, Chinese Academy of Sciences, Shanghai 201203, China
⁵Sino-African Joint Research Center, Chinese Academy of Science, Wuhan 430074, China
⁶Department of Pathology, First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China
⁷Department of Orthopaedic Surgery, First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China
⁸Department of Breast Surgery, Third Affiliated Hospital of Kunming Medical University, Kunming Cancer Center, Kunming 650118, China
⁹Department of Breast Surgery, First Affiliated Hospital of Kunming Medical University, Kunming 650032, China
¹⁰Department of Biosciences, University of Oslo, Oslo 0316, Norway
¹¹Key Laboratory of Environment and Health, Ministry of Education & Ministry of Environmental Protection, And State Key Laboratory of Environmental Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 434000, China
¹²Center for Biosafety Mega-Science, Chinese Academy of Sciences, Wuhan 430071, China

Received 16 September 2021; received in revised form 4 November 2021; accepted 30 November 2021

*Corresponding authors.
E-mail addresses: rlai@mail.kiz.ac.cn (Ren Lai), xiaosheng@hust.edu.cn (Xia Sheng), yangj@ulrik.uio.no (Yang Jin).
¹These 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.2021.12.024
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/).
Abstract Unlike healthy, non-transformed cells, the proteostasis network of cancer cells is taxed to produce proteins involved in tumor development. Cancer cells have a higher dependency on molecular chaperones to maintain proteostasis. The chaperonin T-complex protein ring complex (TRiC) contains eight paralogous subunits (CCT1-8), and assists the folding of as many as 10% of cytosolic proteome. TRiC is essential for the progression of some cancers, but the roles of TRiC subunits in osteosarcoma remain to be explored. Here, we show that CCT4/TRiC is significantly correlated in human osteosarcoma, and plays a critical role in osteosarcoma cell survival. We identify a compound anticarin-β that can specifically bind to and inhibit CCT4. Anticarin-β shows higher selectivity in cancer cells than in normal cells. Mechanistically, anticarin-β potently impedes CCT4-mediated STAT3 maturation. Anticarin-β displays remarkable antitumor efficacy in orthotopic and patient-derived xenograft models of osteosarcoma. Collectively, our data uncover a key role of CCT4 in osteosarcoma, and propose a promising treatment strategy for osteosarcoma by disrupting CCT4 and proteostasis.

© 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

Osteosarcoma (OS) is a primary bone malignancy that mainly affects children and adolescents. Approximately 85% of OS patients have metastasis, and their 5-year survival rate is less than 20%. The mainstay of curative treatment for OS is surgical resection in combination with chemotherapy using drugs such as doxorubicin, cisplatin, and methotrexate, which are effective in patients with localized disease. However, for patients with metastatic or relapsed OS, clinical outcome has barely improved during the past 30 years because of a lack of effective therapeutics. Of the molecular alterations, activation of the receptor tyrosine kinases (RTKs), such as signal transducer and activator of transcription 3 (STAT3) signaling, is frequently observed in OS tumors.

Mounting evidence has suggested that cancer cells are highly dependent on the homeostasis of protein synthesis and degradation, also termed proteostasis. T-complex protein ring complex (TRiC) belongs to type II chaperonin subfamily and is a cylindrically structured protein with two back-to-back rings, each of which is composed of eight paralogous subunits (CCT1–CCT8). Adenosine triphosphate binding and hydrolysis drive conformational change of TRiC to encapsulate and fold substrate polypeptides. TRiC facilitates the folding of 10% of the cytosolic proteome, including many key structural and regulatory proteins, such as β-actin, tubulin, cell cycle regulator CDC20, VHL tumor suppressor, the TORC1 complex, and STAT3. TRiC level is significantly elevated in multiple cancers and is associated with several essential biological processes in cancer progression. Genetic inhibition of TRiC represses cancer cell growth, providing the rationale that TRiC may be a promising therapeutic target. TRiC subunit has a unique ability to assist the folding of select proteins. However, the functional roles of TRiC in tumors have been poorly defined due to the paucity of specific inhibitors.

In this study, we show that CCT4/TRiC activity is tightly correlated and elevated in human OS. We identified a small molecule natural coumarin compound derived from the bark of the traditional Chinese medicine (Antiaris toxicaria Lesch.), anticarin-β, as a potent inhibitor of CCT4/TRiC. Anticarin-β shows remarkable antitumor efficacy in different orthotopic and patient-derived xenograft (PDX) models of OS by impeding CCT4-mediated maturation of STAT3. Therefore, our data implicate an important oncogenic role of CCT4/TRiC in OS and highlight the potential of targeting CCT4 as a novel treatment option for OS.

2. Materials and methods

2.1. Ethical statement and tissue collection

Paraffin-fixed tissue sections of human osteosarcoma (OS) specimens were collected at the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). Frozen tissues of human OS specimens were collected at the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). The samples were obtained upon informed consent from patients and was ethically approved by the Ethics Committee of both hospitals.

2.2. Antibodies

Primary antibodies against caspase-3 (9665, CST), caspase-7 (ab32522, Abcam), CCT4 (ab205013, Abcam), GAPDH (97166, CST), PARP (9532, CST), p-STAT1 (Tyr701) (7649, CST), p-STAT2 (Tyr690) (4441, CST), p-STAT3 (Ser727) (7914, CST), p-STAT5 (Tyr694) (4322, CST), p-STAT6 (Tyr641) (9361, CST), STAT1 (9172, CST), STAT3 (9145, CST), XIAP (ab32522, Abcam), CCT4 (ab129072, Abcam), GAPDH (97166, CST), PARP (9532, CST), p-STAT1 (Tyr701) (7649, CST), p-STAT2 (Tyr690) (4441, CST), p-STAT3 (Ser727) (7914, CST), p-STAT5 (Tyr694) (4322, CST), p-STAT6 (Tyr641) (9361, CST), STAT1 (9172, CST), STAT3 (9145, CST), XIAP (2045, CST), LC3 (12741, CST), β-catenin (8480, CST), N-cadherin (13116, CST), β-actin (3700, CST), and secondary Fluo-rescin AffiniPure goat anti-rabbit IgG (111-095-144, Jackson) and Cy3 AffiniPure goat anti-rabbit IgG (111-165-144, Jackson) antibodies were used for immunofluorescent staining. Primary antibodies against Ki67 (9449, CST), CD3 (ab135372, Abcam), CD68 (76437, CST), CD56 (99746, CST), and secondary Fluorescin AffiniPure goat anti-rabbit IgG (111-095-144, Jackson) antibodies were used for immunofluorescence. Antibodies against p-STAT3 (Tyr705) (9145, CST), STAT3 (9139, CST), cleaved caspase-3 (Asp175) (9661, CST), and CCT4 (ab129072, Abcam) were used for immunohistochemistry.
2.3. Cell culture

The human cell lines EJ-1 (CRL-3380, ATCC), HCT 116 (CCL-247, ATCC), MCF7 (HTB-22, ATCC), MDA-MB-231 (HTB-26, ATCC), NCI-H157 (CRL-5802, ATCC), NCI-H292 (CRL-1848, ATCC), NCI-H460 (HTB-177, ATCC), NCI-H661 (HTB-183, ATCC), and T24 (HTB-4, ATCC) were cultured in RPMI-1640 medium (Cellgro, Corning). Human 801-D (200690YJ, KCB, Kunming, China), HEK-293T (CRL-11268, ATCC), HeLa (CCL-2, ATCC), HL-a (CCL-199, ATCC), HT-29 (HTB-38, ATCC), HUVEC (PCS-100-013, ATCC), LX-2 (337957, BNCC, Beijing, China), SK-MES-1 (HTB-58, ATCC), T98G (CRL-1690, ATCC), and U-87 MG (HTB-14, ATCC) cells were maintained in Dulbecco's modified Eagle medium (DMEM, Cellgro). Human A549 (CCL-185, ATCC), HepG2 (HB-8065, ATCC), K-562 (CCL-243, ATCC), MG-63 (CRL-1427, ATCC), PANC-1 (CRL-1469, ATCC), SaOS2 (HTB-85, ATCC), U2OS (HTB-96, ATCC), and NSC cells were maintained in DMEM: Nutrient Mixture F-12 (Cellgro). The cell lines GBM-18 and GBM-3 were cultured in DMEM (Cellgro).

All culture media was supplemented with 10% fetal bovine serum, 100 unit/mL penicillin and 100 unit/mL streptomycin, and were maintained in a humidified atmosphere containing 5% CO2 in an incubator at 37°C.

2.4. Quantification and statistical analysis

Statistical analysis was conducted with GraphPad Prism 6.0 (GraphPad Software). When needed, a d’Agostino-Pearson normality test was performed and was followed by Student’s t-tests or non-parametric Mann–Whitney tests when the distribution was not normal. Statistical comparisons between groups were conducted with ordinary one-way analysis of variance with multiple comparisons tests. All results are presented as the

Figure 1  The expression of TRiC subunits is increased in human osteosarcoma (OS). (A) Quantitative reverse transcription PCR analysis of the expression of TRiC subunits in OS tissues and matched para-cancerous bone tissues (n = 11). Relative quantification (RQ) for each subunit is presented as a dot-box plot. (B) TRiC expression was compared in patients with or without metastasis in the GSE21257 dataset. (C) Association between TRiC activity score and overall survival of the patients was estimated by Kaplan–Meier analysis. (D) Immunohistochemical analysis of CCT4 in OS tissues (17 cases in TMA (Tissue microarray)) and NBT (normal bone tissue, n = 6). Scale bar, 2 mm. (E) CCT4 knockout MG-63 cells were generated, and then the cells were plated and cultured for colony formation assays. The data are presented as the mean ± SD, n = 3.

*P < 0.05; **P < 0.01, two-tailed t-test.
Figure 2  Anticarin-β inhibits the chaperonin activity of TRiC through interaction with CCT4. (A) Workflow for identification of CCT4 binding compounds, which mainly consists of virtual screening, pharmacophore screening, and experimental validation. The box represented the 3D structural of CCT4 and binding site (red boll) for virtual screening. (B) The viability of MG-63 cells treated with the top 5 candidates on the screen. Dapagliflozin IC50 200 μmol/L, n = 3. (C) Comparison of the 3D pharmacophores of anticarin-β and dapagliflozin, purple/green spheres represent electron acceptor/donor, yellow spheres represent ring aromatic, blue spheres represent hydrophobe group. (D) The luciferase refolding assay was performed to measure the inhibitory effect of anticarin-β on the chaperonin activity of purified human TRiC. Bovine albumin was used as a negative control. The mean SD from three representative experiments is shown. (E) The cellular thermal shift assay was performed on intact MG-63 cells with 2.5 μmol/L anticarin-β. The band intensity values were calculated from three independent experiments. The points and error bars represent the mean SD, n = 3; *P < 0.05, **P < 0.01 versus the control group, two-tailed t-test. (F) CCT4 mutant plasmids were transfected into U2OS cells followed by 1 μmol/L anticarin-β treatment for 20 h. Cell viability was analyzed by trypan blue. The points and error bars represent the mean SD, n = 4; ****P < 0.0001 versus the WT group, two-tailed t-test. CK, blank vector; WT, wild type CCT4. (G) 3D structure of the binding site of anticarin-β with CCT4, the green dotted lines represent hydrogen bonds, and the pink dotted lines represent alkyl hydrophobic bonds. (H) Venn diagrams show the number of overlapped genes among top 100 significantly up-regulated (↑) and down-regulated (↓) genes in OS cells with anticarin-β or siCCT4 treatment. (I) Hallmark GSEA shows a significant overlap of enriched pathways in OS cells between anticarin-β and siCCT4 treatment. Bar plot shows commonly enriched Hallmark pathways with FDR < 0.25. The numbers represent the normalized enrichment scores (NES) for the corresponding gene sets.
Figure 3  Anticarin-β inhibits OS cell growth by inducing apoptosis and disrupting autophagosome degradation. (A) Heatmap showing the selective cytotoxicity of anticarin-β towards a panel of normal and cancer cell lines. The cells were treated with a serial concentration of anticarin-β for 48 h. Cell viability was measured by MTT assay, and the IC50 was determined, n = 4. (B) The real-time cell analysis data show that anticarin-β has selective cytotoxicity on osteosarcoma cells (left, anticarin-β 0.5 μmol/L) compared with non-malignant cells (right, anticarin-β 2 μmol/L). Arrows indicate the time that anticarin-β was applied. (C) OS cells were treated with 0.05 μmol/L anticarin-β for 1 day and then plated for colony formation. Anticarin-β has no effects on the cell cycle distribution of OS cells. OS cells were treated with 0.5 μmol/L anticarin-β for 12 h, and the proportion of cells in G0/G1, S, G2/M, was determined by PI (propidium iodide) staining. The points and error bars represent the mean ± SD, n = 3; ns, not significant; *** P < 0.001 versus the phosphate buffered saline (PBS) group, two-tailed t-test. (D) MG-63 cells were treated with 0.5 μmol/L anticarin-β for the indicated durations before being analyzed by Western blot. (E) BrdU analysis of MG-63 cells treated with either PBS or anticarin-β. Scale bar, 20 μm. The data represent mean ± SD, n = 20; **** P < 0.0001 versus the PBS group, two-tailed t-test. (F) MG-63 cells were loaded with caspase 3/7 green probes and then treated with 0.5 μmol/L anticarin-β or vehicle for 24 h. An increase in fluorescence indicated the occurrence of apoptosis. Scale bar, 1 μm. The data represent mean ± SD, n = 20; **** P < 0.0001 versus the PBS group, two-tailed t-test. (G) After treatment with
means ± standard deviation (SD) or standard error of mean (SEM) from at least three independent measurements. The P values were calculated, and significance is represented as follows: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Additional methods and references are available at Supporting Information.

3. Results

3.1. TRiC signaling is activated in OS and correlates with poor outcome

We first investigated the possible involvement of TRiC signaling in OS by interrogating the publicly available gene expression datasets of OS. The interrogation of the PRédiction of Clinical Outcomes from Genomic Profiles (PRECOG) database showed that gene expression of chaperonins was significantly associated with prognosis in various cancers, particularly in OS (Supporting Information Table S1A). Of note, TRiC showed the highest PRECOG score in OS amongst the chaperonin subfamily (Tables S1B and S1C). The expression of all TRiC subunits was significantly increased in the OS tissues compared to the paired normal bone tissues (Fig. 1A). To further assess whether TRiC level is associated with OS development, we analyzed the correlation of TRiC expression with metastatic status and overall survival. The expression of TRiC subunits was significantly higher in patients with metastasis and the overall expression of TRiC subunits poor overall survival (Fig. 1B and C and Supporting Information Fig. S1A, see Supporting Information Table S2 for primers information). Together, these results indicate that the activity of TRiC signaling is correlated with OS progression and prognosis.

Next, we evaluated the in vitro efficacy of TRiC subunits in OS cell lines. Colony formation analysis revealed that knockdown of CCT4 or CCT7 significantly reduced the clonogenicity of MG-63 cells (Fig. S1B, see Supporting Information Table S3 for detailed siRNA information). This is in line with a recent structure study demonstrating that the CCT4 subunit plays a key role in mediating the function of TRiC19-21. Hereafter, we focused on CCT4 as a surrogate of TRiC in the following characterization. Consistent with the above observation, immunohistochemical analysis revealed significantly elevated level of CCT4 in OS compared to normal tissues (Fig. 1D, see Supporting Information Table S4 for detailed group information). Interestingly, CCT4 knockout using CRISPR/Cas9 approach was lethal to OS cells, with no viable clone obtained after 2 weeks (Fig. 1E). Alternatively, we performed subsequent functional assays by CCT4 knockdown, which significantly affected the migration and invasion of MG-63 cells, but showed little effect on apoptosis, autophagy, and proliferation (Supporting Information Fig. S2). These results corroborated an indispensable role of CCT4/TRiC in OS cell survival and proposed CCT4 as a potential therapeutic target for OS.

3.2. Discovery of anticarin-β as an inhibitor of the CCT4 subunit of TRiC

Despite these findings, the paucity of available inhibitors impeded us from further evaluation. To overcome this barrier, we first performed a model-based virtual screen of the United States Food and Drug Administration-approved drug library, intending to search for potential inhibitors for the CCT4 subunit of TRiC (Fig. 2A). This gave rise to several compounds that potentially bind CCT4 (Supporting Information Table S5). Then, we evaluated the effect of the top 5 compounds on the growth of MG-63 cells, in which the CCT4 level is slightly lower compared to in U2OS and SaOS2 cells (Fig. S2B). We found that dapagliflozin, a drug for type 2 diabetes, showed the strongest inhibitory activity relative to the other candidates, with a half-maximal inhibitory concentration (IC50) of 200 µmol/L (Fig. 2B). To search for more potent inhibitors, we created a three-dimensional pharmacophore model of dapagliflozin (Fig. 2C) and screened an in-house chemical library. A natural compound anticarin-β (19993560, the Cambridge Crystallographic Data Centre) with a 3D pharmacophore highly similar to that of dapagliflozin was identified (Fig. 2C).

To verify the inhibitory effect of anticarin-β, a luciferase refolding assay was performed with purified TRiC, whose activity remains in vitro22. Anticarin-β dose-dependently inhibited the refolding of denatured luciferase by isolated TRiC (Fig. 2D). Furthermore, the surface plasmon resonance-based binding assay showed that anticarin-β bound to immobilized recombinant CCT4 protein with an affinity (Kd ~ 2.2 µmol/L) significantly higher compared to with other TRiC subunits (Supporting Information Fig. S3A), indicating a direct and specific interaction of anticarin-β with CCT4. To assess whether anticarin-β also binds to CCT4 in vivo, we performed the cellular thermal shift assay, an approach to determine the effect of ligand binding on the thermal stability of target proteins in live cells23. Compared with control, anticarin-β treatment resulted in significantly lower thermal stability of CCT4 in MG-63 cells at denaturation temperature from 60 to 68 °C (Fig. 2E). GAPDH was used as a control and exhibited no difference in thermal stability upon anticarin-β treatment, indicating a specific ligand effect on CCT4.

3.3. Anticarin-β inhibits TRiC activity by specifically binding to its CCT4

Subsequently, we mapped the binding site of anticarin-β in CCT4 using both in silico and experimental approaches. Blind docking experiment identified 4 potential binding pockets for anticarin-β in the protein structure of CCT4. Next, a 100 ns molecular dynamics simulation was performed to determine the contribution of specific amino acid residues to the stability of anticarin-β binding (Supporting Information Fig. S3B and Table S6). Mutagenesis analysis revealed that four residues (G105, T107, T464, and T68) are significantly involved in anticarin-β binding.
V508) at site 2 may be critical for anticarin-β binding. This was confirmed by cell viability assay where mutation of these residues in CCT4 significantly prevented the cytotoxicity of anticarin-β in U2OS cells (Fig. 2F and G, see Supporting Information Table S7 for primers information of point mutation). Isothermal titration calorimetry was used to study binding affinity constants of anticarin-β with different point mutants of CCT4. The affinity (Kₐ) between the CCT4 mutants and anticarin-β decreased by 3–26 times compared to the wild-type CCT4 (Supporting Information Fig. S4). 3D structure modeling further suggested that these three mutant residues (T107, T464, and V508) may interact directly with anticarin-β (Fig. 2G). Importantly, transcriptomic analysis revealed that the effect of anticarin-β on global gene expression was highly concordant to that of CCT4 depletion (Fig. 5C). Amongst the top 100 up-regulated genes, there were 71 commonly occurred in both treatments, whereas 41 in the top 100 down-regulated (Fig. 2H). This led to the enrichment of similar oncogenic pathways, such as Hallmark Kras pathway, KRAS signaling, hypoxia, and apoptosis (Fig. 2I). Together, these data demonstrate that anticarin-β inhibits TRiC activity by specifically binding to its CCT4 subunit.

3.4. Anticarin-β inhibits survival and induces apoptosis in OS cells

To assess the potential cytotoxicity of anticarin-β, we explored the effect of anticarin-β on the viability of a series of non-cancerous and cancer cell lines. Interestingly, dose-dependent inhibition of viability was observed for all cancer cell lines tested, whereas no significant cytotoxic effect was detected on the non-malignant cell lines (Fig. 3A). Notably, the OS and glioblastoma cell lines were more sensitive to anticarin-β than the other cancer cell lines (Fig. 3A). In a time-course assay, 0.5 μmol/L anticarin-β decreased the viability of MG-63 cells by ~73% within 24 h, whereas no toxicity was observed in HEK-293T and HUVEC cells (Fig. 3B). Concerning the long-term cell growth, colony-forming assay revealed that anticarin-β inhibited the growth of three OS cell lines, MG-63, U2OS, and SaOS2, by approximately 95% (Fig. 3C). Cell cycle distribution was not significantly altered in response to anticarin-β (Fig. 3C), indicating that anticarin-β may not arrest cell cycle or suppress proliferation in OS cells. These data suggest that anticarin-β effectively inhibits the viability and growth of OS cells in vitro.

Another possible explanation to these findings is that anticarin-β likely induces cell death rather than cell cycle arrest in OS cells. To test this, we examined apoptotic markers by Western analysis in MG-63 cells treated with anticarin-β. There was a sharp increase in the level of cleaved PARP and cleaved caspase-3/7 after 12 h of anticarin-β treatment, which was accompanied by a rapid decline in the expression of XIAP (Fig. 3D). Compared with the control treatment (PBS), anticarin-β treatment significantly inhibits cell proliferation (Fig. 3E), as demonstrated by BrdU staining. Utilizing the CellEvent caspase-3/7 Green probe, we verified that caspase-3/7 cleavage was induced in approximately 80% of MG-63 cells treated with anticarin-β, whereas the ratio was lower than 5% in the control cells (Fig. 3F).

Consistently, Annexin V-FITC assay and propidium iodide fluorescence staining also showed significantly stronger signals in cells exposed to anticarin-β relative to control (Fig. 3G). Importantly, ectopic expression of CCT4 partially rescued MG-63 cells from apoptosis induced by anticarin-β (Fig. 3H). These results indicate that anticarin-β exerts antitumor effect by inducing apoptosis in OS cells.

Since TRiC depletion was recently shown to confer impaired autophagy and sensitivity to apoptotic insults 26, we further determined whether blocked autophagic flux mediates the anticarin-β-induced apoptosis. Transmission electron microscopic analysis of MG-63 cells treated with anticarin-β revealed a dramatic increase in the number of autophagosomes, which did not appear to merge with lysosomes (Fig. 3I). To investigate whether autophagosome-lysosome fusion was hindered, we transfected MG-63 cells with the tandem-labeled mCherry-Green fluorescent protein (GFP)-LC3 construct to distinguish autophagosomes and autolysosomes 25. Indeed, anticarin-β treatment significantly increased the number of autophagosomes and decreased the number of autolysosomes (Fig. 3J). These data implicate that anticarin-β blocks autophagosome—lysosome fusion and impairs autophagy, which may mediate cell death.

3.5. Anticarin-β inhibits TRiC-mediated STAT3 maturation and activation

To uncover the genes or pathways that may contribute to the differential sensitivity to anticarin-β, we analyzed the correlation between the IC₅₀ of the tested cell lines and their gene expression profiles using the Cancer Cell Line Encyclopedia 26. The expression of CLEC11A, a member of the C-type lectin superfamily encoding the stem cell growth factor, showed the most significant correlation with anticarin-β sensitivity (Fig. 4A). Despite the limited knowledge about its role in cancer, further interrogation of the PharmacoDB database 27 showed that CLEC11A expression in cancer cells strongly predicted sensitivity to inhibitors of the RTKs, especially type III RTKs (Fig. 4A). These bioinformatic analyses establish a link between anticarin-β activity and RTKs signaling.

To gain a better insight into this connection in the context of OS, we performed a phospho-kinase antibody array of RTK pathways in MG-63 cells treated with anticarin-β. Interestingly, anticarin-β significantly reduced the levels of phosphorylated (p-) STAT proteins, while it induced AKT and ERK activation (Fig. 4B). Western blot validated that both p- and total STAT3 level was robustly attenuated in MG-63 cells in response to anticarin-β (Fig. 4C), a phenotype that can be rescued by CCT4 overexpression (Fig. 4D). Similar to CCT4 knockdown, anticarin-β significantly reversed the expression of a series of genes directly regulated by STAT3 in the RNA-seq analysis (Fig. 4E), in particular the level of pro-apoptotic genes (Fig. 4F). These data suggest that the apoptosis-inducing effect of anticarin-β may be mediated, at least in part, by repressing STAT3 signaling.

A recent study evidenced that TRiC is critical for the proper folding and activation of STAT3 28. To evaluate whether anticarin-β inhibits this process, we generated MG-63 cells expressing GFP (control), wild-type STAT3 (STAT3-WT), dominant-negative STAT3 (STAT3-DN), or constitutively active STAT3 (STAT3-CA) via lentivirus transduction (Fig. 4G). As expected, compared with GFP control, STAT3-WT and STAT3-DN did not protect the cells from apoptosis induced by anticarin-β. Interestingly, introduction of STAT3-CA only slightly rescued the apoptotic phenotype (Fig. 4G), suggesting that anticarin-β may disrupt the TRiC-mediated folding and maturation of STAT3, including its constitutively active variant.

3.6. Anticarin-β inhibits tumor growth in an orthotopic mouse model of OS

Building on the significant in vitro effect and the mechanism of action, we further evaluated the in vivo efficacy of anticarin-β in preclinical models of OS. To this end, we first determined the maximum tolerated dose of anticarin-β to define the optimal in vivo concentration. No lethality or apparent toxicity was...
observed within 50 days at doses up to 20 mg/kg (Fig. 5A). A pharmacokinetic study on anticarin-β in CD-1 mice showed that anticarin-β had comparable $t_{1/2}$ values in plasma and muscle (1.08−1.46 h), and could be almost eliminated within 8 h (Fig. 5B and Supporting Information Table S8). Therefore, daily administration of anticarin-β at a dose of 5 mg/kg was used for animal experiments.

Based on the favorable in vitro cytotoxicity and in vivo safety results, we first evaluated the efficacy of anticarin-β in an orthotopic xenograft model of OS, in which luciferase-expressing MG-63 cells were transplanted into BALB/c nude mice. The tumor-bearing mice were treated with or without anticarin-β (0.5 or 5 mg/kg/day) and tumor growth was monitored by measurement of bioluminescence. Two commonly used first-line therapeutic drugs for OS, methotrexate (20 mg/kg/twice a week) and cisplatin (5 mg/kg/day), were also included for comparison. Relative to vehicle treatment (control), anticarin-β treatment led to significant dose-dependent tumor regression, whereas methotrexate and cisplatin only slightly
inhibited tumor growth (Fig. 5C and D and Supporting Information Fig. S5A). About 40% of control tumor-bearing mice died within 49 days, suggesting successful transplantation of human MG-63 cells (Fig. 5E). In agreement with the effect on tumor growth, anticarin-β at both 0.5 and 5 mg/kg markedly improved the survival of tumor-bearing mice. In contrast, despite the moderate efficacy, cisplatin treatment did not improve the survival rate, whereas methotrexate treatment even worsened it (Fig. 5E). Following this, methotrexate treatment resulted in remarkable body weight loss of the mice, whereas anticarin-β (5 mg/kg) significantly prevented tumor-caused weight loss compared with the vehicle control (Fig. 5F), indicating no obvious toxicity and improved well-being of the mice. This was further confirmed by histological analysis of major organs, which showed no observable difference between the vehicle and 5 mg/kg anticarin-β treated groups (Fig. 5G).

Next, we examined the effect of anticarin-β on the activity of STAT3 signaling in the transplanted tumors. In line with the in vitro findings, STAT3 and p-STAT3 protein levels were both decreased in the tumor tissues of mice treated with anticarin-β, while cleaved caspase 3 was significantly induced (Fig. 5H, Fig. S5B and S5C). These results corroborate that anticarin-β effectively inhibits tumor growth and STAT3 activation in vivo.

3.7. Anticarin-β inhibits tumor growth in multiple PDX models of OS

To further evaluate the clinical utility of this compound, ex vivo 3D culture and mouse PDX models were generated. Three different PDX models derived from different stages of OS,
SA3849 (post-chemotherapy), SA4061 (post-radiation), and SA4062 (lung metastasis), were used for the ex vivo 3D assay.

Anticarin-β displayed potent inhibitory effect on all cultures, with the strongest effect on the lung metastasis PDX model SA4062 (Fig. 6A). Next, PDX model SA4062 tumors were subcutaneously inoculated into NOD/SCID mice. As shown in Fig. 6B, compared with vehicle administration, daily administration of 5 mg/kg anticarin-β significantly retarded SA4062 tumor growth (Fig. 6B). More interestingly, anticarin-β combined with peripheral blood mononuclear cell has a better inhibitory effect, these results indicated that anticarin-β could assist immune cells to kill tumor cells (Fig. 6C–E). Consistently, PDX tumors treated with anticarin-β displayed significantly decreased STAT3, p-STAT3 level, and increased cleaved PARP (Fig. 6D). Collectively, these results...
demonstrate that anticarin-β markedly impedes tumor growth in different preclinical models of OS without inducing obvious toxicity, indicating that anticarin-β may be effective in treating OS, especially the metastatic/late-stage OS.

4. Discussion

Maintaining proteostasis, including protein synthesis, folding, secretion, and degradation, is critical to the function and viability of cancer cells. The chaperonin TRiC is significantly increased in some cancers and strongly correlates with disease progression. The diversification of TRiC subunits into eight paralogs underlies its unique ability to assist the folding of select proteins and function in oncogenesis, which prompts us to investigate the functional role of TRiC subunit. Here, our bioinformatic analysis suggests that CCT4/TRiC level tightly correlates with OS progression and prognosis. Consistently, genetic ablation of CCT4 robustly impairs growth and survival of OS cells. The in vitro metastatic potential of OS cells is also markedly weakened upon CCT4 inhibition. These findings implicate CCT4/TRiC as a driver of OS progression, and prompt us to search for potential drugs that may specifically inhibit its activity. Encouragingly, after a series of virtual screening and biochemical validation, we identified a natural compound anticarin-β that physically interacts with CCT4. Anticarin-β and CCT4 knockdown lead to the deregulation of numerous common genes and similar pathways in OS cells, advocating that anticarin-β blocks autophagic flux and induces apoptosis in OS cells, again mirroring the effect of genetic inhibition of CCT4.

Interestingly, interrogation of the PRECOG database coincides with the transcriptomic analysis of anticarin-β-treated OS cells, which links anticarin-β efficacy with RTK signaling in OS cells. Results from a comprehensive array of RTK pathways further reveal that STAT3 signaling is robustly repressed in OS cells upon anticarin-β treatment. Western analysis validates that anticarin-β reduces both phospho- and total forms of STAT3. Since TRiC is recently shown to facilitate STAT3 protein folding and full activation, we further verify that anticarin-β impedes the CCT4-mediated maturation of STAT3, leading to the suppressed STAT3 signaling and proteostasis disorder. In keeping with this, the level of CCT4/TRiC and STAT3 is significantly associated in human OS cohorts. Therefore, our results uncover important molecular signaling in OS cells and provide a promising candidate molecule to target it.

Building on the favorable in vitro effect and the mechanism of action, we evaluate the in vivo efficacy of anticarin-β in preclinical mouse models of OS. Anticarin-β shows stronger antitumor efficacy in an orthotopic model of OS compared to clinical cytotoxic agents, with satisfactory profile of pharmacokinetics and toxicity. Furthermore, of the three PDX tumors modeling different stages of OS, the lung metastasis PDX displays the highest sensitivity to anticarin-β. These results propose anticarin-β as a novel therapeutic agent for OS that worth further preclinical and clinical evaluation.

5. Conclusions

This study unveils an essential role of TRiC—STAT3 signaling in OS progression. Importantly, we demonstrate that inhibiting the CCT4 subunit of the TRiC complex with a natural compound anticarin-β effectively disrupts this signaling network and proteostasis and results in profound antitumor effect in multiple preclinical models of OS. Therefore, our results suggest a promising strategy for the development of CCT4/TRiC-targeted therapeutics for OS treatment.

Availability of data and materials

The original data is available at Mendeley Data (https://data.mendeley.com/datasets/kkgty54cmz/1).

Acknowledgments

We thank Dr. Lian Yang for technical advice and assistance with the ITC analysis. We thank Dr. Lin Zeng for technical advice and assistance with the LC–MS/MS spectrum analysis. We thank Dr. Wenhao Yang for writing advice. This work was supported by funding from the National Natural Science Foundation of China (81903666 and 31930015), the Chinese Academy of Sciences (XDB31000000, KFJ-STS-SCYD-304, and K. C. Wong Education Foundation; China), the Science and Technology Department of Yunnan Province (202101AT070301, 2019ZFF003, 202002AA100007, 202003AD150008, and 2019FB103; China), Project of Innovative Research Team of Yunnan Province (2019HC005; China), and the Department of Industry and Information Technology of Yunnan Province (2019-YT-053, China).

Author contributions

Ren Lai, Xia Sheng, Yang Jin, and Gan Wang designed the study and wrote the paper. Gan Wang, Min Zhang, and Ping Meng conducted most of the experiments, analyzed the results, and wrote the paper. Chengbo Long and Yunfei Wang conducted the compound purification experiments. Chengbo Long and Ping Meng conducted the cell-derived xenograft model experiments. Xiaodong Luo, Zhiye Zhang, and Xingwei Yang conducted structural identification experiments. James Mwangi and Peter Muiruri Kamau conducted the RNA experiments. Zunfu Ke, Wenlin Chen, Fei Ge, and Yi Zhang provided clinical samples. Zhi Dai and Xia Sheng conducted the compound identification experiments. Dongsheng Li, Qiumin Lv, and Mingqiang Rong provided technical assistance and contributed to the preparation of the figures. All authors analyzed the results and approved the final version of the manuscript.

Conflicts of interest

The authors have declared that no conflict of interest exists.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.12.024.

References

1. Ottaviani G, Jaffe N. The epidemiology of osteosarcoma. *Cancer Treat Res* 2009;152:3–13.
2. Moore DD, Luu HH. Osteosarcoma. *Cancer Treat Res* 2014;162:65–92.
3. Daw NC, Chou AJ, Jaffe N, Rao BN, Billups CA, Rodriguez-Galindo C, et al. Recurrent osteosarcoma with a single pulmonary
metastasis: a multi-institutional review. Br J Cancer 2015;112:278–82.
4. Faisham WI, Mat Saad AZ, Alsaih LN, Nor Azman MZ, Kamarul Imran M, Biswal BM, et al. Prognostic factors and survival rate of osteosarcoma: a single-institution study. Asia Pac J Clin Oncol 2017;13:e104–10.
5. Isakov MS, Bielack SS, Meltzer P, Gorlick R. Osteosarcoma: current treatment and a collaborative pathway to success. J Clin Oncol 2015;33:3029–35.
6. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin 2018;68:7–30.
7. Han Y, Guo W, Ren T, Huang Y, Wang S, Liu K, et al. Tumor-associated macrophages promote lung metastasis and induce epithelial–mesenchymal transition in osteosarcoma by activating the COX-2/STAT3 axis. Cancer Lett 2019;440–441:116–25.
8. Dai C, Sampson SB. HSF1: guardian of protein stability in cancer. Trends Cell Biol 2016;26:17–28.
9. Frydman J, Nimmesgern E, Erdjument-Bromage H, Wall JS, Tempst P, Hartl FU. Function in protein folding of TRiC, a cytosolic ring complex containing TCP-1 and structurally related subunits. EMBO J 1992;11:4767–78.
10. Joachimiak LA, Walztboeni T, Liu CW, Aebersold R, Frydman J. The structural basis of substrate recognition by the eukaryotic chaperonin TRiC/CCT. Cell 2014;159:1042–55.
11. Sterllicht H, Farr GW, Sternlicht ML, Driscoll JK, Willison K, Yaffe MB. The t-complex polypeptide 1 complex is a chaperonin for tubulin and actin in vivo. Proc Natl Acad Sci U S A 1993;90:9422–6.
12. Connors A, Bogdanova A, Shovenko A, Zachariae W. The CCT chaperonin promotes activation of the anaphase-promoting complex through the generation of functional Cdc20. Mol Cell 2003;12:87–100.
13. Cuellar J, Ludlam WG, Tensmeyer NC, Aoba T, Dhavale M, Santiago C, et al. Structural and functional analysis of the role of the chaperonin CCT in mTOR complex assembly. Nat Commun 2019;10:2865.
14. Kasembi M, Lau WC, Roh SH, Eckols TK, Frydman J, Chiu W, et al. Modulation of STAT3 folding and function by TRiC/CCT chaperonin. PLoS Biol 2014;12:e1001844.
15. Vallin J, Grantham J. The role of the molecular chaperone CCT in protein folding and mediation of cytoskeleton-associated processes: implications for cancer cell biology. Cell Stress Chaperones 2019;24:17–27.
16. Yokota S, Yamamoto Y, Shimizu K, Momoi H, Kamikawa T, Yamaoka Y, et al. Increased expression of cytosolic chaperonin CCT in human hepatocellular and colonic carcinoma. Cell Stress Chaperones 2001;6:345–50.
17. Yokota S, Yanagi H, Yura T, Kubota H. Cytosolic chaperonin is upregulated during cell growth. Preferential expression and binding to tubulin at G1/S transition through early S phase. J Biol Chem 1999;274:37070–8.
18. Guest ST, Kratke ZR, Bollig-Fischer A, Haddad R, Ether SP. Two members of the TRiC chaperonin complex, CCT2 and TCP1 are essential for survival of breast cancer cells and are linked to driving oncogenes. Exp Cell Res 2015;332:223–35.
19. Gestaut D, Roh SH, Ma B, Pintilie G, Joachimiak LA, Leitner A, et al. The chaperonin TRiC/CCT associates with prefoldin through a conserved electrostatic interface essential for cellular proteinostasis. Cell 2019;177:751–765 e15.
20. Jin M, Han W, Liu C, Zang Y, Li J, Wang F, et al. An ensemble of cryo-EM structures of TRiC reveal its conformational landscape and subunit specificity. Proc Natl Acad Sci U S A 2019;116:19513–22.
21. Reissmann S, Joachimiak LA, Chen B, Meyer AS, Nguyen A, Frydman J. A gradient of ATP affinities generates an asymmetric power stroke driving the chaperonin TRiC/CCT folding cycle. Cell Rep 2012;2:866–77.
22. Knee KM, Sergeeva OA, King JA. Human TRiC complex purified from HeLa cells contains all eight CCT subunits and is active in vitro. Cell Stress Chaperones 2013;18:137–44.
23. Martinez Molina D, Jafari R, Ignatashchenko M, Seki T, Larsson EA, Dan C, et al. Monitoring drug target engagement in cells and tissues using the cellular thermal shift assay. Science 2013;341:84–7.
24. Pavel M, Imarisio S, Menzies FM, Jimenez-Sanchez M, Siddiqui FH, Wu X, et al. CCT complex regulates survival pathogenic protein aggregation and autophagy. Nat Commun 2016;7:13821.
25. Kimura S, Noda T, Yoshimori T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autobag 2007;3:452–60.
26. Hafner M, Niepel M, Chung M, Sorger PK. Growth rate inhibition metrics correct for confounders in measuring sensitivity to cancer drugs. Nat Methods 2016;13:521–7.
27. Smirnov P, Kofia V, Maru A, Freeman M, Ho C, El-Hachem N, et al. PharmacoDB: an integrative database for mining in vitro antitumor drug screening studies. Nucleic Acids Res 2018;46:D994–1002.
28. Timofeeva OA, Tarasova NI, Zhang X, Chasovskikh S, Cheema AK, Wang H, et al. STAT3 suppresses transcription of proapoptotic genes in cancer cells with the involvement of its N-terminal domain. Proc Natl Acad Sci U S A 2013;110:1267–72.
29. Dufey E, Urra H, Hetz C. ER proteostasis addiction in cancer biology: novel concepts. Semin Cancer Biol 2015;33:40–7.
30. Wang Y, Wang K, Jin Y, Sheng X. Endoplasmic reticulum proteinostasis control and gastric cancer. Cancer Lett 2019;449:263–71.