Autophagic activation potentiates the antiproliferative effects of tyrosine kinase inhibitors in medullary thyroid cancer

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Background. We hypothesized that autophagy inhibition would enhance the anticancer efficacy of ret protooncogene–targeted therapy in medullary thyroid cancer.

Methods. Medullary thyroid cancer–1.1 and TT cells were treated with sunitinib or sorafenib in the presence or absence of everolimus, trehalose, or small interfering RNA directed against autophagy protein 5.

Results. Sunitinib and sorafenib each robustly induced light chain 3-II expression, indicating autophagy activation. Autophagy protein 5 silencing diminished the antiproliferative effects of sunitinib and sorafenib by 44% (P < .05) and 41% (P < .05), respectively, in medullary thyroid cancer–1.1 cells and by 43% (P < .01) and 39% (P < .05), respectively, in TT cells. In contrast, everolimus increased the antiproliferative effects of sunitinib and sorafenib by 24% (P < .01) and 27% (P < .01), respectively, in medullary thyroid cancer–1.1 cells and by 20% (P < .05) and 23% (P < .05), respectively, in TT cells. Trehalose increased the antiproliferative effects of sunitinib and sorafenib by 26% (P < .01) and 27% (P < .01), respectively, in medullary thyroid cancer–1.1 cells and by 28% (P < .05) and 29% (P < .05), respectively, in TT cells. Autophagy protein 5 silencing abrogated both everolimus- and trehalose-induced increases in tyrosine kinase inhibitor efficacy.

Conclusion. Loss (gain) of autophagy diminishes (improves) the efficacy of sunitinib and sorafenib. Our findings suggest that autophagic activation should be combined with targeted ret protooncogene therapy for patients with advanced medullary thyroid cancer. (Surgery 2012;152:1142-9.)

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AUTOPHAGY is a process involving the degradation of cellular components through lysosomal machinery to regenerate energy by processing long-lived proteins and retired organelles. The totality of evidence indicates that autophagy is cytoprotective in tumors, enabling cells to adapt to increased metabolic demands, a hypoxic microenvironment, or stress from chemotherapy. However, excessive autophagy can lead to a nonapoptotic form of organized cell death called programmed cell death type II (PCD2), particularly in cells that are defective in apoptosis machinery. Ultimately, the effects of autophagic activation on cancer cell survival appear to be context and cell type–specific.

Medullary thyroid cancer (MTC) is a neuroendocrine malignancy that is more aggressive than well-differentiated thyroid cancer of follicular cell origin. The only curative treatment for MTC is surgical resection, but many patients present with locally advanced or metastatic disease that is not amenable to curative thyroidectomy and lymph node dissection. Many sporadic and all familial MTC cases are driven by activating mutations in the ret protooncogene (RET). The application of targeted therapeutics to MTC have largely focused on RET inhibition.

Small molecule inhibitors that reduce RET activity (eg, sorafenib, vandetanib, motesanib, sunitinib, and XL-184) revealed promising anticancer activity against MTC in preclinical studies. Although the US Food and Drug Administration
recently approved the use of vandetanib for advanced MTC based on data from a randomized, phase III trial, there were few radiographic tumor responses and the vast majority of patients achieved only disease stability. The overall impact of RET inhibition in MTC appears to be cytostatic, with no complete responses reported in patients with advanced disease. In the present study, we hypothesized that autophagy inhibitors could potentiate the anticancer activity of tyrosine kinase inhibitors (TKIs) that target RET in MTC.

METHODS

Materials. Mouse anti-human microtubule-associated protein 1 light chain 3 (LC3) antibody was purchased from MBL International Cooperation (Nagoya, Japan). Mouse anti-actin antibody pan Ab-5 was purchased from Neomarker (Fremont, CA). Rabbit anti-human autophagy protein 5 (Atg-5) and cleaved caspase-3 (Asp175) antibodies were purchased from Cell Signaling (Beverly, MA). Secondary horseradish peroxidase-conjugated mouse and rabbit antibodies were obtained from Vector Laboratories (Burlingame, CA). Everolimus was obtained from Novartis Pharmaceutical (East Hanover, NJ). Trehalose, a mammalian target of rapamycin (mTOR)-independent activator, was obtained from Sigma-Aldrich (St. Louis, MO). Sunitinib, sorafenib, and AZD6244 were obtained from Selleck Chemicals LLC (Houston, TX).

Cell culture and RNAi transfections. The human MTC cell lines MTC-1.1 and TT were cultured as described previously. MTC-1.1 cells were obtained from Dr Orlo H. Clark (University of California at San Francisco, San Francisco, CA) and TT cells were obtained from obtained from the American Type Culture Collection (ATCC CRL-1803; Manassas, VA). MTC-1.1 and TT cells were used throughout passages 8 to 14 and 3 to 11, respectively. Cells were subcultured at 80% confluency by trypsinization (in a 0.5% [vol/vol] trypsin solution, supplemented with 0.2% [vol/vol] ethylenediaminetetraacetic acid). Cells were transfected with scrambled siRNA or Atg-5 validated siRNA duplex. The target sequence against human Atg-5 was 5’-AACCTTGGCCCTAGAGAGA AA-3’ (Qiagen, Valencia, CA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. All transfections were performed in 6-well plates, and cells were processed 48 hours after transfection.
Western blotting. Cell lysates were prepared and analyzed by Western blotting as previously described.7

Autophagy determination. We assayed the expression level of the LC3-II protein, which is generated when microtubule-associated protein 1 LC3 undergoes autophagosomal breakdown. Conjugation of ubiquitous cytosolic LC3-I to phosphatidylethanolamine produces the LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes and is a reliable marker for autophagic activity.8 We assayed LC3-II protein expression levels by Western blotting.

MTS cell proliferation assay. Cell proliferation was colorimetrically determined at 490 nm using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay kit (CellTiter 96 AQueous non-radioactive cell proliferation assay; Promega Madison, WI) as previously described.7 After incubation with sunitinib or PD98059 for 48 hours, cells in a 96-well plate were incubated with 333 mg/L MTS and 25 μM phenazine methosulfate solution for 2 hours at 37°C in a humidified, 5% CO2 atmosphere. The absorbance of soluble formazan produced by cellular reduction of MTS was measured at 490 nm using an enzyme-linked immunosorbent assay reader (SpectraMax M5 Multi-Mode Microplate Reader; Molecular Devices, Sunnyvale, CA). Percent proliferation relative to the controls was calculated based on the MTS readout. Experiments were repeated 4 times, and each had quadruplicate samples.

Statistical analysis. Differences between treatment groups were evaluated with a 2-tailed independent Student t test. Each assay was performed in triplicate, and P < .05 was considered significant.

RESULTS

Sunitinib and sorafenib, but not AZD6244, activate autophagy. We first determined the dose of each drug that inhibits cell proliferation by 50% (IC50) for sunitinib and sorafenib. Using the MTS assay, IC50 of sunitinib and sorafenib were 50 and 10 nM, respectively, in both MTC-1.1 and TT cells (Fig 1). Sunitinib and sorafenib treatment at the IC50 increased LC3-II protein levels in both MTC-1.1 and TT cells (Fig 2, A and B). Treatment of AZD6244, a non-RET TKI, showed no effects on
LC3-II expression. The siRNA-mediated knockdown of Atg-5, a key molecule for autophagosome formation,9 abrogated the induction of LC3-II after sunitinib and sorafenib exposure (Fig 2, C). These results indicated that both sunitinib and sorafenib promote autophagic activation in MTC cells. Sunitinib and sorafenib exposure also increased the expression level of cleaved caspase-3, a marker of apoptosis,10 indicating that these TKIs also activate apoptosis.

Fig 3. Autophagy inhibition blocks the antiproliferative effects of sunitinib and sorafenib but not AZD6244. Medullary thyroid cancer–1.1 (MTC-1.1) and TT cells were transfected transiently with scrambled or autophagy protein 5 (Atg-5) small interfering RNA. After transfection, cells with and without Atg-5 knockdown were exposed to sunitinib (50 nM), sorafenib (10 nM), and AZD6244 (30 nM) for 48 hours. Treated cells were subjected to a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium proliferation assay. Similar experiments were repeated 3 times. Histograms represent the relative percent of OD490 nM absorbance. The asterisk indicates significance versus scrambled small interfering RNA–treated control (P < .05). All data are relative multiples of expression compared to untreated cells. The data are representative of 3 experiments and are expressed as the mean ± the standard error.

Fig 4. Sunitinib or sorafenib in addition to everolimus treatment exhibits synergistic effects on cell proliferation. Medullary thyroid cancer–1.1 (MTC-1.1) and TT cells were transiently transfected with scrambled or autophagy protein 5 (Atg-5) small interfering RNA. After transfection, cells with and without Atg-5 knockdown were exposed to sunitinib (50 nM), sorafenib (10 nM), or AZD6244 (30 nM) in the presence or absence of everolimus (20 nM) for 48 hours. Treated cells were subjected to a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium proliferation assay. Similar experiments were repeated 3 times. Histograms represent the relative percent of OD490 nM absorbance. The asterisk indicates significance versus scrambled small interfering RNA–treated control (P < .05). The pound sign indicates significance at P < .05 against sunitinib or sorafenib without everolimus. All data are relative multiples of expression compared to untreated cells. The data are representative of 3 experiments and are expressed as the mean ± the standard error.
Autophagy inhibition decreases the antiproliferative effects of sunitinib and sorafenib. Scrambled siRNA treatment did not change the antiproliferative efficacy of sunitinib and sorafenib at the IC50 dose. However, Atg-5 siRNA treatment decreased the antiproliferative effects of sunitinib and sorafenib by 44% ($P < .05$) and 41% ($P < .05$), respectively, in MTC-1.1 cells and by 39% ($P < .05$) and 36% ($P < .05$), respectively, in TT cells (Fig 3). In contrast, AZD6244 treatment had no antiproliferative effects in both scrambled and Atg-5 siRNA-transfected cells (Fig 3). This suggests that autophagic cell death plays a significant role in the antiproliferative effects of TKIs that inhibit RET in the context of MTC.

Everolimus potentiates the antiproliferative activity of sunitinib and sorafenib. We determined the IC50 of everolimus as 20 nM. At the IC50 dose, everolimus increased the antiproliferative effects of sunitinib and sorafenib by 24% ($P < .01$) and 27% ($P < .01$), respectively, in MTC-1.1 cells and by 20% ($P < .05$) and 29% ($P < .05$), respectively, in TT cells (Fig 4). To determine if the effects of everolimus involved the activation of autophagy, we performed RNA interference for Atg-5, which is an essential component of the autophagosome. Atg-5 silencing abrogated the antiproliferative effects of adding everolimus to sunitinib and sorafenib (Fig 4). Because Atg-5 has no known activity outside of its role in autophagy, these data suggest that the effects of everolimus are largely caused by the activation of autophagy (Fig 5).

mTOR-independent autophagic activation potentiates the antiproliferative activity of sunitinib and sorafenib. To show the effects of autophagic activation without mTOR inhibition on TKI efficacy, we used trehalose to activate autophagy. We determined that the IC50 of trehalose to be 100 mM (data not shown). Trehalose increased the antiproliferative effects of sunitinib and sorafenib by 26% ($P < .01$) and 29% ($P < .05$), respectively, in MTC-1.1 cells and by 28% ($P < .05$) and 29% ($P < .05$), respectively, in TT cells (Fig 6). Atg-5 silencing diminished the antiproliferative effects of adding trehalose to sunitinib or sorafenib (Fig 7).

**DISCUSSION**

Autophagic activation potentiates the anticancer effects of sunitinib and sorafenib in MTC. This concept is supported by the observation that the loss of autophagic activity via Atg-5 knockdown reduces the efficacy of sunitinib and sorafenib. Conversely, agents that activate autophagy...
Trehalose and everolimus enhance the antiproliferative effects of sunitinib and sorafenib. The effects of trehalose and everolimus on TKI cytotoxicity are abrogated by Atg-5 knockdown. Because Atg-5 is an essential component of the autophagosome with no other function, this suggests that the enhancement of TKI efficacy by trehalose and everolimus are mediated largely through the activation of autophagy. This finding is consistent with previous findings in papillary thyroid cancer that autophagic activation potentiates the antiproliferative effects of doxorubicin, suggesting that the cytotoxicity can be enhanced through the promotion of PCD2. A recent article found that everolimus plus erlotinib, a TKI targeting epidermal growth factor receptor (EGFR), synergistically inhibits cell proliferation and increases autophagic activity in lung cancer cells. The loss of EGFR also activates autophagy and increases chemosensitivity to doxorubicin in prostate cancer cells.

In contrast, other studies have reported that targeting autophagy improves TKI sensitivity. In non–small cell lung cancer, autophagic inhibition—either through the pharmacologic inhibitor chloroquine or RNA interference of essential autophagosome elements—augments the growth inhibitory effect of EGFR-directed TKIs. In prostate cancer, autophagy inhibition enhanced the cytotoxicity of Src family kinase inhibitors. Similarly, autophagic inhibition promotes cell death in gastrointestinal stromal tumors treated with KIT-targeted therapy. One recent study reported that chloroquine, an inhibitor of late autophagy, augments the cytotoxicity of TKIs including imatinib, nilotinib, and dasatinib in chronic myeloid leukemia cells. Ultimately, autophagic activation can either promote or suppress TKI sensitivity, depending on cell type. Our findings raise additional questions that should be addressed in future studies. The concept of PCD2 is controversial, and it is difficult to prove that PCD2 is the definitive mechanism of cell death. Although the effects of adding everolimus could be attributed to the effects of mTOR inhibition that are independent of autophagy, our data suggest that the promotion of PCD2 is largely responsible for the effects of everolimus. Finally, our work did not include in vivo studies, and as such, we did not investigate issues related to efficacy and toxicity in animals. Future studies should test the efficacy of sunitinib/sorafenib plus everolimus in animal models. Despite these drawbacks, we believe that our principal finding remains valid: in the context of RET-directed TKI therapy, autophagic activation improves treatment response in MTC.

In summary, autophagic activation enhances the anticancer effects of TKIs that target RET in MTC.
We propose that autophagy activators could play an important role in combination therapy and that future clinical trials should use such a strategy for patients with advanced MTC.

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REFERENCES


Fig 7. Sunitinib or sorafenib in addition to trehalose exhibits synergistic effects on autophagic activation. Medullary thyroid cancer–1.1 (MTC-1.1) and TT cells were transiently transfected with scrambled or autophagy protein 5 (Atg-5) small interfering RNA. After transfection, cells with and without Atg-5 knockdown were exposed to sunitinib (50 nM), sorafenib (10 nM), or AZD6244 (30 nM) in the presence or absence of trehalose (100 mM) for 48 hours. Cell lysates were prepared and light chain 3 (LC3)-I and -II protein expression levels were monitored by Western blotting. Reprobing against Atg-5 was performed to monitor Atg-5 knockdown efficiency. Reprobing against actin was performed to ensure equal protein loading. Densitometry analysis of LC3-II protein expression normalized to actin is shown.


DISCUSSION

Dr Mark Cohen (Kansas City, KS): Did you confirm autophagy by any other means besides LC3? Did you look at transmission microscopy or anything?

Dr Chi-Lou Lin (Boston, MA): Actually, in this study, we did not look at the electron microscopy imaging, but your suggestion is good because the autophagosome formation observed by electron microscopy imaging is important evidence of autophagic activation.

Dr John Phay (Columbus, OH): As you may know, Matt Ringel’s group recently published data on TT cells and MZ cells, not the MTC1. And, interestingly, they found that everolimus actually increased RET activity at these low doses. Did you, by chance, take a look at that?

Dr Chi-Lou Lin (Boston, MA): In this study, we did not look at that. We found that if you inhibit RET activity, perhaps autophagic activation is an additional way to kill the cells. Your suggestion is very good because maybe we should look at how everolimus effects RET activity.

Dr Jessica Gosnell (San Francisco, CA): I have 2 quick questions.

One, I wondered how you chose the 2 TKIs that were used.

And the second follow-up question: have you considered using ZD6474 or vandetanib, since that’s recently been approved for patients with medullary thyroid cancer?

Dr Chi-Lou Lin (Boston, MA): The reason why we picked those 2 drugs is because they are RET inhibitors. And those inhibitors not only target RET, they are multi-targeted small molecule inhibitors. We picked those 2 drugs, because they are RET inhibitors.

The second question, to test the vandetanib, actually, we have tested cells with vandetanib, but we did not look at autophagy. In the future, we could see if autophagic activation could potentiate vandetanib efficacy.