Heme Oxygenase-1 Promotes Survival of Renal Cancer Cells through Modulation of Apoptosis- and Autophagy-regulating Molecules

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Background: The cytoprotective enzyme HO-1 promotes tumor growth.

Results: HO-1 down-regulated apoptosis- and autophagy-regulating proteins, and attenuated Rapamycin- and Sorafenib-induced apoptosis and autophagy in renal cancer cells.

Conclusion: HO-1 protects cancer cells from chemotherapeutic drug-induced death by down-regulating apoptosis and autophagy.

Significance: Inhibition of HO-1 augments efficiency of chemotherapeutic agents to kill cancer cells by promoting both apoptosis and autophagy.

The cytoprotective enzyme heme oxygenase-1 (HO-1) is often overexpressed in different types of cancers and promotes cancer progression. We have recently shown that the Ras-Raf-ERK pathway induces HO-1 to promote survival of renal cancer cells. Here, we examined the possible mechanisms underlying HO-1-mediated cell survival. Considering the growing evidence about the significance of apoptosis and autophagy in cancer, we tried to investigate how HO-1 controls these events to regulate survival of cancer cells. Rapamycin (RAPA) and sorafenib, two commonly used drugs for renal cancer treatment, were found to induce HO-1 expression in renal cancer cells Caki-1 and 786-O; and the apoptotic effect of these drugs was markedly enhanced upon HO-1 knockdown. Overexpression of HO-1 protected the cells from RAPA- and sorafenib-induced apoptosis and also averted drug-mediated inhibition of cell proliferation. HO-1 induced the expression of anti-apoptotic Bcl-xL and decreased the expression of autophagic proteins Beclin-1 and LC3B-II; while knockdown of HO-1 down-regulated Bcl-xL and markedly increased LC3B-II. Moreover, HO-1 promoted the association of Beclin-1 with Bcl-xL and Rubicon, a novel negative regulator of autophagy. Drug-induced dissociation of Beclin-1 from Rubicon and the induction of autophagy were also inhibited by HO-1. Together, our data signify that HO-1 is up-regulated in renal cancer cells as a survival strategy against chemotherapeutic drugs and promotes growth of tumor cells by inhibiting both apoptosis and autophagy. Thus, application of chemotherapeutic drugs along with HO-1 inhibitor may elevate therapeutic efficiency by reducing the cytoprotective effects of HO-1 and by simultaneous induction of both apoptosis and autophagy.

Heme oxygenase-1 (HO-1) is a stress-inducible intracellular enzyme that degrades heme into carbon monoxide (CO), biliverdin, and ferrous iron (1). HO-1 classically functions to maintain cellular homeostasis under stress conditions. This cytoprotective effect may be attributed to the removal of a potent inflammatory agent heme, and to the functions of biologically active products generated during HO-1-mediated heme degradation. The by-products of heme degradation play a crucial role in the adaptive response of cells to oxidative and cellular stresses by reducing inflammation and apoptosis, and inducing cell proliferation and angiogenesis (1–3). Inhibition of apoptosis seems to be one of the major mechanisms underlying the cytoprotective function of HO-1. HO-1 confers protection from ischemia-reperfusion injury, cisplatin nephrotoxicity and endotoxic shock by attenuating apoptotic signals. Thus, HO-1 is a critical player in the survival machinery of cell and plays the role of savior in many human diseases (4, 5).

Despite its cytoprotective properties, recent evidences suggest a role for HO-1 in promoting cancer (6–9). HO-1 is overexpressed in different types of cancers and is further induced by radiation and chemotherapy (6, 10, 11). HO-1 endorses survival of various cancers, including hepatoma, melanoma, thyroid and lung carcinoma, while inhibition of HO-1 reduces viability of colon carcinoma, acute myeloid leukemia and induces apoptosis of renal cancer cells (12, 13). The protective function of HO-1 thus turns out to be a double-edged sword, as cancer cells utilize the anti-apoptotic effects of HO-1 as a shield from chemotherapeutic agents to survive treatment. Here, we examined the possible mechanisms underlying HO-1-dependent cell survival.

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The abbreviations used are: HO-1, heme oxygenase-1; CoPP, cobalt protoporphyrin; CORM, CO-releasing molecule; siRNA, small interfering RNA; PI, propidium iodide.
mammalian cell cultures (6, 10, 14). However, there are few reports about anti-proliferative role of HO-1 in prostate and breast cancer (15, 16). Revelation of its effects on tumor growth and metastasis has undoubtedly increased the significance of HO-1 in the field of cancer biology. Many studies have targeted inhibition of this enzyme as an approach to sensitize cancer cells to therapies (14, 17).

While most of the studies have tried to investigate the anti-apoptotic role of HO-1 in promoting cancer cell survival, it is of interest to also explore the regulation of autophagy by the enzyme. Recent studies show that along with apoptosis, type II cell death or autophagy plays a vital role in ascertaining the fate of cancer cells (18–20). Autophagy is a “self-eating” process responsible for intracellular degradation of damaged and dysfunctional organelles and protein aggregates. It is a non-selective process where cytoplasm and organelles are encapsulated within double-membrane vesicles called autophagosomes, and are eventually degraded as the autophagosomes fuse with lysosomes (18). Protein degradation is thought to be a protein turnover process. Thus, by ensuring a constant recycling of essential nutrients and cellular components, the intracellular degradation process autophagy serves a cytoprotective role, especially under nutrient-deficient conditions. Autophagy is induced in tumor cells during starvation and hypoxia and can protect the cells from apoptosis under such metabolic and oxidative stress conditions (21, 22). Regardless of its proposed protective functions, the role of autophagy in cancer is a matter of debate.

In contrast to studies that suggest its pro-tumorigenic function, many observations indicate a tumor-suppressing role for autophagy. An anti-cancer role for autophagy first came under consideration when Beclin-1, a core component of the autophagosome nucleation complex was identified as a tumor suppressor gene (23). Beclin-1 is monoallelically deleted in human breast, ovarian and prostate cancers, and Beclin-1−/− mice have been found to develop spontaneous tumors (23–26). The anti-cancer function of autophagy is also supported by facts that the tumor suppressor genes p53 and PTEN induce autophagy (27, 28), while pro-survival Bcl family members, Bcl-2 and Bcl-xL, inhibit autophagy by directly associating with Beclin-1 (29). Controversy regarding the role of autophagy in cancer is further augmented by conflicting reports about the effect of autophagy on chemotherapeutic treatment. Several chemotherapeutic drugs, including sorafenib, rapamycin (RAPA) and histone deacetylase inhibitors have been reported to induce autophagy (18, 30, 31). While some groups suggest that autophagy augments toxicity of the drugs to facilitate apoptosis of cancer cells, others propose that autophagy is induced in cancer cells as a survival strategy against these drugs. Considering the contradictory evidence regarding the role of autophagy in cancer, it is believed that autophagy suppresses the growth and progression of early tumors, while it supports the survival of established tumors and promotes cancer at later stages (18, 32).

We have recently demonstrated that HO-1 is induced in human renal cancer cells via the activation of Ras-Raf-ERK pathway; and overexpression of HO-1 plays a pivotal role in promoting cell survival (13). In the present study, we investigated the mechanism(s) underlying HO-1-mediated cell survival. We observe that HO-1 protects renal cancer cells from apoptosis and autophagy induced by chemotherapeutic drugs RAPA and sorafenib; and HO-1-mediated cancer cell survival involves modulation of regulatory proteins (particularly, Bcl-xL, Beclin-1, LC3B-II and Rubicon) for both apoptosis and autophagy.

**EXPERIMENTAL PROCEDURES**

Reagents—Cobalt protoporphyrin (CoPP) was obtained from Frontier Scientific. CO-releasing molecule (CORM) tricarbonyldichlororuthenium (II) dimmer (CORM-2) was obtained from Sigma-Aldrich. RAPA and sorafenib were purchased from LC Laboratories. The gene-specific small interfering RNA (siRNA) for HO-1 along with its control was purchased from Qiagen. The transfection of siRNA was performed using LifeTect 2000 (Invitrogen).

Cell Lines—The human renal cancer cell lines (786–0 and Caki-1) were obtained from American Type Culture Collection. 786–0 cells were grown in RPMI 1640, and Caki-1 cells were grown in McCoy’s medium supplemented with 10% fetal bovine serum (GIBCO).

Tissue Samples—Tissue samples of human renal cell cancer (RCC) were obtained from surgical specimens of patients who underwent surgery at the University Hospital (Wurzburg, Germany). The protocol to obtain tissue samples was approved by the review board of the hospital. Tumor tissues were graded (stages I through IV) according to Robson staging system. Tumor cells were isolated from RCC tissues by Cytospin preparations.

Plasmid—The pCMV-HO-1 cDNA plasmid was used in transfection studies to overexpress human HO-1.

Transfection Assays—786–0 or Caki-1 (2.5 × 10⁵ cells) were transfected with the human HO-1 overexpression plasmid using Effectene Transfection Reagent (Qiagen), according to the manufacturer’s protocol. The total amount of transfected plasmid DNA was normalized using a control empty expression vector. Transfection efficiency was determined by co-transfection of the β-galactosidase gene under control of cytomegalovirus immediate early promoter and by measurement of β-galactosidase activity using standard assay system (Promega).

Immunoprecipitation Assays—Immunoprecipitations were performed with 0.5 mg of total protein at antibody excess using anti-Bcl-xL (Cell Signaling Technology) or anti-Rubicon (Abcam). Immunocomplexes were captured with protein A-Sepharose beads (GE Healthcare), and bead-bound proteins were subjected to Western blot analysis using anti-Beclin-1 (Santa Cruz Biotechnology), anti-Bcl-xL or anti-Rubicon.

Western Blot Analysis—Protein samples were run on SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore Corp.). The membranes were incubated with anti-HO-1 (R&D Systems), anti-β-Actin (Sigma-Aldrich), anti-Bcl-xL, anti-Bcl-2 (Cell Signaling Technology), anti-Beclin-1, anti-LC3B (Sigma-Aldrich), or anti-Rubicon; and subsequently incubated with peroxidase-linked secondary antibody. The reactive bands were detected by using chemiluminescent substrate (Pierce).
**Cell Proliferation Assay**—Cell proliferation was measured by MTT Cell Proliferation Assay (ATCC) following the manufacturer’s protocol. Cells were plated in 96-well plates. Following treatment, 10 μl of MTT reagent was added to each well. Once purple crystals of formazan became clearly visible under microscope, 100 μl of Detergent Reagent was added and the cells were incubated at dark for 4 h. Absorbance was measured at 570 nm and corrected against blanks, which consisted of culture medium processed in the same way as above in the absence of cells. The reading at 570 nm is directly proportional to cell proliferation (number of viable cells).

**Apoptosis Assay**—Cellular apoptosis was measured by Annexin-V and propidium iodide (PI) staining using APC Annexin-V Apoptosis Detection Kit (eBioscience) according to the manufacturer’s protocol. Following staining, the cells were analyzed by flow cytometry on a FACSCalibur.

**Autophagy Assay**—Cellular autophagy was monitored using Cyto-ID Autophagy Detection Kit (Enzo Life Sciences) following manufacturer’s protocol. The 488 nm excitable Cyto-ID Green Autophagy Detection Reagent supplied in the kit becomes brightly fluorescent in vesicles produced during autophagy and thus serves as a convenient tool to detect autophagy at cellular level. Following treatment, the cells were trypsinized, washed in phosphate-buffered saline (PBS) and resuspended in 2000× dilution of the Detection Reagent. After 30 min of incubation at 37 °C, the cells were washed and analyzed by flow cytometry.

**Immunohistochemistry**—Immunohistochemistry was performed on either frozen sections of RCC or tumor cells isolated from RCC tissues by Cytospin preparations. Briefly, acetone-fixed sections/cells were first stained with anti-Rubicon (Abcam), and then incubated with a species-specific Cy3-conjugated secondary antibody (Dianova). In a second step, the sections/cells were stained with FITC-conjugated anti-Beclin-1 (Bioss). Specimens were washed thoroughly in between incubations and counterstained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich). The sections were mounted with polyvinyl alcohol mounting medium with DABCO (Sigma-Aldrich) and visualized under fluorescence microscope. Co-localization of Rubicon and Beclin-1 was evaluated by merged images.

** Statistical Analysis**—Statistical significance was determined by Student’s t test. Differences with \( p < 0.05 \) were considered statistically significant.

**RESULTS**

**HO-1 Is Overexpressed in Renal Cancer Cells Following RAPA and Sorafenib Treatment**—We have recently shown that the cytoprotective enzyme HO-1 is overexpressed in human renal cancer cells and promotes cell survival (13). In addition, tumor cells may bypass the killing effects of different chemotherapeutic agents because of overexpression of HO-1 (6, 14). Here, we examined if there is any change in HO-1 expression in renal cancer cells (786-0 and Caki-1) following treatments with RAPA and sorafenib, two approved drugs that are being used to treat renal cancer. The cells were treated with different concentrations of either RAPA (10 and 20 ng/ml) or sorafenib (10 and 20 μM); control cells were treated with vehicle alone. Western blot analysis showed that treatments with both RAPA and sorafenib were associated with a marked increase in HO-1 protein expression compared with vehicle-treated controls (Fig. 1, A and B). In accordance with current literature, it is expected that the overexpressed HO-1 may mediate a protective function against RAPA- and sorafenib-mediated killing of renal cancer cells.

**Inhibition of HO-1 Augments RAPA- and Sorafenib-induced Apoptosis of Renal Cancer Cells**—Treatments with RAPA and sorafenib can promote apoptosis of cancer cells. As our previ-
ous experiment suggested that treatments with RAPA and sorafenib are associated with HO-1 overexpression, here we wished to evaluate if the knockdown of HO-1 could facilitate RAPA- and sorafenib-induced apoptosis of renal cancer cells. To this end, 786-O cells were transfected with either HO-1 siRNA or control siRNA. Cells were then treated with either RAPA or sorafenib; and control cells were treated with vehicle alone. The cells were stained with Annexin-V and propidium iodide and analyzed by flow cytometry to check the apoptotic index. As shown in Fig. 2A, RAPA treatment increased cellular apoptosis in control siRNA-transfected renal cancer cells compared with vehicle-treated controls; the percentage of apoptotic cells (early + late apoptotic cells) increased from 3.29% (1.79 + 1.5%) to 13.7% (10.5 + 3.2%). However, the knockdown of HO-1 significantly increased cellular apoptosis in RAPA-treated cells; the percentage of apoptotic cells increased from 13.7% (control siRNA-transfected and RAPA-treated cells) to 30.44% (HO-1 siRNA-transfected and RAPA-treated cells).

Similarly, as shown in Fig. 2B, sorafenib treatment increased cellular apoptosis in control siRNA-transfected renal cancer cells compared with vehicle-treated controls; the percentage of apoptotic cells (early + late) increased from 2.73% (vehicle-treated control cells) to 13.56% (control siRNA-transfected and sorafenib-treated cells). However, the knockdown of HO-1 significantly increased cellular apoptosis in sorafenib-treated cells; the percentage of apoptotic cells increased from 13.56% (control siRNA-transfected and sorafenib-treated cells) to 36.69% (HO-1 siRNA-transfected and sorafenib-treated cells). The knockdown of HO-1 was confirmed by Western blot (Fig. 2C). These observations clearly suggest that the knockdown of HO-1 can augment RAPA- and sorafenib-induced apoptosis of renal cancer cells.

**Overexpression of HO-1 Inhibits RAPA- and Sorafenib-induced Apoptosis of Renal Cancer Cells**—In our earlier experiment, we measured the status of RAPA- and sorafenib-induced apoptotic cells following HO-1 knockdown. Here, we examined the effect of HO-1 overexpression on RAPA- and sorafenib-induced renal cancer cell apoptosis. We used a HO-1 plasmid (pCMV-HO-1) that overexpresses human HO-1. 786-O cells were transfected with either the HO-1 plasmid or empty vector. As shown in Fig. 3A, treatment with RAPA increased cellular apoptosis in vector-transfected renal cancer cells compared with vehicle-treated controls; the percentage of apoptotic cells (early + late) increased from 1.81% (vehicle-treated control cells) to 10.95% (vector-transfected and RAPA-treated cells).

FIGURE 2. Inhibition of HO-1 promotes RAPA- and sorafenib-induced apoptosis. A and B, 786-O cells were transfected with either HO-1 siRNA (50 nM) or control siRNA. After 24 h of siRNA transfection, the cells were treated with either 10 ng/ml RAPA (A) or 20 μM sorafenib (B) for 48 h; control cells were treated with vehicle alone. Apoptotic index of the cells was determined by Annexin-V (APC) and propidium iodide staining. C, knockdown of HO-1 in siRNA-transfected cells was confirmed by Western blot. Data shown are representative of three independent experiments.
HO-1 Down-regulates Apoptosis and Autophagy

Similarly (Fig. 3B), the overexpression of HO-1 significantly decreased sorafenib-induced apoptosis of renal cancer cells; the percentage of apoptotic cells (early + late) decreased from 8.0% (vector-transfected and sorafenib-treated cells) to 2.96% (HO-1 plasmid-transfected and sorafenib-treated cells). Overexpression of HO-1 following transfection with the HO-1 plasmid was confirmed by Western blot (Fig. 3C). Together, these observations suggest that HO-1 can play a major role in limiting cellular apoptosis of renal cancer cells following treatments with RAPA and sorafenib.

Overexpression of HO-1 Prevents RAPA- and Sorafenib-mediated Down-regulation of Renal Cancer Cell Proliferation—We sought to determine if overexpression of HO-1 could modulate the effect of RAPA and sorafenib on renal cancer cell proliferation. Caki-1 cells were first transfected with either the HO-1 overexpression plasmid or empty vector, and then treated with either RAPA or sorafenib; control cells were treated with vehicle alone. Following treatments, cells were subjected to a cell proliferation assay. As shown in Fig. 4 and supplemental Fig. S1, treatment with either RAPA or sorafenib decreased the proliferation of renal cancer cells compared with vehicle-treated controls; however, following overexpression of HO-1, treatments with RAPA or sorafenib could not decrease cell proliferation up to similar level. We found a similar effect of HO-1 in 786-O cells; however, the effect was more prominent and significant in Caki-1 cells. These results suggest that the overexpres-

FIGURE 3. Overexpression of HO-1 inhibits RAPA- and sorafenib-induced apoptosis. A and B, 786-O cells were transfected with either HO-1 overexpression plasmid (0.5 μg) or the empty vector. After 24 h of plasmid transfection, the cells were treated with either 10 ng/ml RAPA (A) or 20 μM sorafenib (B) for 48 h; control cells were treated with vehicle alone. Apoptotic index of the cells was determined by Annexin-V (APC) and propidium iodide staining. C, overexpression of HO-1 in plasmid-transfected cells was confirmed by Western blot. Data shown are representative of three independent experiments.

FIGURE 4. Overexpression of HO-1 prevents RAPA- and sorafenib-mediated inhibition of cell proliferation. Caki-1 cells were transfected with either HO-1 overexpression plasmid (1.0 μg) or the empty vector. After 24 h of transfection, the cells were treated with 10 ng/ml RAPA, 20 μM sorafenib or vehicle alone for 48 h. Cell proliferation was measured by MTT assay. Data shown are representative of three independent experiments. Columns, average of triplicate readings of two different samples; bars, S.E. *, p < 0.05, **, p < 0.005.

**Induction of HO-1 Is Associated with Increase in the Expression of Anti-apoptotic Bcl-xL in Renal Cancer Cells**—Our earlier experiments suggested that the overexpression of HO-1 in renal cancer cells can significantly down-regulate cellular apoptosis induced by chemotherapeutic agents. It has been shown that with increased expression of Bcl-2 gene family (Bcl-2 or Bcl-xL), levels of apoptosis are minimal in renal cell cancer, which may assist in cancer progression and resistance to chemotherapeutic treatments (33). Here, we tested whether induction of HO-1 in renal cancer cells is also associated with modulation of the expression of Bcl-2 family proteins. HO-1 was overexpressed in Caki-1 cells by either CoPP treatment (1–20 μM) or transfection with the HO-1 plasmid (0.5–1.0 μg); control cells were either treated with vehicle or transfected with empty vector. We observed that overexpression of HO-1 in Caki-1 cells was associated with a marked decrease in the expression of Bcl-2 family proteins. HO-1 was overexpressed in Caki-1 cells by either CoPP treatment (1–20 μM) or transfection with the HO-1 plasmid (0.5–1.0 μg); control cells were either treated with vehicle or transfected with empty vector. We observed that overexpression of HO-1 promoted marked induction of Bcl-xL (Fig. 5, A and B, top panels); however, there was no significant change in the expression of Bcl-2 (Fig. 5, A and B, second panel; and supplemental Fig. S2, A and B). We also confirmed that siRNA-mediated knockdown of HO-1 markedly decreased the expression of Bcl-xL without any significant change in the expression of Bcl-2 (Fig. 5C, top two panels, and supplemental Fig. S2C).

These findings prompted us to investigate if increased level of CO, which arises from HO-1-mediated heme degradation, can also regulate Bcl-xL. We treated 786-O cells with the CO releasing molecule CORM-2. As illustrated in Fig. 5D (top panel) and supplemental Fig. S2D, CORM-2 treatment indeed increased the expression of Bcl-xL compared with vehicle-treated control. Together, our results suggest that induced expression of Bcl-xL can be one of the possible mechanisms for the anti-apoptotic effect of HO-1 in renal cancer cells.

**Induction of HO-1 Down-regulates the Expression of Autophagy-regulating Molecules Beclin-1 and LC3B in Renal Cancer Cells**—Recent studies show that along with apoptosis, autophagy also plays a vital role in regulating the fate of cancer cells (18). Interestingly, it has been shown that sorafenib can promote the killing of cancer cells through autophagy (30). Thus, we wanted to determine if the induction of HO-1 in renal cancer cells could also modulate the expression of two autophagy-regulating genes, Beclin-1 and LC3B. To this end, HO-1 was overexpressed in Caki-1 cells by either CoPP treatment (1–20 μM) or transfection with the HO-1 plasmid (0.5–1.0 μg); control cells were either treated with vehicle or transfected with empty vector. We observed that overexpression of HO-1 promoted marked induction of Bcl-xL (Fig. 5, A and B, top panels); however, there was no significant change in the expression of Bcl-2 (Fig. 5, A and B, second panel; and supplemental Fig. S2, A and B). We also confirmed that siRNA-mediated knockdown of HO-1 markedly decreased the expression of Bcl-xL without any significant change in the expression of Bcl-2 (Fig. 5C, top two panels, and supplemental Fig. S2C).

We also checked the effect of CO on the expression of autophagy-regulating genes in renal cancer cells. As shown in

![Figure 5](image-url)
Fig. 5D (second and third panels) and supplemental Fig. S2D, the treatment with CORM-2 markedly decreased the expression of Beclin-1 and LC3B-II compared with vehicle-treated control. Together, these observations suggest that the overexpressed HO-1 in part also promotes survival of renal cancer cells through down-regulation of autophagy-regulating proteins.

**Overexpression of HO-1 Promotes the Association between Bcl-xL and Beclin-1**—The Bcl-2 family members (Bcl-2 and Bcl-xL) classically function as anti-autophagy proteins by directly associating with Beclin-1 (29, 34). In our earlier experiments, we observed that induction of HO-1 in renal cancer cells promotes overexpression of Bcl-xL. Here, we sought to evaluate if there is any change in the status of Bcl-xL-Beclin-1 complex following HO-1 overexpression. Caki-1 and 786-0 cells were transfected with either the HO-1 plasmid or empty vector. We observed that induction of HO-1 in both cell types markedly increased the complex formation between Bcl-xL and Beclin-1 compared with vector-transfected controls (Fig. 5E, top panel). As observed in our earlier experiments, the expression of Bcl-xL was also increased following HO-1 induction (second panel). These findings suggest that the overexpression of HO-1 in renal cancer cells leads to increased association between Bcl-xL and Beclin-1, which may promote cell survival through decreased autophagy.

**There Is Co-localization of Beclin-1 and Rubicon in Human Renal Cancer Cells, and This Association Is Increased Following Induction of HO-1**—A novel molecule Rubicon has recently been identified as a Beclin-1-interacting protein that suppresses autophagosome maturation and inhibits autophagy (35, 36). We first checked the expression of Beclin-1 and Rubicon in human renal cancer tissues and tumor cells (Cytospin preparations). As shown in Fig. 6, A and B, we found that both proteins were expressed and co-localized in cancer tissues and tumor cells. However, the co-localization was greater in low-stage renal tumor cells compared with high stage tumor cells.

Next, we examined if the induction of HO-1 could also modulate the association between Beclin-1 and Rubicon. Caki-1 and 786-0 cells were transfected with either the HO-1 plasmid or empty vector. We observed that overexpression of HO-1 in renal cancer cells promotes cell survival through decreased autophagy.
both cell types was associated with increased complex formation between Beclin-1 and Rubicon compared with vector-transfected controls (Fig. 7A). Thus, our observations suggest that induction of HO-1 in renal cancer cells may down-regulate autophagy through increased association between Beclin-1 and Rubicon.

**HO-1 Inhibits RAPA- and Sorafenib-induced Dissociation of Beclin-1 from Rubicon**—We sought to determine the effect of RAPA and sorafenib treatment on the complex formation between Beclin-1 and Rubicon, and how HO-1 may modulate the process. Caki-1 cells were transfected with either HO-1 overexpression plasmid or empty vector, and treated with RAPA or sorafenib. As shown in Fig. 7B (top panel), both RAPA (lane 3) and sorafenib (lane 5) treatment down-regulated the complex formation between Beclin-1 and Rubicon compared with vehicle-treated control (lane 1); however, RAPA and sorafenib-mediated down-regulation of this complex formation (Beclin-1-Rubicon) was markedly inhibited following knockdown of HO-1 (lane 4).

We also confirmed this finding by using siRNA. Caki-1 cells were transfected with either HO-1 siRNA or control siRNA, and then treated with sorafenib or vehicle alone. As shown in Fig. 7C (top panel), both knockdown of HO-1 (lane 2) and sorafenib treatment (lane 3) down-regulated the complex formation between Beclin-1 and Rubicon; however, there was further and marked increase in sorafenib-mediated down-regulation of Beclin-1-Rubicon complex formation following knockdown of HO-1 (lane 4). We found similar results (data not shown) in RAPA-treated cells following HO-1 knockdown. Our observations suggest that HO-1 may inhibit RAPA and sorafenib-induced autophagy in renal cancer cells through the modulation of Beclin-1 and Rubicon complex.

**Overexpression of HO-1 Inhibits Autophagy**—To confirm our earlier observations that HO-1 can inhibit therapeutic drug-induced autophagy in renal cancer cells, we investigated the status of autophagy (functional readout) in RAPA-treated cells following HO-1 overexpression. 786-0 cells were treated with different combinations of RAPA and CoPP, an inducer of HO-1. The cells were stained with Cyto-ID Green Autophagy Detection Reagent and analyzed by flow cytometry. As shown in Fig. 7D and supplemental Fig. S3, RAPA significantly promoted autophagy in the cells, while induction of HO-1 markedly attenuated both basal as well as RAPA-induced autophagy. Thus, our data show that HO-1 protects renal cancer cells from autophagy induction by therapeutic drugs.
both apoptosis and autophagy induced by chemotherapeutic drugs.

**DISCUSSION**

The cytoprotective enzyme HO-1, which plays an essential role in maintaining cellular homeostasis under stress conditions, is often highly up-regulated in tumor tissues and can facilitate tumor growth and metastasis. In this study, we show that the overexpression of HO-1 can promote survival of renal cancer cells through regulation of both apoptosis and autophagy. HO-1 significantly attenuates RAPA- and sorafenib-induced apoptosis of cancer cells; and HO-1 overexpression is associated with an induction of Bcl-xL and inhibition of Beclin-1 and LC3B-II. In addition, HO-1 promotes the association of Beclin-1 with Bcl-xL and Rubicon, which is a novel negative regulator of autophagy. Finally, we show that RAPA-induced autophagy is significantly inhibited by HO-1.

Revelation of its role in cancer has given a new dimension to studies involving HO-1. We have recently shown that activation of the Ras-Raf-ERK pathway promotes upregression of HO-1 and survival of renal cancer cells (13). Induction of HO-1 in cancer is often associated with resistance of cancer cells to chemotherapeutic drugs (6, 37, 38); and inhibition of HO-1 in combination with chemotherapy can be a possible and effective therapeutic strategy to enhance efficiency of cancer treatment (8, 10, 14). In this study, we demonstrate that knockdown of HO-1 significantly augments RAPA- and sorafenib-induced apoptosis of renal cancer cells. It has been reported that in addition to induction of apoptosis, the knockdown of HO-1 can also decrease angiogenesis, proliferation and growth of various tumors (7, 39, 40). Importantly HO-1 inhibition has been shown to sensitize cancer cells to cisplatin, gemcitabine and other drug treatments (11, 12, 14). Thus, the mechanisms of HO-1-mediated cell survival need to be explored to understand how the function of HO-1 can be modulated to increase the efficacy of cancer therapy.

The Bcl2 family, comprised of both pro- (Bax, Bak, Bad) and anti-apoptotic (Bcl-2, Bcl-xL) members, is a key regulator of apoptosis (41). It is believed that along with enhanced cell proliferation, impaired apoptosis is a crucial factor in tumor development. Thus, overexpression of anti-apoptotic members of the Bcl-2 family, or loss of their pro-apoptotic relatives can result in oncogenesis and tumor progression. Overexpression of Bcl-xL and other anti-apoptotic Bcl-2 members has been found to be associated with resistance to chemotherapeutic drugs (42, 43). Interestingly, we found that HO-1 markedly induces Bcl-xL in renal cancer cells, with no significant change in expression of Bcl-2. Although both Bcl-xL and Bcl-2 are known to promote anti-apoptotic signals, it is not uncommon when only one of these pro-survival members takes active part in protecting the cells from apoptosis (44, 45). Considering the anti-apoptotic role of Bcl-xL and its involvement in chemoresistance, it is possible that inhibition of apoptosis by modulation of Bcl-xL is one of the possible mechanisms underlying HO-1-mediated cancer cell survival.

While apoptosis has long been known to play a major regulatory role in tumorigenesis, autophagy is fast gaining importance (18–20). Recent studies indicate that cancer cell survival is controlled by a complex interplay of these two cell death pathways. The precise role of autophagy in cancer has always evoked controversy; there are enough evidences to support its role as a killer and also as a savior of cancer cells. It is reasonable to believe that autophagy serves a “quality control” task in the early stages of cancer, by eliminating cells with defective organelles and proteins, thereby inhibiting oncogenesis and tumor growth (18, 23, 46). However, during late or advanced stages of cancer, when rapidly dividing tumor cells are under metabolic stress due to hypoxia and nutrient deficiency, the “protein turnover” process of autophagy comes as a savior; it supplements the growing needs of the cells with excess supply of oxygen and nutrients, thus supporting tumor growth and progression (21, 22, 32). The fact that autophagy is promoted with chemotherapeutic drugs has also been justified by two conflicting rationales. While some groups believe that autophagy (primarily toxic autophagy) promotes apoptotic functions of chemotherapeutic drugs, others suggest that it may act as a survival strategy of cancer cells to protect themselves from toxic effects of the drugs (18, 30, 31). Our data in this study show that HO-1 downregulates some key mediators (Beclin-1 and LC3B-II) of the autophagy pathway, and also inhibits drug-induced autophagy in renal cancer cells. Of several molecules participating in the autophagy pathway, one of the most critical roles is served by Beclin-1. Beclin-1 is an integral part of a class III PI-3K multiprotein complex that is essential for autophagosome nucleation. Binding of Beclin-1 to anti-apoptotic members of Bcl-2 family abrogates autophagy-promoting functions of Beclin-1, while the dissociation of Beclin-1 from these anti-apoptotic proteins promotes autophagy (29, 34). Our findings indicate that HO-1 promotes the association of Beclin-1 with its inhibitor Bcl-xL, and thereby may down-regulate autophagy. Recently, Rubicon was identified as another novel Beclin-1-binding partner that negatively regulates autophagy and endocytosis (35, 36). Rubicon associates with UVRAG-Beclin-1 complex and suppresses autophagosome maturation and endocytic trafficking. Interestingly, our data show a critical role of HO-1 in inhibiting RAPA- and sorafenib-induced dissociation of Rubicon from Beclin-1. Thus, increased association of Rubicon and Beclin-1 can act as one of the possible mechanisms for HO-1-mediated down-regulation of drug-induced autophagy.

Although apoptosis and autophagy are completely different events, convergence of these two pathways has been reported. Inducers of apoptosis can also regulate autophagy, and members of Beclin-1 and Bcl-2 family may serve as a point of crosstalk between these two events (47). It is important to note that under specific circumstances, instead of protecting cells from apoptosis, autophagy can mediate cell death (48). As discussed earlier, chemotherapeutic treatments can promote autophagy to kill tumor cells (30), and it has been suggested that autophagic cell death can occur in cells that may not die by apoptosis (47). We observe that inhibition of both apoptosis and autophagy play a key role in HO-1-mediated survival of renal cancer cells. In support of our findings, it has been shown that the anti-autophagic property of Bcl-2 may play a key role in tumor growth and progression (49).

In summary, this study elucidates an important mechanism underlying HO-1-mediated survival of renal cancer cells. It
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explains how HO-1 can protect cells from death by suppressing both apoptosis and autophagy induced by chemotherapeutic drugs. Thus, our work highlights the significance of HO-1 as a possible therapeutic target for renal cancer treatment. Application of chemotherapeutic drugs along with an HO-1 inhibitor may augment the efficiency of therapy by promoting both apoptosis and autophagy induced by these drugs.

REFERENCES

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