Strategic combination therapy overcomes tyrosine kinase coactivation in adrenocortical carcinoma

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Background. Coactivation of tyrosine kinase limits the efficacy of tyrosine kinase inhibitors. We hypothesized that a strategic combination therapy could overcome tyrosine kinase coactivation and compensatory oncogenic signaling in patients with adrenocortical carcinoma (ACC).

Methods. We profiled 88 tyrosine kinases before and after treatment with sunitinib in H295R and SW13 ACC cells. The effects of monotherapy and strategic combination regimens were determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

Results. The minimum inhibitory concentrations (IC50) of sunitinib quenched its primary targets: FLT-3, VEGFR-2, and RET. In contrast, ERK, HCK, Chk2, YES, CREB, MEK, MSK, p38, FGR, and AXL were hyperactivated. Monotherapy with sunitinib or PD98059 at their IC50 reduced proliferation by 23% and 19%, respectively, in H295R cells and by 25% and 24%, respectively, in SW13 cells. Sunitinib and PD98059 in combination decreased proliferation by 68% and 64%, respectively, in H295R and in SW13 cells, respectively (P < .05 versus monotherapy). The effects of combination treatment exceeded the sum of the effects observed with each individual agent alone.

Conclusion. We describe the first preclinical model to develop strategic combination therapy to overcome tyrosine kinase coactivation in ACC. Because many tyrosine kinase inhibitors are readily available, this model can be immediately tested in clinical trials for patients with advanced ACC. (Surgery 2012;152:1045-50.)

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Adrenocortical carcinoma (ACC) is a rare but aggressive malignancy that accounts for 0.02% of cancers reported in the United States.1 Although long-term survival can be achieved after complete operative resection, approximately 70% of patients present in advanced stages that are not amenable to curative surgery. For patients with metastatic disease or in whom complete resection is not feasible, operation is not indicated. Although systemic therapy is the principal therapy for advanced ACC, it is generally ineffective and does not improve overall survival.5

The adrenolytic agent mitotane is the first-line therapy for advanced ACC. Although mitotane treatment has not improved overall survival in clinical trials, it can decrease cortisol levels and provide some symptomatic relief for patients. However, mitotane therapy often is limited because of drug toxicity or disease progression.3 Cytotoxic drugs, such as cisplatin4 and irinotecan,5 have minimal efficacy in ACC. The highly resistant nature of ACC to conventional chemotherapeutics has prompted efforts to target the molecular defects that drive oncogenesis.

Sunitinib is tyrosine kinase inhibitor (TKI) that targets multiple pro-oncogenic—signaling pathways. In a recent case report, treatment with sunitinib resulted in a partial response on radiography in a patient with ACC.6 Currently, a phase 2 study evaluating the efficacy of sunitinib mono-therapy as second-line therapy in advanced ACC is underway.

However, monotherapy with sunitinib in other cancer contexts generally is ineffective. In multiple studies investigators have found that monotherapy with TKIs results in the coactivation of compensatory oncogenic signaling pathways.7 We hypothesized that the characterization of compensatory
signaling activated by sunitinib treatment could enable development of rational combination regimens to overcome tyrosine kinase coactivation in ACC.

METHODS

Materials. Mouse anti-actin antibody, pan Ab-5, was purchased from Neomarker (Fremont, CA). Rabbit antihuman phospho-ERK1/2, Thr202/Tyr204, extracellular signal-regulated kinase (ERK), phospho-FLT-3 Tyr591, FLT-3, phospho-RET Tyr905, RET antibodies were purchased from Cell Signaling (Beverly, MA). Secondary horseradish peroxidase–conjugated mouse and rabbit antibodies were obtained from Vector Laboratories (Burlingame, CA). PD98059, an ERK inhibitor, was obtained from Calbiochem (La Jolla, CA). Sunitinib was obtained from Selleck Chemicals LLC (Houston, TX).

Cell culture. The human ACC cell lines NCIH295R (H295R) and SW13, originally obtained from ATCC, were generous gifts from Dr Gary D. Hammer (University of Michigan Medical School, Ann Arbor, MI). H295R cells were cultured in complete media containing Dulbecco’s modified Eagle’s medium/F12 (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 250 µg/mL fungizone, 10% fetal bovine serum (Invitrogen), 2.5% ITS+ supplement (Sigma-Aldrich, St. Louis, MO). SW13 cells were cultured in complete media containing Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 250 µg/mL fungizone, and 10% fetal bovine serum. SW13 and H295R cells were used throughout the study between passages 3 and 11 and 7 to 18, respectively.

Western Blotting and receptor tyrosine kinase arrays. Lysates from ACC cells treated with sunitinib were subjected to Western Blotting. Human Phospho-RTK Array (ARY001) and Human Phospho-Kinase Array (ARY003) kits (R&D Systems, Minneapolis, MN) were used to assay the relative level of tyrosine phosphorylation. Two membranes (Membrane #1 and #2) in array ARY001 contained spotted antibodies corresponding to 42 distinct receptor tyrosine kinases (RTKs). One membrane (Membrane #3) in array ARY003 contained spotted antibodies corresponding to 46 distinct RTKs other than the RTKs in ARY001. Both positive and negative controls were plotted in both arrays ARY001 and ARY003. ARY001 and ARY003 profile 202 unique tyrosine phosphorylation residues of 88 RTKs. Cells were lysed by NP-40 lysis buffer according to the manufacturer’s protocol. The arrays were exposed to blocking buffer and incubated with 450 µg of cell lysate overnight at 4°C. The arrays were washed, incubated with a horseradish peroxidase–conjugated phospho-tyrosine detection antibody, treated with ECL solution, and exposed to film.

MTS cell proliferation assay. Cell proliferation was colorimetrically determined at 490 nm using a MTS cell proliferation assay kit (CellTiter 96 AQueous nonradioactive cell proliferation assay; Promega, Madison, WI) as previously described. After incubation with sunitinib or PD98059 for 48 hours, cells in a 96-well plate were incubated with 333 mg/L 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and 25 µm of phenazine methosulfate solution for 2 hours at 37°C in a humidified, 50 mL/liter CO2 atmosphere. The absorbance of soluble formazan produced by cellular reduction of MTS was measured at 490 nm with an ELISA reader (SpectraMax M5 Multi-Mode Microplate Reader; Molecular Devices, Synnyvale, CA). Percent proliferation relative to the controls was calculated on the basis of the MTS readout. Experiments were repeated 4 times, and each had quadruplicate samples.

Statistical analysis. To determine the minimum concentration of each agent necessary to achieve a significant effect (ICmin), we performed the MTS assay as described previously with various drug concentrations. Each assay was done in triplicate. The ICmin was determined as the lowest concentration of drug with a statistically significant 2-tailed Student t test when compared with the negative control.

RESULTS

Treatment with sunitinib activates multiple tyrosine kinases. We first sought to determine whether treatment with sunitinib resulted in the compensatory hyperactivation of any tyrosine kinase. We performed sunitinib dose- and time-response experiments in H295R and SW13 cells (Fig 1). We could not achieve greater than a 50% decrease in proliferation at concentrations lower than 20 nM, even after 120 hours of treatment (data not shown). A 50% decrease in proliferation was achieved by 48 hours at 20 nM, and we determined that this would be an ideal dose and time point for analysis.

The principal targets of sunitinib, including platelet-derived growth factor receptors, vascular endothelial growth factor receptors, and RET, showed no phosphorylation after 48 hours of exposure to sunitinib at 10 nM (Fig 2). Treatment
with sunitinib resulted in increased phosphorylation of ERK, HCK, Chk2, YES, CREB, MEK, MSK, p38, FGR, and AXL (Fig 2). Although there were many differences in the phosphorylation status of individual kinases in H295R compared with SW13 cells before treatment, the pattern of tyrosine kinase activation after sunitinib exposure were strikingly similar in both cell lines (Fig 2).

ERK is the most activated tyrosine kinase after sunitinib treatment. We compared the degree of phosphorylation before and after 48 hours of sunitinib and found that ERK1/2 was the most hyperactivated by sunitinib in both cell lines (Fig 2). Although there were many differences in the phosphorylation status of individual kinases in H295R compared with SW13 cells before treatment, the pattern of tyrosine kinase activation after sunitinib exposure were strikingly similar in both cell lines (Fig 2).

Sunitinib plus ERK inhibition has additive antiproliferative effects. Because monotherapy with sunitinib resulted in the compensatory activation of ERK, we next determined the effect of combining sunitinib with PD98059, a TKI that specifically inhibits ERK. Monotherapy with sunitinib or PD98059 at their IC_{min} concentrations (10 nM for sunitinib and 50 nM for PD98059) for 48 hours reduced proliferation by 23% and 19%, respectively, in H295R cells, and by 25% and 24%, respectively, in SW13 cells (Fig 4). However, strategic combination treatment with these agents decreased proliferation by 68% and 64% at 48 hours in H295R and SW13 cells, respectively (P < .05 versus monotherapy; Fig 4).

DISCUSSION

A trial to evaluate the efficacy of second-line sunitinib in advanced ACC is now underway. There is reason to believe that sunitinib monotherapy will fail to improve overall survival in patients with advanced ACC. In other cancer contexts, tyrosine kinase coactivation is an important mechanism of resistance to TKI monotherapy. Our premise is that combination regimens that inhibit multiple targets simultaneously are needed to overcome coactivation. We sought to develop a preclinical model to select strategic combinatorial regimens to overcome compensatory oncogenic signaling observed after monotherapy with sunitinib. The results from such modeling could be used to predict which combinatorial regimens are most likely to be successful in clinical trials for this rare malignancy.

We performed an unbiased global assessment of the kinome and found that different ACC cell lines have substantial differences in the pattern of kinase activation under baseline conditions. However, treatment with sunitinib resulted in a specific pattern of compensatory signaling in which ERK was the most hyperactivated tyrosine kinase. The antiproliferative effects of combination therapy with sunitinib and the ERK inhibitor PD98059 exceeded the sum of the effects observed with each individual agent alone.

In other contexts, a number of distinct mechanisms can result in resistance to sunitinib. In gastrointestinal stromal tumors, mutations in the critical c-KIT gene result in sunitinib resistance.9 In renal cell carcinoma, the autocrine secretion of interleukin-8 can drive cell proliferation and diminish the antiproliferative effects of sunitinib.10 In a recent report, investigators demonstrated that lysosomal drug sequestration can diminish sensitivity to sunitinib.11 We are the first to characterize compensatory tyrosine kinase coactivation in ACC as a principal mechanism of TKI resistance.

Interestingly, Gotink et al11 reported that treatment with sunitinib increased ERK phosphorylation levels in sunitinib-resistant colorectal and renal carcinoma cell lines. A recent study showed that combination treatment of sunitinib...
with mitotane results in the rapid metabolism of sunitinib. Specifically, treatment with mitotane up-regulates CYP3A4, which results in diminished sunitinib levels and chemoresistance. This study of combination therapy with mitotane and sunitinib highlights the fact that not all combination drug regimens offer additive or synergistic effects and supports the use of preclinical models to predict which drugs should be used together.

Fig 2. Receptor tyrosine kinase array analysis of sunitinib-treated cells. (A) The film images exhibit the phosphorylation status of RTKs identified by the Human Phospho-RTK Array Kits. Whole-cell extracts from H295R and SW13 cells exposed for 48 hours to sunitinib (10 nM) were incubated in the RTK arrays, and the phosphorylation status was determined by subsequent incubation with a horseradish peroxidase–conjugated phospho-tyrosine detection antibody. Each RTK was spotted in duplicate and the pairs of dots in each corner are the positive controls. (B) The table demonstrates the list of RTKs with phosphorylation status changing in response to sunitinib treatment. The rightmost column represents mean phosphorylation change ± SD (n = 4). (Color version of figure is available online.)

Fig 3. Sunitinib decreases FLT-3 and RET phosphorylation but increases ERK phosphorylation in a time-dependent manner. H295R and SW13 cells were treated with sunitinib (10 nM) for various time points as indicated. Cell lysates were prepared and phospho-FLT-3, RET, and ERK levels were monitored by Western Blotting. Re-probing against FLT-3, RET, and ERK was done to ensure equal protein loading.

Fig 4. Sunitinib plus PD98059 has synergistic antiproliferative effects. H295R and SW13 cells were treated with or without 10 nM of sunitinib (Sun) in the presence or absence of PD98059 (PD, 50 nM) for 48 hours. Treated cells were subjected to the MTS proliferation assay. Similar experiments were repeated 3 times. Histograms represent relative % of OD-490 nm absorbance (*P < .05). All data are relative multiples of expression compared to untreated cells. The data are representative of three experiments and are expressed as the mean ± SE.

Sunitinib highlights the fact that not all combination drug regimens offer additive or synergistic effects and supports the use of preclinical models to predict which drugs should be used together. There are several issues that remain to be addressed. First, administration of multiple TKIs may be associated with additive toxicity risks.
However, if combination therapy has synergistic anticancer effects, a lower dose of each agent could be used with equal efficacy and a more favorable side effect profile. Although low-dose TKIs in combination could be well-tolerated, the use of a second class of targeted agents could also be used. For instance, monoclonal antibodies, rather than TKIs, could be used to target specific signaling pathways. Our work did not investigate the efficacy and toxicity of the proposed combination therapy in animals. Future studies should test the efficacy of sunitinib in combination with and ERK-targeting agent in animals to confirm the safety and synergistic anticancer activity in vivo.

In summary, preclinical modeling of tyrosine kinase coactivation is a useful tool to develop rational combinatorial therapeutics. Combination treatment with sunitinib and PD98059 is a promising therapeutic strategy for patients with advanced ACC and future studies should test this strategic combination regimen in clinical trials.

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REFERENCES

Dr Michael Demeure (Scottsdale, AZ): I have a question methodologically, primarily. You used the term “synergy.” But I don’t know that you’ve established synergy or that the effects are just additive. Can you clarify why you think they are synergistic?

Dr Daniel Ruan: Yes. The way we defined synergy is—

Dr Michael Demeure: It’s clearly defined in the literature, so I don’t think you have a license to redefine synergy as you wish, so please tell me—

Dr Daniel Ruan: The definition that we adapted from the literature is that if you take the minimum inhibitory concentration of different agents and add them together, their added net effect is greater than the sum of their individual components.

The reason why we used this definition is because it’s very simple and straightforward, and you don’t have to do multiple time- and dose-response curves, which is prohibitively expensive.