Tumor-selective proteotoxicity of verteporfin inhibits colon cancer progression independently of YAP1

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Yes-associated protein 1 (YAP1) is a transcriptional coactivator in the Hippo signaling pathway. Increased YAP1 activity promotes the growth of tumors, including that of colorectal cancer (CRC). Verteporfin, a drug that enhances phototherapy to treat neovascular macular degeneration, is an inhibitor of YAP1. We found that verteporfin inhibited tumor growth independently of its effects on YAP1 or the related protein TAZ in genetically or chemically induced mouse models of CRC, in patient-derived xenografts, and in enteroid models of CRC. Instead, verteporfin exhibited in vivo selectivity for killing tumor cells in part by impairing the global clearance of high-molecular weight oligomerized proteins, particularly p62 (a sequestrome involved in autophagy) and STAT3 (signal transducer and activator of transcription 3; a transcription factor). Verteporfin inhibited cytokine-induced STAT3 activity and cell proliferation and reduced the viability of cultured CRC cells. Although verteporfin accumulated to a greater extent in normal cells than in tumor cells in vivo, experiments with cultured cells indicated that the normal cells efficiently cleared verteporfin-induced protein oligomers through autophagic and proteasomal pathways. Culturing CRC cells under hypoxic or nutrient-deprived conditions (modeling a typical CRC microenvironment) impaired the clearance of protein oligomers and resulted in cell death, whereas culturing cells under normoxic or glucose-replete conditions protected cell viability and proliferation in the presence of verteporfin. Furthermore, verteporfin suppressed the proliferation of other cancer cell lines even in the absence of YAP1, suggesting that verteporfin may be effective against multiple types of solid cancers.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer type and the third leading cause of cancer deaths for both men and women in the United States (1). A key contributing event to the pathogenesis of greater than 80% of sporadic CRCs is inactivation of the APC (adenomatous polyposis coli) tumor suppressor gene. APC inactivation appears to be an early genetic event in the multistep pathway to CRC, and mutations affecting KRAS and TP53 are observed in a significant proportion of CRC cases (2). Whereas the bulk of CRC cells are termed sporadic and are not associated with any clear predisposing factors, a small subset of CRCs arise in patients with inflammatory bowel disease (IBD), a chronic relapsing inflammatory disease of the intestine. CRCs arising in IBD patients are often termed as colitis-associated colon cancers (CACs) (3). Recent work has demonstrated that the growth-promoting role of the transcription coactivator Yes-associated protein 1 (YAP1) is critical for the proliferative response observed during intestinal inflammation as well as in sporadic CRC (4–6).

YAP1 is a downstream effector in the Hippo signaling pathway, which functions as an essential regulator of proliferation, organ size, and cell differentiation (7, 8). In response to a decrease in Hippo signaling, YAP1 translocates into the nucleus and acts as transcriptional coactivator of TEA domain (TEAD) family members (TEAD1 to TEAD4). TEADs regulate various genes that encode proteins with critical roles in proliferation and inhibition of apoptosis (9–11). Activation of YAP1, through disruption of upstream kinases, leads to hyperproliferation and spontaneous tumor formation in certain tissues, including the intestine (6, 12). Recently, it has been shown that YAP1 is an essential component of the β-catenin degradation complex. Upon activation of Wnt signaling, both β-catenin and YAP1 signaling are activated, and YAP1 plays a key role in the hyperproliferative response upon disruption of Apc or activation of β-catenin (13–15). Because YAP1 is a critical component of the β-catenin degradation complex, disruption of YAP1 leads to enhanced β-catenin activation and increased proliferation in the intestine (16). Thus, through its scaffolding role in β-catenin regulation, YAP1 can function as a tumor suppressor. On the other hand, through its transcriptional coactivator role, YAP1 can function as an oncogene (17). Therefore, approaches that specifically inhibit YAP1 transcriptional activity without affecting YAP1’s role in β-catenin turnover could have a therapeutic role in CRC. Several inhibitors that interfere with the YAP1-TEAD complex have been identified (18), including selected porphyrin-containing drugs that potently inhibit YAP1 transcriptional activity (18). Verteporfin (VP), a drug approved by the U.S. Food and Drug Administration as a photosensitizer for photodynamic therapy in patients with age-related macular degeneration (AMD), potently inhibits YAP1 transcriptional activity, independent of light activation (18, 19). Currently, VP has been the main pharmacological tool to understand the role of YAP1 in various cancers (18, 20–26).

Here, we investigated the mechanism of action of VP in CRC using CRC-derived cell lines, mouse models, and patient-derived adenoma and adenocarcinoma enteroid models. Unexpectedly, we uncovered a tumor-suppressive function of VP that was independent of its effects on YAP1.
RESULTS

YAP1 signaling is activated in colon-derived cell lines and CRC

To explore the role of YAP1 signaling in CRC cells, we assessed the abundance of total and phosphorylated YAP1 in various CRC-derived cell lines as well as in the human embryonic kidney (HEK) 293 cell line, which is known to express active YAP1. All CRC cell lines studied had high abundance of total and phosphorylated YAP1 (Fig. 1A and fig. S1A). YAP1 was also assessed in dysplastic and hyperplrophic colon tissues from mice where both Apc alleles were inactivated by a colon epithelial-specific tamoxifen-inducible Cre transgene (Apc<sup>fl/fl</sup>:CDX2<sup>Cre</sup>) (27). Seven days after colon-specific disruption of Apc, the abundance of total and phosphorylated YAP1 increased in the colon of Apc<sup>fl/fl</sup>:CDX2<sup>Cre</sup> mice compared to that of littermate controls (Fig. 1B and fig. S1B). In addition, the abundance of total and phosphorylated YAP1 was increased in the colon tumor epithelium of a littermate controls (Fig. 1B and fig. S1B). In addition, the abundance of total and phosphorylated YAP1 (pYAP1) (A) in human colon cancer cell lines and HEK293A cells, (B) in the colon-specific disruption of Apc<sup>fl/fl</sup>:CDX2<sup>Cre</sup> mice compared to that of littermate controls (Fig. 1B and fig. S1B). To further evaluate the function of YAP1 in CRC, we first explored the effects on CRC cell growth in vitro mouse CAC model (Fig. 1C and fig. S1C). To further evaluate the function and effects of inhibiting YAP1. Data are means ± SEM. (* P < 0.05, ** P < 0.01 versus siScr. All cell line experiments were done in triplicates and repeated at least three times. RLU, relative light units. ***)

YAP1 transcriptional inhibitor VP inhibits CRC growth in vivo

VP, previously identified as a transcriptional inhibitor of YAP1 (18), was assessed in an azoxymethane (AOM)/dextran sulfate sodium (DSS)–induced model of colon cancer. After three cycles of DSS, colon tumors were observed in all treated C57BL/6 mice. Treatment was then initiated with VP (100 mg/kg) injected intraperitoneally at 3-day intervals, and the mice were assessed after six doses of VP (Fig. 2A). VP treatment significantly and rapidly reduced tumor multiplicity and tumor size compared to vehicle treatment (Fig. 2, B and C). Histological analyses demonstrated that the tumors present in the VP-treated mice were minimally dysplastic adenomas as compared to the more advanced adenomas in vehicle-treated mice (Fig. 2D). A significant decrease in the expression of YAP1 target genes (CTGF and CYR61) and in cell proliferation and an increase in apoptosis (assessed by cleaved caspase-3 abundance) were observed in tumors from VP-treated mice (Fig. 2, E to G). However, VP treatment did not affect normal intestinal homeostasis as assessed by histological examination and Ki67 staining (Fig. 2H). In addition, kidney, liver, small intestine, and colon tissues did not exhibit any apparent toxic effects upon VP treatment (fig. S2).

We further assessed the efficacy of VP to decrease cell proliferation in patient-derived colon adenoma and adenocarcinoma enteroid cultures and patient-derived xenograft models. Three-dimensional patient-derived enteroid culture systems recapitulate many relevant aspects of colon tumor growth, in addition to reflecting the phenotypic and genetic changes seen in primary patient tumors, compared to cultured cell lines. Moreover, the enteroid cultures are enriched in presumptive tumor-initiating cells, which have been suggested to be resistant to chemotherapy- and radiation-based treatments (28). The growth of both adenoma and adenocarcinoma enteroid cultures decreased upon exposure to VP in a dose- and time-dependent manner (Fig. 3, A to D). VP was further assessed in CRC patient–derived xenograft model.
When tumors reached an average size of 150 mm², the mice were injected at 3-day intervals for 3 weeks with vehicle or VP. A significant decrease in tumor size, tumor weight, necrosis, and proliferation and an increase in apoptosis were observed in xenografts from VP-treated mice (Fig. 3, E to H). These results were consistent with the effects seen in CRC-derived cell lines and an additional xenograft model using the mouse CRC-derived CT-26 cells (Fig. S3, A to D). VP had a more potent response in activating apoptosis, as assessed by the abundance of cleaved poly(adenosine diphosphate–ribose) polymerase (PARP) and cleaved caspase 3, compared to the effects on cell proliferation as assessed by MTT assay and analysis of proliferating cell nuclear antigen and Ki67 in tumor enteroids and CRC-derived cell lines (Fig. 3, C and D, and fig. S3, E to G). Together, the data suggest that the use of VP has substantial antitumor effects and minimal toxicity in vivo.

VP inhibits STAT3 signaling in colon tumors and colon tumor–derived cell lines

YAP1-dependent transcriptional activation is critical for colon tumor progression; therefore, we investigated what downstream mechanisms might be involved. We found that the phosphorylation of the pro-proliferative kinases such as AKT and ERK (extracellular signal–regulated kinase) was significantly increased in tumors from AOM/DSS mice that were treated with VP (Fig. 2A and fig. S4A). Decreased phosphorylation of STAT3 (signal transducer and activator of transcription 3) after VP treatment was also observed (Fig. 4A and fig. S4B). This is consistent with previous data demonstrating that increased nuclear abundance of YAP1 increases STAT3 phosphorylation (6). STAT3 signaling plays an important role in the survival and proliferation of intestinal epithelial cells in CRC (29, 30). Moreover, crosstalk between the interleukin-6 (IL-6) co-receptor gp130 and YAP1 was recently identified as a major mechanism leading to epithelial cell proliferation in the colon in response to inflammation (5, 31). The expression of STAT3 target genes Socs3, Bcl2, and Il-1β was decreased in tumor tissues after VP treatment (Fig. 4B). Furthermore, IL-6–stimulated STAT3 phosphorylation was ablated after VP exposure in a panel of CRC–derived cells (Fig. 4, C and D, and fig. S4, C to E). In addition, VP also significantly decreased total STAT3 abundance in the cell

Fig. 2. VP treatment decreases colon tumors in a mouse model of colon cancer. (A) Schematic representation of AOM/DSS CAC mouse model that were treated with VP (100 mg/kg) twice a week for 3 weeks. (B to D) Representative images of tumors (B); colon length and the number, size, and volume of colon tumors (C); and hematoxylin and eosin (H&E) staining (D) of representative tumors from mice treated with vehicle (Veh) (n = 9 mice per group) or VP (n = 8). Scale bar, 50 μm. (E and F) Quantitative real-time polymerase chain reaction (qPCR) analysis of CTGF and CYR61 expression in normal (NT) and tumor (Tum) colon tissues from vehicle- or VP-treated mice, normalized to mRNA encoding β-actin. Data are means ± SEM (n = 4 mice per group). (G and H) Immunostaining for the Ki67 or cleaved caspase 3 (c-Caspase 3) in (G) tumor and (H) normal colon tissues from mice treated with vehicle or VP. Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle. Scale bars, 50 μm. DAPI, 4′,6-diamidino-2-phenylindole.
lines studied (Fig. 4, C and D, and fig. S4, C to E) and decreased IL-6–induced STAT3 signaling in a dose- and time-dependent manner (Fig. 4, E and F, and fig. S4, F to H). Furthermore, VP significantly decreased IL-6–mediated induction of a luciferase construct containing a STAT3 response element as well as induction of Socs3 in HCT-116 cells (fig. S4, I and J). The collective data suggest that VP may antagonize colon tumorigenesis by inhibiting STAT3 signaling.

**VP inhibits cell proliferation through YAP1-independent mechanisms**

The antitumor role of VP has been previously reported to be through its inhibition of YAP1 signaling, independent of light activation (18, 26). Given the association of YAP1 and STAT3 (18, 26), this mechanism may occur through its inhibitory effects on YAP1. However, knockdown of YAP1 using siRNA or stable cells expressing two different doxycycline-inducible short hairpin RNAs (shRNAs) against YAP1 (TripZ-YAP1-1 and TripZ-YAP1-2) did not affect IL-6–induced phospho-rylation of STAT3 in HCT-116 cells (Fig. 5, A and B). It is possible that VP could also inhibit TAZ, a close homolog with redundant functions, which also binds to TEAD (13, 14). However, expression of shRNA against TAZ alone or with siRNA against YAP did not affect IL-6–induced phosphorylation of STAT3 in HCT-116 cells (Fig. 5, A and B). It is possible that VP could also inhibit TAZ, a close homolog with redundant functions, which also binds to TEAD (13, 14). However, expression of shRNA against TAZ alone or with siRNA against YAP did not affect IL-6–induced phosphorylation of STAT3 in HCT-116 cells (Fig. 5, A and B). VP repressed the IL-6–mediated phosphorylation of STAT3 even in the absence of YAP1 (Fig. 5E and fig. S5E). These data clearly demonstrate that inhibition of STAT3 signaling by VP is independent of YAP1.
analysis revealed that VP could decrease cell proliferation in cells treated with a STAT3 inhibitor (fig. S5F), suggesting that VP does not inhibit cell proliferation solely by repressing STAT3 signaling. These data prompted us to further assess whether the growth inhibitory role of VP was independent of YAP1. Surprisingly and in contrast to what has been previously reported (18, 25, 26, 32), VP decreased cell proliferation in colon cancer–derived cell lines in the absence of YAP1, TAZ, or YAP1 and TAZ (Fig. 5, F to I, and fig. S5G). Moreover, the growth inhibitory effects of VP were independent of YAP1 in breast, cervical, bone, and liver cancer–derived cell lines (fig. S5H). Therefore, our data demonstrate that VP reduces cell growth in cancer cells through a YAP1-independent mechanism.

**VP increases apoptosis by inducing high–molecular weight oligomerization of proteins**

Besides its reported effects on YAP1, VP has been shown to inhibit autophagy (32, 33). Autophagy is a critical mechanism in many tumors for nutrient recycling and growth, and autophagy inhibitors have been shown to decrease CRC growth (34–36). To assess whether VP decreases CRC cell growth through inhibition of autophagy, the well-characterized autophagy inhibitors bafilomycin A1 or chloroquine were used alone or in conjunction with VP in cultures of HCT-116 cells. Treatment with bafilomycin A1 or chloroquine in combination with VP did not significantly alter growth compared to VP alone (fig. S6, A and B). Structurally, VP belongs to the porphyrin class of drugs. Intracellular porphyrins can cross-link proteins, leading to formation of high–molecular weight oligomers of proteins (33, 37). Therefore, to assess whether the loss of STAT3 signaling by VP was due to oligomerization of STAT3, HCT-116 cells were treated with VP in a dose- and time-dependent manner. VP treatment led to a shift in the molecular weights of both phosphorylated and total STAT3 toward less monomer abundance at the higher VP concentrations or incubation periods (Fig. 6A and fig. S6C). Similarly, proteins such as p62 and lamin A/C that oligomerize upon porphyrin treatment were also found to form protein oligomers after VP treatment (Fig. 6B and fig. S6D). Although YAP1 protein decreased with VP treatment, we could not detect high–molecular weight oligomers of YAP1, possibly because the YAP1 epitope was masked among the protein oligomers. In addition, we also observed oligomerization of p62, STAT3, and lamin A/C in the lysates from VP-treated patient-derived xenografts (fig. S6E). The VP structural analogs protoporphyrin IX (PPIX) and mesoporphyrin (Meso) also caused protein oligomerization, whereas the analog hemin did not induce protein oligomers (Fig. 6C and fig. S6F). Protein oligomerization was strongly correlated with inhibition of cell proliferation because exposure to VP, PPIX, or Meso, but not hemin, resulted in significantly decreased cell proliferation (Fig. 6D). Protein oligomerization by VP was associated with induction of endoplasmic reticulum stress markers in HCT-116 cells in a time- and dose-dependent manner (fig. S6G). However, pretreatment with tauroursodeoxycholic acid, a chemical chaperone that reduces endoplasmic reticulum stress (38), did not protect HCT-116 cells from VP-mediated decrease in cell proliferation (fig. S6H). Inhibition of autophagy by chloroquine did not induce the formation of protein oligomers, demonstrating that the oligomerization of proteins by VP is independent of its function as an autophagy inhibitor but is rather due to cross-linking of protein (Fig. 6E and fig. S6I) (32, 33, 35).

To further understand the mechanism by which VP decreases cell growth, we used a proteomic approach to globally assess VP-induced high–molecular weight oligomers in HCT-116 and SW480 cells. High–molecular weight proteins (>100 kD) were isolated, and proteins that were identified with the predicted size of less than 60 kD were considered as VP-induced high–molecular weight oligomerized proteins (Fig. 6F). The common proteins identified in both HCT-116 cells and SW480 were listed in table S1. p62 and lamin A/C were identified in the proteomic analysis, thus increasing the confidence that this method could identify novel VP-induced oligomers. Numerous proteins involved in various cellular processes, such as apoptosis, signaling, bioenergetics, structural proteins, and cellular biogenesis, were present in the high–molecular weight fraction upon treatment of HCT-116 and SW480 cells with VP (fig. 6F). To assess whether cells with increased VP-induced oligomerized protein could be identified by immunofluorescence, we visualized p62 after VP treatment in HCT-116 cells. In untreated cells, p62 was diffusely stained throughout the cytosol, whereas p62 protein was highly increased in the perinuclear region in response to VP.
treatment (fig. S6J). To assess whether VP-induced apoptosis was caused specifically by protein oligomerization, we performed dual staining for cleaved caspase 3 (a marker of apoptosis) and p62 (a marker of oligomerization) in HCT-116 cells, human enteroid cultures, and patient-derived xenografts (Fig. 6, G to I). More than 70% of the cells were costained for p62 oligomerization and cleaved caspase 3 in VP-treated colon tumor mouse models. Together, the data suggest that protein oligomerization by VP is associated with apoptotic cell death.

Tumors exhibit impaired clearance of VP-induced protein oligomers, resulting in apoptotic cell death

VP has very low toxicity, and in vivo treatment resulted in decreased tumor growth without affecting normal tissues (39, 40). To understand the mechanism by which VP elicits tumor-specific growth inhibition, mice with AOM/DSS-induced colon tumors were treated with VP and then assessed for protein oligomerization. Notably, VP treatment induced significantly greater oligomerization of p62 and STAT3 in the tumor tissue compared to adjacent normal tissue (Fig. 7A and fig. S7A). Previous work has demonstrated that oxidative stress after exposure to porphyrins could lead to increased protein oligomerization (41). However, protein oligomers induced by VP were only slightly attenuated by the antioxidants N-acetyl cysteine, butylated hydroxyanisole, and sodium azide (fig. S7, B and C). To assess whether there was a difference in VP uptake between the tumor and normal adjacent tissue, we assessed free intracellular VP by high performance liquid chromatography (HPLC). VP has two regioisomers, which eluted at 21 and 22 s, respectively. Surprisingly, the abundance of the free form of VP was significantly less in tumors than in adjacent normal tissue (Fig. 7B). This may be explained by increased VP in oligomerized proteins in tumors, but it also clearly demonstrates that normal tissues take up VP.

We then assessed whether the protein oligomer clearance mechanism is impaired in tumors using explants of the tumor and adjacent normal tissue that were then treated with VP ex vivo, followed by replacing the medium without VP (washout period). Western blot analysis revealed effective clearance of high–molecular weight p62 protein oligomers in normal tissues but not in tumors (Fig. 7C and fig. S7D). Unlike in vivo tumors, colon cancer–derived cell lines were able to effectively clear the high–molecular weight oligomers (fig. S7E). However, inhibition of proteasomal [MG-132 (N-carbobenzyloxy-L-leucyl-L-leucinal)] and autophagic pathways using an early-autophagy inhibitor (wortmannin) or a late-autophagy inhibitor (bafilomycin) impaired the clearance of protein oligomers after a washout period (fig. S7E).

In vivo, tumors grow in nutrient-deprived and hypoxic environments; ex vivo, although grown in nutrient-rich medium, tumors still exhibit significant hypoxia as assessed by increased abundance of the hypoxic transcription factors hypoxia-inducible factor 1α (HIF1α) and HIF2α (Fig. 7D and fig. S7F). Tumors under nutrient-deprived or hypoxic conditions heavily rely on....

Fig. 5. VP inhibits growth of CRC-derived cells independent of YAP1. (A and B) Western blot analysis of HCT-116 cells transfected with (A) siScr or siYAP1. (B) HCT-116 cells stably expressing doxycycline (Doxy)–inducible shRNA against YAP1 (clone 1 and 2). Cells were untreated or exposed to IL-6 (10 ng/ml, 20 min). (C and D) Western blotting of lysates from HCT-116 cells stably expressing (C) control (shScr) or shRNA against TAZ (shTAZ clones 1 to 3) or (D) shTAZ (clones 1 and 2) and either siScr or siYAP1 and treated with IL-6 (10 ng/ml, 20 min). (E) Western blot analysis of HCT-116 cells transfected with siScr or siYAP1 and treated with VP (1 μg/ml) and then IL-6 (10 ng/ml) for 20 min. (F to I) MTT assay in HCT-116 cells transfected with siScr or siYAP1 and treated with vehicle or VP (1 μg/ml). (G to I) MTT assay in VP (1 μg/ml)–treated HCT-116 cells stably expressing (G) doxycycline-inducible shYAP1, (H) shTAZ, or (I) shTAZ and siScr or siYAP1. Data are means ± SEM of at least three independent experiments, each done in triplicate. ***P < 0.001 versus vehicle.
Fig. 6. VP-induced protein oligomerization causes apoptotic cell death. (A) Abundance of monomers and high–molecular weight (MW) oligomers of phosphorylated and total STAT3 in response to IL-6 (10 ng/ml) in HCT-116 cells pretreated with VP (left, 1 μg/ml VP for the indicated times; right, treatment for 2 hours). (B) Abundance of monomers and high–molecular weight oligomers of p62, lamin A/C, and STAT3 in CRC cell lines treated with VP (1 μg/ml) for 2 hours. (C and D) HCT-116 and RKO cells were exposed for 2 hours to vehicle, VP (1 μg/ml), 5 μM PPIX, or 5 μM hemin. Both cell lines were then assessed for high–molecular weight oligomers or monomers by Western blot (C), and HCT-116 cells were additionally assessed for proliferation (D). (E) As in (C), also in response to 2-hour exposure to 100 μM chloroquine (CQ). (F) Identification and classification of VP-induced oligomers in HCT-116 (HCT) and SW480 (SW) by mass spectrometry and Panther Gene Ontology. (G to I) Coimmunostaining for cleaved caspase 3 and p62 in (G) HCT-116 cells treated with VP (1 μg/ml) for 8 hours. Scale bar, 50 μm. Data are means ± SEM of at least three independent experiments, each performed in triplicate. Blots and images are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle.
recycled and scavenged nutrients and metabolites through proteasomal and autophagic turnover (34–36, 42). The role of hypoxia and nutrient deprivation in the clearance of protein oligomers was assessed in cultured cells. HCT-116 cells were treated with VP for 2 hours followed by a washout period of up to 8 hours, under normoxic or hypoxic conditions. Normoxic HCT-116 cells rapidly cleared the protein oligomers compared to hypoxic cells (Fig. 7E and fig. S7G). Similarly, glucose deprivation decreased the clearance of VP-induced protein oligomers (Fig. 7F and fig. S7H). Consistent with the clearance of high–molecular weight oligomers, the washout period partially restored cell proliferation in HCT-116 and RKO rectal carcinoma cells (Fig. 7G). However, hypoxic cells were highly susceptible to a decrease in cell proliferation after exposure to VP (Fig. 7G). Replacing the cells in normal medium without VP did not restore their proliferation under hypoxic conditions (Fig. 7G). Similar studies could not be performed in glucose-deprived medium, because the cells could not be cultured for time periods amenable for growth assays.

**Protein oligomerization is a critical mechanism in VP-induced growth inhibition**

To further establish a cause-effect relationship between protein oligomerization and growth inhibition by VP, we determined whether decreasing VP-induced protein oligomers could protect cells from VP-mediated inhibition of proliferation. Pretreating HCT-116 cells with the autophagy activator rapamycin or the proteasome activator betulinic acid decreased the abundance of protein oligomers after cells were exposed to VP (Fig. 8A and fig. S8A). However, rapamycin and betulinic acid could not resolve VP-induced oligomers after longer-term VP treatment (fig. S8B), suggesting that VP oligomerization may eventually saturate the clearance pathways. Moreover, treatment with proteasomal regulators such as trichostatin A (a histone deacetylase inhibitor) or geldanamycin (a heat shock protein 90 inhibitor) did not prevent VP-induced protein oligomerization (fig. S8C), possibly because of the weak regulation of proteasome activity by these regulators compared to that of betulinic acid. Because activation of autophagic and proteasomal pathways resolved acute VP-induced oligomerization, we assessed early parameters of cell proliferation to confirm that protein oligomerization is a major mechanism by which VP decreases cell proliferation. Decreasing the abundance of protein oligomers using rapamycin or betulinic acid prevented the effects of VP on apoptosis (assessed by cleaved PARP abundance) and cell proliferation (Fig. 8, B and C, and fig. S8D). Collectively, the data suggest that normoxic cells in a nutrient-rich environment could efficiently use the autophagic and proteasomal machinery to clear VP cross-linked proteins. However, hypoxic or nutrient-deprived tumor cells, which depend on autophagic and proteosomal pathways for nutrient supply, fail to clear VP cross-linked protein oligomers, resulting in apoptotic cell death and cancer regression (Fig. 8D).

### DISCUSSION

VP photodynamic therapy is frequently used as a first-line therapy for AMD, and a meta-analysis has demonstrated that VP photodynamic therapy is frequently used for preventing vision loss in patients (39). Currently, antiangiogenic treatments targeting vascular endothelial growth factors are the...
most common therapy for neovascular AMD (43). However, the characterization of VP as a YAP1 transcriptional inhibitor has revived interest in VP as a potential therapy for cancer. VP potently limits tumor growth in many different tumor models (18, 25, 26). Consistent with these reports, the data presented here expand these findings to CRC. Using CRC-derived cell lines, patient-derived enteroids and xenografts, and a mouse model of CRC, we demonstrate that VP exerts its tumor inhibitory effects through inhibition of cell proliferation and induction of apoptosis. Our studies demonstrate that growth inhibition after VP treatment was independent of YAP1 or the related Hippo effector TAZ. The present work identifies a VP-induced mechanism that is toxic specifically to tumors, initiating cell death by inducing high–molecular weight oligomerization of numerous proteins involved in key cellular pathways.

A potential generalized mechanism for VP inhibition of CRC growth involves high–molecular weight protein oligomers

VP and other related porphyrin molecules induce high–molecular weight oligomers (33, 37). However, heme, which also inhibits YAP1, could not form protein oligomers (18). We show that the VP-induced cell death correlates with the ability of porphyrins to induce high–molecular weight protein oligomers. In addition, cells with cytosolic protein oligomers in colon cell lines, CRC enteroids, and xenografts exhibited an increase in markers of apoptosis, and decreasing oligomers by activating autophagic and proteasomal pathways further supports protein oligomer–induced proteotoxicity as an important mechanism by which VP induces cell death. Porphyrin-related molecules induce oligomerization through a direct nonenzymatic cross-linking of proteins forming monomeric high–molecular weight oligomers potentiated by oxidative stress (33, 37). Through proteomic analysis, we identified VP to oligomerize nearly 250 proteins involved in various biological pathways in two different colon cancer–derived cell lines. VP-induced protein oligomerization appears to be a selective phenomenon observed with specific proteins such as p62, STAT3, and lamin A/C. Further efforts will be needed to better understand protein features and possible motifs that are critical in initiating oligomerization and to identify specific proteins and mechanisms that are essential for proteotoxicity-induced apoptosis.

Impaired clearance of protein oligomers contributes to tumor-selective cell death in CRC

Colon tumors are exquisitely sensitive to VP-induced cell death. Cancer-derived cell lines in culture and normal tissues are relatively less energetically stressed and do not rely on autophagic or proteasomal recycling for their key nutrients. Therefore, they can efficiently use these pathways to remove high–molecular weight protein oligomers. However, when colon cancer cell lines are energetically stressed with hypoxia or glucose deprivation, mimicking the conditions of tumors in vivo, high–molecular weight protein oligomers are not cleared efficiently. Rescuing cell growth by decreasing protein oligomers through activation of autophagy or proteasome in cells treated with VP suggests that VP inhibits cell growth partly by inducing high–molecular oligomerization of key proteins involved in various biological processes. The inability to clear protein oligomers correlates to increased cell death. Cancer cells heavily depend on the autophagic and proteasomal pathways to scavenge nutrients that are essential for their growth and survival (35, 36, 42). Therefore, rerouting of autophagic and proteasomal pathways to promote clearance of oligomers results in nutrient deprivation of cancer cells. On the other hand, failure to clear the protein oligomers results in persistent accumulation of toxic protein oligomers. Thus, the compromised cellular function both energetically and in the clearance of protein oligomers results in tumor-specific increase in apoptotic cell death by VP (Fig. 9).

VP is a critical tool for understanding the role of YAP1 signaling in cancer (18, 26). The present data clearly identify a novel mechanism of action for VP in inhibiting cancer growth that is independent of its previously known photoactivation or YAP1 inhibition. Our data demonstrating that VP treatment leads to tumor-specific proteotoxicity provide an alternative treatment strategy for CRC. Moreover, the present

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**A potential generalized mechanism for VP inhibition of CRC growth involves high–molecular weight protein oligomers**

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work provides evidence that VP might have potential for treatment approaches in some patients diagnosed with CRC.

**MATERIALS AND METHODS**

**Animals and treatments**

C57BL/6 mice were used and maintained in standard cages in a light- and temperature-controlled room and were fed with standard chow and water ad libitum. For the AOM/DSS tumor model, 6-week-old mice were injected intraperitoneally with AOM (10 mg/kg body weight). One week after AOM injection, the mice were given drinking water with 1.5% DSS for 7 days (inflammatory phase) and regular drinking water for 14 days (recovery phase); this was repeated three times. The mice were assessed 4 weeks after the last cycle of DSS treatment. For the VP treatment [U.S. Pharmacopeial Convention (USP) Reference Standard], VP dissolved in dimethyl sulfoxide (DMSO) was diluted using phosphate-buffered saline (PBS), and the mice were injected intraperitoneally with VP (100 mg/kg) as described in the figure legends. Control mice were injected with DMSO diluted with PBS. All animal studies were carried out in accordance with the Institute of Laboratory Animal Resources guidelines and were approved by the University Committee on Use and Care of Animals at the University of Michigan.

**Cell culture and proliferation assays**

Cells were maintained in DMEM (supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic agent) at 37°C in 5% CO₂ and 21% O₂. For hypoxia experiments, the cells were incubated in a hypoxia chamber (5% CO₂ and vector in which scrambled or two shRNAs against YAP1 were cloned (gifts expressing cells were generated using the doxycycline-inducible pTRIPZ after the last injection.**

**HPLC analysis**

VP was measured at 430 nm (excitation) and 690 nm (emission). The column was eluted at a flow rate of 1.0 ml/min with linear gradients of solvents A and B [A, 0.05 mM monobasic sodium phosphate (pH 3.5 in water); B, methanol]. The solvent gradients were as follows: 0 to 4 min, 50 to 65% B; 4 to 8 min, 65 to 85% B; 8 to 22 min, 85 to 99% B; 22 to 27 min, 99 to 10% B; 27 to 30 min, 10 to 50% B. Cell/tissue lysates were diluted 10-fold in HPLC injection solvent [50% methanol, 50% 0.05 mM monobasic sodium phosphate (pH 3.5)].

**Histology and immunofluorescence**

Formalin-fixed sections were stained with H&E, and frozen sections were used for immunofluorescence analysis as previously described (44). Primary antibodies against Ki67 (1:100, Vector Laboratories Inc.), cleaved caspase 3 (1:200, Cell Signaling Technology), and p62 (1:100, Santa Cruz Biotechnology) were used.

**Quantitative real-time RT-PCR (qPCR)**

RNA extraction, reverse transcription, and qPCR were previously described (44). The primers used in the study are listed in table S2.

**Statistical analysis**

Results are expressed as means ± SD or means ± SEM. Significance between two groups was calculated by independent t test, and significance

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among different groups was tested using one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc comparisons.

SUPPLEMENTARY MATERIALS

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Fig. S1. YAP1 signaling is activated in human colon cancer cell lines and tissues. Fig. S2. VP treatment does not induce toxicity in normal tissues. Fig. S3. VP treatment decreases growth in CT-26 xenograft model and colon cancer cell lines. Fig. S4. VP treatment inhibits STAT3 signaling. Fig. S5. The growth inhibitory effect of VP is YAP1-independent. Fig. S6. The growth inhibitory effect of VP is associated with oligomerization of proteins. Fig. S7. Hypoxia and nutrient deprivation impair clearance of VP-induced protein oligomers. Fig. S8. Activating autophagic and proteasomal pathways partially clears protein oligomers. Table S1. Proteins that are oligomerized in response to VP. Table S2. List of primers.

REFERENCES AND NOTES

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Tumor-selective proteotoxicity of verteporfin inhibits colon cancer progression independently of YAP1


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Aggregates kill cancer cells

The drug verteporfin is used clinically to enhance phototherapy and may also inhibit the transcription factor YAP1, which is often active in cancers. However, Zhang et al. found a different path to toxicity for verteporfin-mediated death of colorectal cancer cells. Verteporfin triggered the accumulation of toxic amounts of protein oligomers that selectively killed colorectal cancer cells in mice and in cells cultured under hypoxic and nutrient-deprived conditions. Normal cells in culture and in tumor-adjacent tissue sections from mice cleared these aggregates through autophagy and survived. Thus, verteporfin produces tumor-selective proteotoxicity, which may be a useful therapeutic for patients with solid tumors.