Ancient drug curcumin impedes 26S proteasome activity by direct inhibition of dual-specificity tyrosine-regulated kinase 2

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Curcumin, the active ingredient in Curcuma longa, has been in medicinal use since ancient times. However, the therapeutic targets and signaling cascades modulated by curcumin have been enigmatic despite extensive research. Here we identify dual-specificity tyrosine-regulated kinase 2 (DYRK2), a positive regulator of the 26S proteasome, as a direct target of curcumin. Curcumin occupies the ATP-binding pocket of DYRK2 in the cocrystal structure, and it potently and specifically inhibits DYRK2 over 139 other kinases tested in vitro. As a result, curcumin diminishes DYRK2-mediated 26S proteasome phosphorylation in cells, leading to reduced proteasome activity and impaired cell proliferation. Interestingly, curcumin synergizes with the therapeutic proteasome inhibitor carfilzomib to induce apoptosis in a variety of proteasome-addicted cancer cells, while this drug combination exhibits modest to no cytotoxicity to noncancerous cells. In a breast cancer xenograft model, curcumin treatment significantly reduces tumor burden in immunocompromised mice, showing a similar antitumor effect as CRISPR/Cas9-mediated DYRK2 depletion. These results reveal an unexpected role of curcumin in DYRK2-proteasome inhibition and provide a proof-of-concept that pharmacological manipulation of proteasome regulators may offer new opportunities for anticancer treatment.

Significance

Curcumin is an ancient drug derived from turmeric and has been found to exhibit potent anticancer properties albeit through controversial mechanisms of action. Using a biochemical model, mouse cancer model, and cellular models, we show that curcumin is a highly potent and selective inhibitor of dual-specificity tyrosine-regulated kinase 2 (DYRK2), a positive regulator of the 26S proteasome. Curcumin perturbs 26S proteasome activity via DYRK2 inhibition in various cancer cells and in the mouse cancer model leading to impairment of cell proliferation and reduction of cancer burden in mice. This novel mechanism of action of curcumin opens up new avenues for potential preventative or therapeutic strategies in proteasome-addicted cancers like triple-negative breast cancer and multiple myeloma.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID code 5ZTN).

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“Achilles’ heel” of the aggressive basal-like triple-negative breast cancer (TNBC) (30) and the devastating plasma cell malignancy, multiple myeloma (31). Therapeutic proteasome inhibitors bortezomib (Velcade) (31, 32), carfilzomib (Kyprolis) (33), and ixazomib (Ninlaro) (34) are Food and Drug Administration-approved with proven clinical benefit in treating early stage and refractory multiple myeloma.

Given the proteasome’s biological complexity coupled with the normal cell toxicity of proteasome inhibitor drugs, recent work has focused on inhibiting the proteasome indirectly by identifying and targeting proteasome regulators (35, 36). Recently, our laboratory reported a proteasome regulator, dual-specificity tyrosine-regulated kinase 2 (DYRK2) that directly phosphorylates the conserved Thr25 of the ATPase RPT3 subunit of the proteasome (37). In that study we demonstrated that DYRK2 depletion impairs proteasome activity and results in accumulation of numerous proteins involved in diverse cellular processes (37). These DYRK2-depleted cells exhibited a slower proliferation rate and significantly reduced tumor burden in a mouse xenograft model (37). Taken together, we established that DYRK2 is a molecular target with promising anticancer potential not only for chemosensitive but also for proteasome inhibitor-resistant/adapted cancers.

In the current study, we provide evidence that curcumin is a specific and potent inhibitor of DYRK2 and regulates the proteasome activity via DYRK2 inhibition. Cryocrystal structure of curcumin with DYRK2 reveals that curcumin binds potently to the active site of DYRK2 via hydrophobic and hydrogen bonds. Furthermore, curcumin was found to not effect the proteasome activity of cells with DYRK2 deletion. Notably, curcumin treatment significantly reduced tumor volume in a TNBC mouse xenograft model, and the tumor volume was comparable to DYRK2-depleted tumors. The results establish that the inhibition of the DYRK2–proteasome axis is the primary mode of action of curcumin with expanded therapeutic utility in proteasome inhibitor-resistant cancer burdens.

Results

Curcumin Is a Potent and Selective Inhibitor of DYRK2. The structure of curcumin is shown in Fig. 1A. Curcumin inhibits DYRK2 with an IC$_{50}$ of 5 nM (Fig. 1B) in vitro. To evaluate whether curcumin could suppress cellular DYRK2 activity, we treated HEK293T cells with stable DYRK2-FLAG overexpressing with increasing concentrations of curcumin and assessed curcumin-mediated changes of RPT3 phosphorylation at Thr25, the major site of DYRK2 phosphorylation on the proteasome. We observed that curcumin treatment suppressed pT25 RPT3 phosphorylation in a dose-dependent manner, with maximal effects observed at inhibitor concentrations of 3–10 μM (Fig. 1D). To evaluate the specificity of curcumin, we also examined the activity of 140 protein kinases, including CMGC kinase family members that are closely related to DYRKs (Fig. 1C and SI Appendix, Table S1). Curcumin targeted DYRK2 specifically; curcumin treatment was found to also inhibit DYRK1A, DYRK3, PIM, MLK, and PHK kinases albeit to a lesser extent compared with DYRK2. Curcumin had off-target effects on DYRK1A with an IC$_{50}$ of 190 nM (Fig. 1E) and on DYRK3 with an IC$_{50}$ of 20 nM (Fig. 1F). Contrary to previous literature, curcumin did not exhibit potent inhibition of purified IKKβ (IC$_{50}>10$ μM) (Fig. 1G) or purified GSK3β (IC$_{50}>3$ μM) (Fig. 1H) in vitro. The results reveal that curcumin is a highly potent inhibitor of DYRK2 with off-target effects on related DYRK isoforms.

Structure of DYRK2 in Complex with Curcumin. To elucidate how curcumin specifically inhibits DYRK2, we crystallized DYRK2 in the presence of curcumin and determined the structure at 2.5 Å (SI Appendix, Table S2) [Protein Data Bank (PDB) ID code: 5ZTN]. Strong electron densities are present for curcumin, with critical interactions with the active site of DYRK2. The structure of curcumin in complex with DYRK2 is shown in Fig. 1C.
allowing confident interpretation of its position (Fig. 2A). Curcumin occupies the ATP-binding pocket of DYRK2 (Fig. 2B). One of the 4-hydroxy-3-methoxyphenyl groups of curcumin forms hydrogen bonds with the Lys251 (the ion pair Lys), Glu266 (the ion pair Glu), and Asp368 (the DFG Asp) of DYRK2 that anchors curcumin deep within the ATP-binding pocket of DYRK2 (Fig. 2C). Ile228, Ala249, Ile285, Phe301, Leu303, Leu355, and Ile367 are involved in making hydrophobic interactions with curcumin (Fig. 2C). The cocrystal structure of DYRK2-curcumin clearly reveals that curcumin imparts its inhibitory effect on DYRK2 by directly binding to the DYRK2 ATP-binding pocket.

**Curcumin Inhibits 26S Proteasome Activity.** We reasoned that curcumin, by inhibiting DYRK2, may reduce proteasome activity in cells. Indeed, curcumin treatment of MDA-MB-231 and HaCaT cells caused a 25–40% decrease of proteasome activity toward the fluorogenic peptide substrate Suc-LLVY-AMC without altering proteasome abundance as indicated by Western blotting (Fig. 3A and B). Notably, the same degree of proteasome inhibition was observed with CRISPR/Cas9-mediated DYRK2 knockout (37), and yet the DYRK2-null MDA-MB-231 (Fig. 3A) or HaCaT (Fig. 3B) cells showed no further decrease of proteasome activity with curcumin treatment. These results strongly support that curcumin down-regulates proteasome activity via DYRK2 inhibition. Moreover, in 293T cells, curcumin treatment reduced all three types of proteasome peptidase activities (Fig. 3C) and also stabilized UBL-YFP-PEST (37), a short-lived reporter protein that undergoes rapid ubiquitination and proteasome degradation (Fig. 3D). Remarkable accumulation and stabilization of two other well-established proteasome substrates, p21CIP1 and IκBα, was also evident in curcumin-treated cells compared with control (Fig. 3D). Furthermore, the inhibitory effect of curcumin on proteasome activity was observed across a panel of TNBC and multiple myeloma cell lines (Fig. 3F). Importantly, cotreatment of the TNBC MDA-MB-231 cell line with curcumin and carfilzomib resulted in a much stronger proteasome inhibition than either compound alone (Fig. 3E). Together, these results demonstrate that the antiproteasome activity of curcumin, due to DYRK2 inhibition, is similar in different cancer cell types and exhibits synergistic inhibition with carfilzomib.

**Curcumin Impedes Cell Proliferation and Invasion and Induces Apoptosis.** Next, we wanted to determine if curcumin-carfilzomib-mediated synergistic impairment of proteasome activity could have an effect on cancer cell viability. Interestingly, a marked synergistic cytotoxicity was observed across four different multiple myeloma cell lines with a combination index (CI) of <1 (Fig. 4A and SI Appendix, Table S3). However, noncancerous myeloid cell AH11 exhibited modest cytotoxicity toward the combination (Fig. 4B). Similar data were observed in four different triple-negative breast cancer cell lines with a curcumin-carfilzomib CI <1 for cytotoxicity in all cancer cells with modest to no effect in noncancerous mammary cell line MCF10A (Fig. 4B and SI Appendix, Table S3). We next used bortezomib-resistant cell lines that were generated by adaptation to continuous proteasome inhibition (38). RPMI8226.BR and MM.1S.BR cells were treated with 10 μM curcumin in parallel with their bortezomib-sensitive WT counterparts. RPMI8226.BR and MM.1S.BR cells exhibited comparable cytotoxicity to 10 μM of curcumin although these cells were 20- to 100-fold more resistant to bortezomib than their respective bortezomib-sensitive cells (Fig. 4C). Treatment with curcumin induced apoptotic cell death with a significant increase in caspase3/7 activity in multiple myeloma cell lysates (Fig. 4D). DYRK2-mediated p125-RPT3 phosphorylation is essential for proteasomal degradation of cell-cycle regulators and timely progression of S-phase (37). Curcumin treatment clearly perturbs cell proliferation that is consistent with DYRK2 inhibition. MDA-MB-231
Curcumin Reduces Tumor Burden at a Rate Consistent with DYRK2 Inhibition. MDA-MB-231 is a basal-like TNBC cell line shown to be resistant to proteasome inhibition-mediated cytotoxicity (30). Our results on cell viability and biochemistry made us wonder whether curcumin could inhibit the tumorigenic growth of these cells in vivo. It was documented previously that curcumin reduces tumor burden in various cancer models at 200 mg/kg–1 gm/kg body weight (12–15). We have previously reported that tumors derived from DYRK2 KO cells grow at a significantly lower rate than those formed by the parental cells (37). To compare the effect of curcumin on tumor growth to DYRK2-depleted tumor growth, parental and DYRK2 KO MDA-MB-231 cells were injected s.c. into NOD scid gamma (NSG) immunocompromised mice to induce tumor formation. Parental MDA-MB-231 bearing mice with palpable tumors were randomized into two groups of n = 5 each, and vehicle control or 300 mg/kg curcumin was injected intraperitoneally every alternate day. Indeed, curcumin treatment significantly reduced tumor burden after 2 wk of treatment (Fig. 5A), and curcumin-treated tumor weights (Fig. 5B) were comparable with tumors derived from MDA-MB-231 DYRK2 KO cells. Histological examination of the xenograft tumors also showed greatly attenuated Ki-67 staining (a cellular marker for proliferation) in both curcumin-treated and DYRK2 KO tumors (Fig. 5C). Furthermore, the total proteasome activity in tumors treated with curcumin alone was significantly lower compared with vehicle-treated tumor lysates (Fig. 5D), thus suggesting the direct contribution of DYRK2 inhibition in tumor regression. These data strongly support the role of curcumin-mediated inhibition of the DYRK2–proteasome axis in regulating cell proliferation in vivo and suggest that targeting proteasome regulators (such as DYRK2) in combination with proteasome inhibitors may be a promising approach of anticancer therapy.

Discussion
This current study is a comprehensive report of a major mechanism of action of curcumin in alleviating proteasome-addicted tumor burden. Results show that curcumin potently binds and inhibits DYRK2 (Figs. 1 and 2), resulting in reduced 26S proteasome activity, (Fig. 3) which leads to impaired cell proliferation with induction of apoptotic cell death, and (Fig. 4) culminating in reduced tumor growth (Fig. 5). We also report a comprehensive kinase specificity profile of curcumin (Fig. 1D and SI Appendix, Table S1), which clearly shows that curcumin is a highly specific and potent inhibitor of DYRK2 with a >10-fold higher potency for DYRK2 compared with other DYRK isoforms (Fig. 1E and F). Higher concentrations of AHH1 and 5TGM1-GFP cells treated with curcumin exhibited a markedly slower rate of cell proliferation compared with DMSO-treated controls (Fig. 4E). However, curcumin treatment of MDA-MB-231 DYRK2 KO cells did not exhibit a further decrease in cell proliferation (Fig. 4E), suggesting that the curcumin-mediated impairment of cell proliferation is directly linked to DYRK2 inhibition. Furthermore, curcumin treatment largely blocked the ability of TNBC cells to migrate in transwell chemotaxis and 3D matrigel invasion assays (Fig. 4F). These results support that curcumin-mediated DYRK2-proteasome activity impairment has a direct effect on cytotoxicity, proliferation, and invasion of proteasome-dependent TNBC and multiple myeloma cells. It has been previously shown that curcumin shows synergism with proteasome inhibitors resulting in cytotoxicity in cancer cells (38); however, our data clearly indicate that curcumin-mediated cytotoxicity is predominantly rendered by curcumin-mediated inhibition of DYRK2.
Curcumin reduces tumor burden to a similar extent as DYRK2 depletion.

(A) Tumor xenograft studies were carried out with or without 300 mg/kg of curcumin treatment. MDA-MB-231 parental or genome-edited (DYRK2 KO) cells were injected s.c. into NSG mice. MDA-MB-231 parental cells bearing mice with palpable tumors (16 d postinjection marked by an ‘*’) were treated with vehicle control of curcumin two to three times a week by i.p. injection, and tumor volume was measured twice a week (n = 5 per condition). (B) Forty-two days postinjection, tumors were resected and tumor weight was measured. **p < 0.01, ***p < 0.001 (compared with vehicle-treated, ordinary one-way ANOVA, mean ± SD from n = 5 mice each). (C) Histological examination of consecutive sections of the tumors with H&E and Ki67-staining. (Scale bar, 100 μm.) (D) Proteasome activity in whole-tumor lysates from vehicle or curcumin-treated tumor-bearing mice was measured with Suc-LLVY-AMC. Immunoblotting of the whole-tumor lysates was carried out with indicated antibodies. **p < 0.01 (compared with control treated, two-tailed paired Student’s t test, mean ± SD from n = 3 different tumors for each treatment).

Fig. 5. Curcumin reduces tumor burden to a similar extent as DYRK2 depletion. (A) Tumor xenograft studies were carried out with or without 300 mg/kg of curcumin treatment. MDA-MB-231 parental or genome-edited (DYRK2 KO) cells were injected s.c. into NSG mice. MDA-MB-231 parental cells bearing mice with palpable tumors (16 d postinjection marked by an ‘*’) were treated with vehicle control of curcumin two to three times a week by i.p. injection, and tumor volume was measured twice a week (n = 5 per condition). (B) Forty-two days postinjection, tumors were resected and tumor weight was measured. **p < 0.01, ***p < 0.001 (compared with vehicle-treated, ordinary one-way ANOVA, mean ± SD from n = 5 mice each). (C) Histological examination of consecutive sections of the tumors with H&E and Ki67-staining. (Scale bar, 100 μm.) (D) Proteasome activity in whole-tumor lysates from vehicle or curcumin-treated tumor-bearing mice was measured with Suc-LLVY-AMC. Immunoblotting of the whole-tumor lysates was carried out with indicated antibodies. **p < 0.01 (compared with control treated, two-tailed paired Student’s t test, mean ± SD from n = 3 different tumors for each treatment).

Tumor volume cm

Bar

0

0.5

1

1.5

VEH

Cur

Cur KO

A

Vehicle treated

Curcumin 300 mg/Kg

DYRK2 KO

Fig. 5. Curcumin reduces tumor burden to a similar extent as DYRK2 depletion. (A) Tumor xenograft studies were carried out with or without 300 mg/kg of curcumin treatment. MDA-MB-231 parental or genome-edited (DYRK2 KO) cells were injected s.c. into NSG mice. MDA-MB-231 parental cells bearing mice with palpable tumors (16 d postinjection marked by an ‘*’) were treated with vehicle control of curcumin two to three times a week by i.p. injection, and tumor volume was measured twice a week (n = 5 per condition). (B) Forty-two days postinjection, tumors were resected and tumor weight was measured. **p < 0.01, ***p < 0.001 (compared with vehicle-treated, ordinary one-way ANOVA, mean ± SD from n = 5 mice each). (C) Histological examination of consecutive sections of the tumors with H&E and Ki67-staining. (Scale bar, 100 μm.) (D) Proteasome activity in whole-tumor lysates from vehicle or curcumin-treated tumor-bearing mice was measured with Suc-LLVY-AMC. Immunoblotting of the whole-tumor lysates was carried out with indicated antibodies. **p < 0.01 (compared with control treated, two-tailed paired Student’s t test, mean ± SD from n = 3 different tumors for each treatment).

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Fig. 5. Curcumin reduces tumor burden to a similar extent as DYRK2 depletion. (A) Tumor xenograft studies were carried out with or without 300 mg/kg of curcumin treatment. MDA-MB-231 parental or genome-edited (DYRK2 KO) cells were injected s.c. into NSG mice. MDA-MB-231 parental cells bearing mice with palpable tumors (16 d postinjection marked by an ‘*’) were treated with vehicle control of curcumin two to three times a week by i.p. injection, and tumor volume was measured twice a week (n = 5 per condition). (B) Forty-two days postinjection, tumors were resected and tumor weight was measured. **p < 0.01, ***p < 0.001 (compared with vehicle-treated, ordinary one-way ANOVA, mean ± SD from n = 5 mice each). (C) Histological examination of consecutive sections of the tumors with H&E and Ki67-staining. (Scale bar, 100 μm.) (D) Proteasome activity in whole-tumor lysates from vehicle or curcumin-treated tumor-bearing mice was measured with Suc-LLVY-AMC. Immunoblotting of the whole-tumor lysates was carried out with indicated antibodies. **p < 0.01 (compared with control treated, two-tailed paired Student’s t test, mean ± SD from n = 3 different tumors for each treatment).
Considering the poor pharmacokinetics/pharmacodynamics (PK/PD) of curcumin, there is an unresolved debate on curcumin treatment as a viable therapeutic option (6). Our current work establishes curcumin not only as an excellent research tool for DYRK2 inhibition, but also as a potential therapeutic possibility. Derivatives of curcumin with improved PK/PD could be viable treatment options especially for proteasome-dependent highly refractory TNBC or multiple myeloma in the future. With respect to the vast body of literature on potential targets of curcumin, we do not claim inhibition of the DYSRK2–proteasome axis as the only possible mechanism of action of curcumin; nevertheless, we have conclusively established that impairment of proteasome activity by DYSRK2 inhibition is a major mechanism of action for curcumin in the context of the alleviation of proteasome-dependent neoplastic malignancies.

**Materials and Methods**

Details on the general methods, antibodies, reagents, curcumin preparation and treatment, IC₅₀ determination, protein kinase inhibitor specificity screen, cell lines, transfaction, lysis, genome editing, protein purification, crystallography, alignment studies, genome editing, proteasome assays, cell proliferation, viability, invasion, and tumor xenograft studies are presented in **SI Appendix**.

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