Sorafenib attenuates p21 in kidney cancer cells and augments cell death in combination with DNA-damaging chemotherapy

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Abbreviations: RCC, renal cell carcinoma; sEH, soluble epoxide hydrolase

There are few effective therapeutic options for metastatic renal cell carcinoma (RCC). Conventional chemotherapeutic agents are ineffective since these tumors are unusually resistant to DNA damage, likely due to an exuberant DNA repair response. Sorafenib, as one of the few available effective therapeutic options for metastatic RCC, has been shown to inhibit cell proliferation by inhibition of tyrosine kinases. We have recently shown that sorafenib inhibits soluble epoxide hydrolase, which catalyzes metabolism of the anti-inflammatory epoxyeicosatrienoic acids. Given previous work demonstrating the anti-apoptotic role of p21 in RCC as a potential mechanism for its drug resistance, we asked whether sorafenib signals through this pathway. We now show that sorafenib markedly decreases p21 levels in several RCC and hepatocellular carcinoma cells. Neither the MEK inhibitor PD98059 nor the sEH inhibitor t-AUCB, which represent known sorafenib-targeted signaling pathways, alter p21 levels, demonstrating that the p21 inhibitory effect of sorafenib is independent of these signaling cascades. In cells treated with doxorubicin to augment p21, sorafenib markedly decreases this protein, and the combinations of paclitaxel or doxorubicin with sorafenib show additive cytotoxicity as a function of the VHL status of the cells, suggesting that lower doses of each agent could be used in the clinical setting. In summary, we show a novel signaling pathway by which sorafenib exerts its salutary effects in RCC; future work will focus on the use of these drug combinations in the context of conventional therapeutics, and novel compounds and protocols targeting p21 in conjunction with sorafenib should be pursued.

Introduction

Renal cell carcinoma (RCC) is the sixth most common cancer in the United States and one of the few cancers whose incidence is increasing, and survival of patients with metastatic RCC is dismal (26% 5-y survival of TNM Stage IV based on 2005 statistics). For the one-third of patients who present with metastatic disease, there are few therapeutic options available since conventional chemotherapeutic and immunomodulatory approaches are ineffective. An important area of research in our and other laboratories relates to the mechanism of RCC chemotherapy resistance, which is a serious clinical problem and likely due to an exuberant DNA repair mechanism mediated by the p53 tumor suppressor pathway, and subsequent induction of the downstream anti-apoptotic molecule p21.

We have previously shown that p21, a cyclin-dependent kinase inhibitor intimately involved in p53 signaling, can direct cells into the growth suppressive or anti-apoptotic pathways. Consistent with this finding, p21 has been shown to be a prognostic marker indicating worse survival when cytosolically located in both RCC and breast cancer. In addition, forced cytosolic localization of p21 results in anti-apoptosis and growth promotion in different cell types. Indeed, and likely for this reason, p21 induction has been shown to be an early event in oncogenesis.

Sorafenib is a multi-kinase inhibitor which targets both angiogenic and non-angiogenic targets in cancer. This agent is in current clinical use to treat advanced RCC as well as unresectable hepatocellular carcinoma. However, there are severe, although rare, substantial adverse events associated with the use of this drug, such as cardiac ischemia, left ventricular dysfunction, neutropenia and hypertension. Thus, novel mechanisms of sorafenib are being evaluated in order to narrow the molecular targets associated with its therapeutic application thereby decreasing adverse events. Sorafenib has not previously been shown to have a specific effect upon p21, which lies downstream of p53 and which conveys the survival effect necessary for DNA repair. Due to the likely pivotal role of p21 induction in chemotherapy failure in RCC as well as other cancers, we asked whether one of the mechanisms by which sorafenib exerts its beneficial therapeutic effect is via inhibition of p21.
The soluble epoxide hydrolase (sEH) converts epoxyeicosatrienoic acids (EETs) to the less active dihydroxyeicosatrienoic acids (DHETs). The EETs have been demonstrated to be vasodilators in various animal models and play an important role in regulation of blood pressure as well as control and prevention of heart disease. The sEH inhibitors have been shown to stabilize the EET levels and thus have beneficial effects on hypertension, occlusion, atherosclerosis and inflammation through increasing endogenous levels of EETs and other lipid epoxides. Previous work in our laboratory has demonstrated that sorafenib is a potent inhibitor \( K_i = 17 \pm 4 \, \text{nM} \) of the soluble epoxide hydrolase (sEH), an enzyme with pleiotropic effects on inflammation and vascular disease, this finding suggests that sEH inhibition may account for at least part of the effect of sorafenib on RCC. In our ongoing studies of unexpected mechanisms of sorafenib signaling in RCC, we now show that p21 is markedly inhibited by sorafenib in both kidney and liver cancer cell lines, and that this result is independent of this drug’s known effects on both MEK/ERK and sEH pathways. Furthermore, despite the substantial induction of p21 by the doxorubicin, sorafenib is still able to markedly downregulate p21 and thereby contribute to the cytotoxicity of DNA-damaging chemotherapy in this combination therapy.

Our finding of the unexpected attenuation of a major anti-apoptotic protein which is induced by chemotherapy by a currently available drug suggests the need for further exploration of combination therapy with sorafenib, or inhibitors of p21, and DNA-damaging agents in RCC, both in vitro and in vivo. In light of this data, additional studies directed at novel and specific methods of p21 attenuation as a therapeutic manipulation in kidney and other cancers are currently underway in our laboratory.

Results

Sorafenib decreases levels of p21 independently from MEK/ERK and sEH pathways. The cyclin kinase inhibitor p21 is a p53-pathway protein induced by DNA damage or cellular stress and possesses differential effects on apoptosis depending on cell lines and conditions (reviewed in ref. 2 and 3); we have previously demonstrated an anti-apoptotic effect of p21 in RCC cell lines. This survival function of p21 likely maintains cell viability during the DNA repair process, such that attenuation of p21 can subvert this repair process and thereby sensitize RCC and other cancer cells to conventional chemotherapy. Due to sorafenib’s efficacy in metastatic RCC, a disease with substantial resistance to conventional chemotherapy, we asked whether at least part of this drug’s salutary effect is mediated by p21 modulation. Given the fact that steady-state plasma levels of sorafenib of up to 10 mg/l (16 \, \text{µM}) can be achieved by oral administration, we incubated two separate RCC and hepatocellular carcinoma cell lines for 24 h with sorafenib at concentrations ranging from 1–20 \, \text{µM}. Substantial decrease in p21 was seen in all cell lines tested, and occurred in a dose-dependent manner and at pharmacological plasma levels (Fig. 1).

To determine specificity of sorafenib for p21 downregulation, we examined the levels of several other proteins relevant to p21 signaling, including p53, the cyclin kinase inhibitor p27, and p-Akt which has been shown to phosphorylate and thereby stabilize p21 to promote cell survival. Despite the fact that p21 was markedly attenuated in all four cell lines, both p-Akt and p27 showed inconsistent levels of decrease or induction when all cell lines were evaluated in aggregate (Fig. 1), suggesting that the sorafenib effect on p21 is specific and independent of the Akt/ PKB signaling pathway. In addition, despite the parallel attenuation of p53 to p21 observed in some of the cell lines, the mechanism of p21 downregulation by sorafenib is unlikely to be solely through p53, since p27, which also lies downstream of p53, is not consistently decreased after sorafenib treatment among all cell lines (i.e., it is increased in Huh-7 cells).

To confirm the immunoblotting data showing that p21 is decreased by sorafenib in RCC cells, we performed immunofluorescence in both of the RCC cell lines used in this study. Basal levels of p21 were seen in both cytosolic and nuclear compartments in ACHN cells, with higher levels in the nucleus as expected since p21 was not induced by cellular stress. When incubated with sorafenib at 10 \, \text{µM}, there was a marked decrement of p21 in both compartments (Fig. 2), consistent with the immunoblotting data (Fig. 1). In addition, this finding is consistent with at least partial p53 independence of sorafenib-induced p21 attenuation, since 786-O cells are mutant for the von Hippel-Lindau tumor suppressor gene (which is seen in most sporadic cases of clear cell RCC and contributes to its pathogenesis), hence have unstable p53, and appear to have quantitatively less p21 using similar confocal microscopy settings.

Sorafenib is well known as a multi-kinase inhibitor of Raf, VEGFR and PDGFR kinases and has growth inhibitory effects attributed to its effects on these signaling cascades through MEK/ERK pathway attenuation. Thus, in light of this fact and our earlier findings that sorafenib possesses sEH inhibitory effects, we next asked whether the mechanism of sorafenib’s inhibition of p21 expression is related to alteration of these signaling pathways. Both p53-wild type ACHN and VHL mutant 786-O cells were treated with the specific MEK inhibitor, PD98059 or a specific sEH inhibitor, t-AUCB. While p-ERK, which lies downstream of MEK, was decreased by both sorafenib and PD98059 in both cell lines, p21 was decreased only by sorafenib and not by either PD98059 or t-AUCB (Fig. 3). Thus, downregulation of p21 levels by sorafenib occurs independently of the MEK/ERK and sEH pathways.

The cytotoxicity of sorafenib is greater than that of MEK/ERK and sEH inhibitors. As a multi-kinase inhibitor, sorafenib has profound effects on cell viability, but it is not clear which kinases or other signaling proteins are actually responsible for its cytotoxic effect in RCC cells. As we have already shown that p21 inhibition was independent of MEK/ERK pathway proteins and sEH inhibition, we next asked whether these pathways are required for sorafenib’s cytotoxic effects in RCC by performing MTT assays of sorafenib in parallel with inhibitors of these individual signaling pathways. When ACHN cells were incubated with PD98059, t-AUCB, or sorafenib at the same concentrations, sorafenib alone showed greater than 50% cell death at 10 \, \text{µM}, while, surprisingly, PD98059 and t-AUCB showed...
non-significant cytotoxicity at this dose (Fig. 4). Thus, sorafenib causes cytotoxicity either independently of the MEK/ERK and sEH signaling pathways, or via a combination of these and other pathways; these data, in light of our previous work showing that p21 attenuation shows cytotoxicity in these cell lines, suggest that the decrease in p21 protein is at least in part responsible for the profound cytotoxicity of sorafenib.

Sorafenib attenuates p21 despite chemotherapeutic induction by doxorubicin and leads to additive cytotoxicity. Both doxorubicin and paclitaxel cause DNA damage which is presumed to contribute to their anticancer effects. However, in order for this mechanism to be effective, the DNA repair process has to be subverted or overwhelmed, or DNA damage will be repaired and the cancer cells will survive due to lack of apoptosis. In addition, given the frequency and/or severity of adverse events using high doses of most chemotherapeutic agents when used alone, it is often desirable to reduce the dose by means of combination chemotherapy. Thus, we next asked whether p21 can remain attenuated by sorafenib even when it is augmented as a result of DNA damage from chemotherapy. When ACHN and 786-O cells were incubated with doxorubicin, p21 was increased as expected due to DNA damage (or other cellular stress) (Fig. 5A), yet despite this marked increase, p21 was still markedly decreased by sorafenib in both cell lines (Fig. 5B). Interestingly, there was no induction of p21 by paclitaxel at the same concentrations (Fig. 5C), possibly due to less quantitative DNA damage by this agent at given concentrations as compared with doxorubicin. As with doxorubicin, however, p21 was similarly reduced by the combination of sorafenib and paclitaxel (Fig. 5D).

Given our finding that p21 is decreased by sorafenib, we next asked whether sorafenib capitalizes on the anti-apoptotic effect of this protein for its in vitro cytotoxicity in the face of DNA-damaging chemotherapeutics. Such a finding would be evidenced by increasing cytotoxicity with the combination of DNA damaging chemotherapy and p21-attenuating sorafenib, and would be expected to be at least partially dependent on the p53 status of the cell lines investigated. Doxorubicin alone at 0.1 μM caused the expected cytotoxic effect in both ACHN and 786-O cells as assessed by an MTT assay (Fig. 6A). Upon incubation with the combination of doxorubicin (0.1 μM) and sorafenib (at 10 μM), there was an additive effect on cytotoxicity of doxorubicin in both cell lines (Fig. 6A). Lower concentrations of doxorubicin did not elicit an additive effect with sorafenib (data not shown). At 1 μM of doxorubicin, toxicity was too great to observe an additive effect with sorafenib (data not shown).

In contrast, in the case of paclitaxel, which did not augment p21 (Fig. 5), there was an additive effect of the combination of this drug (10 μM) and sorafenib (10 μM) in ACHN cells, but there was no additive effect at any concentration tested in the VHL-mutant 786-O cells (Fig. 6B). As in the case of doxorubicin, lower concentrations of paclitaxel did not elicit additive
effects with sorafenib (10 μM; data not shown). Thus, the combination of sorafenib and doxorubicin led to a consistent additive cytotoxic effect in p53 wild-type ACHN cells, whereas the combination of sorafenib and paclitaxel, which did not increase p21, resulted in cytotoxicity in just one RCC cell line (p53-wt ACHN), consistent with the necessity of p21 downregulation for the full cytotoxic effect of sorafenib. In addition, in the VHL mutant and hence p53 unstable cell line, 786-O, there was no additive at any concentrations in response to paclitaxel. Thus, the additive effect of sorafenib and DNA-damaging therapeutics in RCC cells is a function of the p21 inducing property of the chemotherapy as well as of the p53 status of the cells examined; furthermore, this data support the necessity of p21 attenuation for the full apoptotic effect of sorafenib in RCC cells.

Discussion

For many cancers, treatment with DNA damaging agents, at doses required for clinical efficacy, are associated with unacceptable adverse effects as well as inadequate cure rates. Kidney cancer is notoriously resistant to chemotherapy as well as conventional immunotherapy, although recent work with kinase inhibitors has shown promise for late-stage disease. A possible reason for chemotherapy resistance is failure of DNA damaging agents, when used alone, to cause cancer cell apoptosis, since inactivation of apoptosis pathways after cellular stress is essential for cancer development. The mechanism by which a cell escapes apoptosis after being subjected to stresses leading, for example, to DNA strand breakage is assumed to be through induction of the anti-apoptotic protein p21. This p21 induction, either dependent or independent of p53, functions to arrest growth in G1 such that DNA damage can be repaired. By exploiting the anti-apoptotic function of p21 to therapeutic advantage in several previous studies in RCC cells using both antisense oligodeoxynucleotides and small molecule inhibitors, we have identified another paradigm with which to attack therapy-resistant kidney cancers at their Achilles’ heel. Attenuation of p21 causing increase cell killing, and for which the paradigm described here would be expected to hold, is not limited to kidney cancer, as this finding has also been shown in colon and breast cancer, and in malignant mesothelioma. The importance of the current work lies not only in our demonstration of a novel and unexpected mechanism of action of sorafenib, but also in the fact that we now show that an already approved and currently utilized drug has p21 attenuating effects and could therefore be rapidly moved to the clinic.

Sorafenib was originally described as a multi-kinase inhibitor with specificity for the Raf, VEGFR and PDGFR kinases, and we have recently shown that this drug also inhibits activity of sEH, an enzyme which catalyzes Figure 2. Sorafenib inhibits both cytosolic and nuclear p21 in RCC cells. ACHN and 786-O cells were grown to confluence on 8 well chamber slides and treated with sorafenib or vehicle (DMSO) at the indicated concentrations for 24 h. The cells were subjected to immunofluorescence visualization by confocal microscopy as described in Materials and Methods. p21 is green and the nuclear dye (DAPI) is blue.
An Akt inhibitor (Akt Inhibitor V, Triciribine) was evaluated but failed to decrease p-Akt without substantial toxicity in the RCC cell lines used in this study. So it remains possible that p21 lies at the distal end of several signaling cascades impacted by sorafenib in RCC, and we are currently evaluating other possible indirect mechanisms of p21 inhibition by sorafenib, but, based on the hepatocellular carcinoma data (Fig. 1), it is clear that p-Akt attenuation is not universally required for the downregulation of p21.

Others have examined combinations of sorafenib and conventional chemotherapies, although not DNA-damaging agents, in RCC. An unpublished Phase II report with gemcitabine showed that this combination showed no significant side effects, but efficacy was not reported. Sorafenib and doxorubicin showed advantages as compared with each agent separately in HCC patients, yet sorafenib and cisplatin showed decreased cellular cytotoxicity in human colorectal carcinoma cell lines. Interestingly, the latter study also demonstrated decreased levels of p21 in these cells without comment on the potential significance of this finding or any further experiments related to it. We could find no published studies evaluating the combination of a DNA intercalating agent (such as doxorubicin) or a microtubule inhibiting agent (such as paclitaxel) in RCC. RCCs treated with doxorubicin, by virtue of the DNA damage seen with this agent, would be expected to rely heavily on p21 induction to escape an apoptotic fate, and surprisingly, paclitaxel has been shown to have similar properties. This reliance on p21 by both of these agents is consistent with the additive effect of sorafenib and both doxorubicin and paclitaxel in the p53 wild-type ACHN cells seen in our study, with less of an effect in the VHL mutant 786-O cells.

In summary, our data provide a plausible argument that sorafenib can act as a sensitizing agent for conventional chemotherapeutics in the treatment of RCC by attenuating the metabolism of the anti-inflammatory epoxyeicosatrienoic acids. The affected growth factor receptors activate the Raf/MEK/ERK pathway which induces cell proliferation. Furthermore, in clear cell RCC (ccRCC), which is by far the most common RCC histology type in humans, loss of VHL function, which occurs in the majority of ccRCCs, results in constitutive activation of the HIF-1α transcript leading to abnormal activation of VEGFR. Thus the mechanism of sorafenib’s action as targeted therapy for ccRCC has been assumed to involve mainly inhibition of Raf and VEGFR and thereby reduction of cell proliferation. In this study, we provide a novel mechanism by which sorafenib influences the apoptotic state of RCC cells: through attenuation of p21. To our knowledge, this is the first comprehensive study of sorafenib or any approved drug as a p21 inhibitor.

Sorafenib, at doses that attenuate p21, is considerably more cytotoxic than the MEK/ERK pathway and sEH inhibitors, both of which when added separately did not show a decrease in p21. In light of previous work showing RCC cell cytotoxicity using antisense and small molecule inhibitor approaches to p21 attenuation, these results strongly suggest that downregulation of p21, independent of MEK/ERK or sEH inhibition, confers a substantial part of the cytotoxicity, and hence the clinical efficacy, of sorafenib. These findings are supported by earlier reports that cytotoxic p21 interferes with the activities of caspas, thereby interfering with the apoptotic process, and that higher cytotoxic expression of p21 is associated with a worse outcome in patients with metastatic RCC.

In RCC, but not in hepatoma cells, p-Akt was decreased by sorafenib in a dose dependent fashion. As it has been shown that p-Akt stabilizes p21 and causes it to remain p21 in the cytosol, this finding suggests that a possible mechanism of p21 inhibition by sorafenib, at least in RCC, is indirect and occurs via p-Akt inhibition by sorafenib. In an attempt to address this concern, an Akt inhibitor (Akt Inhibitor V, Triciribine) was evaluated but failed to decrease p-Akt without substantial toxicity in the RCC cell lines used in this study. So it remains possible that p21 lies at the distal end of several signaling cascades impacted by sorafenib in RCC, and we are currently evaluating other possible indirect mechanisms of p21 inhibition by sorafenib, but, based on the hepatocellular carcinoma data (Fig. 1), it is clear that p-Akt attenuation is not universally required for the downregulation of p21.

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In summary, our data provide a plausible argument that sorafenib can act as a sensitizing agent for conventional chemotherapeutics in the treatment of RCC by attenuating the
compounds and combinations shown here in vitro should lead to further evaluations of p21 attenuation, by sorafenib as well as other p21-inhibitry methods, in animal and human trials.

Materials and Methods

Materials. Sorafenib (free base) was purchased from LC Laboratories. Paclitaxel, doxorubicin, and the p-MEK inhibitor (PD98059) were purchased from Sigma. The sEH inhibitor trans-4-[4-(3-adamantan-1-yl-ureido)-cyclo-hexyloxy]-benzoic acid (t-AUCB) was synthesized according to our previously described method. Paclitaxel, doxorubicin, PD98059, sorafenib and t-AUCB were dissolved in dimethyl sulfoxide (DMSO). Mouse monoclonal anti-p21WAF1/Cip antibody and anti-GAPDH antibody were obtained from Millipore. Mouse monoclonal anti-phosphor Akt antibody and rabbit monoclonal anti-phospho extracellular signal-regulated kinase (ERK) antibody were obtained from Cell Signaling Technology, Inc. Goat anti-mouse and goat anti-rabbit HRP conjugated IgG were obtained from Bio-Rad. ECL Plus Western Blotting Detection Reagents was obtained from GE Healthcare. Normal goat serum, rabbit monoclonal anti-p21WAF1/Cip antibody, and Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) were purchased from Cell Signaling Technology, Inc., VECTASHIELD HardSet Mounting Medium with DAPI was purchased from Vector Laboratories.

Cell lines. Two human proximal tubule epithelial cancer cell lines, ACHN and 786-O, and the human liver cancer cell line, HepG2, were obtained from the American Type Culture Collection. The human liver cancer cell line, Huh-7, was generously provided by Dr. Jian Wu (Division of Hepatology and Gastroenterology, UC, Davis). ACHN cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), and 100 units/mL streptomycin and 100 mg/mL penicillin. 786-O cells grown to confluence in complete growth media (10% serum) and treated with sorafenib, t-AUCB, PD98059 or vehicle (DMSO) for 72 h. Subsequently, an MTT assay was performed as described in Materials and Methods. Error bars are standard deviations in quadruplicate. The experiment shown is representative of at least three separate experiments. *p < 0.05 of sorafenib compared with both other additions.

Figure 4. Sorafenib demonstrates greater cytotoxicity than p-MEK or sEH inhibition. ACHN and 786-O cells grown to confluence in complete growth media (10% serum) and treated with sorafenib, t-AUCB, PD98059 or vehicle (DMSO) for 72 h. Subsequently, an MTT assay was performed as described in Materials and Methods. Error bars are standard deviations in quadruplicate. The experiment shown is representative of at least three separate experiments. *p < 0.05 of sorafenib compared with both other additions.
Immunofluorescence. After appropriate treatments in 8-well chamber slides, the cells were washed with PBS and fixed in 2% paraformaldehyde for 1 h at room temperature. The cells were washed with PBS and blocked in the blocking buffer, 5% normal goat serum and 0.3% Triton X-100 in PBS, for 1 h at room temperature. After blocking, the cells were incubated with rabbit monoclonal anti-p21WAF1/Cip antibody overnight at 4°C. The cells were washed with PBS and incubated with anti-rabbit IgG (H+L), F(ab’)2 Fragment Alexa Fluor® 488 Conjugate, diluted 1:1,000 in antibody dilution buffer (1% BSA and 0.3% Triton X-100 in PBS) for two hours in the dark at room temperature. The cells were washed with PBS and coverslipped with vectashield with DAPI. The specimens were examined by confocal microscopy. Three to four randomly selected fields were examined in each of three separate experiments.

MTT assay. Five x 10⁴ cells were plated in 96-well plates and incubated for 16 h at 5% CO₂ at 37°C. After appropriate treatments, the cells were incubated in 20 μl of thiazolyl blue
tetrazolium bromide (MTT) solution (5 mg/ml in PBS) with 180 μl of the growth media for 3 h. Then, the MTT solution was removed and the blue crystalline precipitate in each well was dissolved in DMSO (200 μl). Visible absorbance of each well at 540 nm was quantified using a microplate reader.

Statistical analysis. Comparisons of mean values were performed using the independent samples t-test. A p value of less than 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

**Figure 6.** Sorafenib has additive effect to chemotherapy treatment. ACHN and 786-O cells were plated in 10% serum-containing media and treated with (A) doxorubicin (0.1 μM) or (B) paclitaxel (10 μM) in the presence or absence of sorafenib (10 μM) in complete (10% serum) growth media for 72 h. Subsequently, an MTT assay was performed as described in Materials and Methods. The experiment shown is representative of at least three separate experiments. * p < 0.05 comparing single vs. double treatment.

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