Carfilzomib is an effective anticancer agent in anaplastic thyroid cancer

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Abstract

Anaplastic thyroid cancer (ATC) is one of the most aggressive human malignancies. Currently, there is no standard or effective therapy for ATC. Drug repurposing for cancer treatment is an emerging approach for identifying compounds that may have antineoplastic effects. The aim of this study was to use high-throughput drug library screening to identify and subsequently validate novel therapeutic agents with anticancer effects in ATC. We performed quantitative high-throughput screening (qHTS) in ATC cell lines (SW-1736, 8505C, and C-643), using a compound library of 3282 drugs. qHTS identified 100 compounds that were active in all three ATC cell lines. Proteasome inhibitors were one of the most active drug categories according to enrichment analysis. Of the three proteasome inhibitors screened, a second-generation proteasome inhibitor, carfilzomib, was the most active. Treatment of ATC cells with carfilzomib significantly inhibited cellular proliferation and induced G2/M cell cycle arrest and caspase-dependent apoptosis. Mechanistically, carfilzomib increased expression of p27 (CDKN1B) and decreased expression of the anti-apoptotic protein ATF4. Pretreatment with carfilzomib reduced in vivo metastases (lung, bone, liver, and kidney) and disease progression, and decreased N-cadherin expression. Carfilzomib treatment of mice with established, widely metastatic disease significantly increased their survival, without significant toxicity. Our findings support the use or clinical study of carfilzomib as a therapeutic option in patients with advanced and metastatic ATC.

Key Words

- anaplastic thyroid cancer
- high-throughput screening
- carfilzomib
- proteasome inhibitor

Introduction

Thyroid cancer is the most common endocrine malignancy (Howlader et al. 2013). Anaplastic thyroid cancer (ATC) accounts for 1–2% of all thyroid cancers, but is the most common cause of thyroid-cancer-related deaths (Kebebew et al. 2005, Nagaiah et al. 2011). Patients with ATC present with advanced disease with local invasion and metastasis (90% of all cases), and even when the disease is localized (10% of all cases), recurrence and metastatic disease develop in most patients even after complete initial tumor resection. ATC is one of the most aggressive human cancers and has a median survival of only 5 months, and fewer than 20% of patients with ATC survive longer than 1 year. Current treatment regimens fail to provide durable, long-term clinical benefits (Kebebew et al. 2005, Granata et al. 2013), and so there is a major need to develop new, effective treatments.

Development of new therapies for rare cancers such as ATC has been hindered for several reasons. Traditional
approaches to drug discovery require an often-prohibitive investment of time and funding, which is an issue that is even more significant for orphan cancers. The drug development process is also further restricted by the decreasing number of compounds entering development phases, as well as the high rate of late-stage drug failures (Munos 2009). A new and inventive approach to cancer therapy development is to exploit the multitude of already established compounds approved for clinical use for other indications (Shahinas et al. 2010, Shum et al. 2010, Miller et al. 2014). Recycling established agents for new indications has proven a far more attractive approach, especially for rare cancers such as ATC. Given that these compounds have known pharmacokinetic, pharmacodynamic, and toxicity profiles, promising drugs can be rapidly transitioned into phase II or III clinical trials to test for efficacy.

In this study, we completed a quantitative high-throughput screening (qHTS) of 3282 clinically approved drugs in ATC cell lines. Active agents were filtered based on both enrichment and pharmacokinetic analyses. We found the proteasome inhibitor drug category to be one of the most active categories, and validated the anticancer activity of carfilzomib in both in vitro and in vivo studies.

**Materials and methods**

**Cell lines**

Human ATC cell lines 8505C (European Collection of Cell Cultures, Salisbury, UK), C-643 (CLS Cell Lines Service GmbH, Eppelheim, Germany), and SW-1736 (CLS Cell Lines Service GmbH) were maintained in DMEM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), fungizone (250 ng/ml), and insulin (10 µg/ml) in a 5% CO₂ atmosphere at 37 °C. The 8505C cell line has BRAF V600E and TP53 mutations, SW-1736 has a BRAF V600E mutation, and C-643 has an HRAS mutation. The cell lines were authenticated by short tandem repeat profiling. The passage number of the cells used in the experiments ranged from 10 to 20 passages.

**National Institutes of Health Chemical Genomics Center Pharmaceutical Collection library screening**

The National Institutes of Health Chemical Genomics Center Pharmaceutical Collection (NPC) consists of 3282 small-molecule compounds, of which 46% are drugs approved for human or animal use by the U.S. Food and Drug Administration (Huang et al. 2011). Detailed information on the drugs in this collection can be found at http://spotlite.nih.govnpc. Test compounds from the NPC library were prepared as described previously (Zhang et al. 2012).

**Quantitative high-throughput proliferation assay**

Cell viability after compound treatment was measured using a luciferase-coupled ATP quantitation assay (Cell-Titer-Glo; Promega) on the 8505C, C-643, and SW-1736 cells as described previously (Nilubol et al. 2012, Zhang et al. 2012).

**Cell proliferation**

Cell proliferation assays were performed in quadruplicate. 8505C, SW-1736, and C-643 cells were plated in black 96-well plates at a concentration of 2–4×10⁴ cells/well, depending on the cell line, in 100 µl of culture medium. After 24 h (day 0), 100 µl of fresh culture medium containing carfilzomib or vehicle were added to each well. CyQUANT (Invitrogen) proliferation assays were performed at 0, 2, 4, and 6 days, according to the manufacturer’s instructions. The cell number was determined using a 96-well fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) at an absorbance of 485 nm/538 nm.

**Cell cycle assay**

Cells were plated in six-well plates at a density of 6×10⁴ to 12×10⁴ cells/well, depending on the cell line, in 2 ml of culture medium. After 24 h, fresh culture medium containing carfilzomib or vehicle was added to each well. Following treatment for 18 h, the cells were harvested, fixed with cold 70% ethanol for 30 min at 4 °C, and incubated in the dark with RNase (100 mg/ml) and propidium iodide (50 mg/ml) for 30 min at 37 °C. A total of 20,000 nuclei were examined by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and the resulting data were analyzed using the ModFit Software (Verity Software House, Topsham, ME, USA).

**Apoptosis assay**

To determine whether drug treatment resulted in apoptosis, we used the APO-BrdU flow cytometry analysis assay (BD Pharmingen, San Jose, CA, USA). We also used the Caspase-Glo 3/7 assay (Promega) to measure caspase
activity. 8505C, SW-1736, and C-643 cells were plated in white 96-well plates at a density of 2.2–4×10³ cells/well, depending on the cell line. After 24 h, fresh culture medium containing carfilzomib or vehicle was added. After 24–48 h, cells were analyzed by APO-BrdU flow cytometry analysis and for caspase 3/7 activity using the Caspase-Glo 3/7 assay kit according to the manufacturer’s instructions. The relative luminescence (which is proportional to caspase 3/7 activity) was calculated and normalized to the total cell number.

**Western blot analysis**

Total protein lysates were separated by SDS–PAGE, transferred onto a nitrocellulose membrane, and immunostained with the following antibodies overnight at 4 °C: anti-p27kip1 (1:250 dilution, BD Transduction Laboratories, San Jose, CA, USA); anti-ATF4 (1:250 dilution, Santa Cruz Biotechnology); anti-N-cadherin (1:1000 dilution, EMD Millipore, Billerica, MA, USA); and anti-β-actin (1:3000 dilution, Santa Cruz Biotechnology). Anti-human β-actin was used as a loading control. The membranes were incubated with the appropriate HRP-conjugated IgG (anti-rabbit antibody at 1:3000 dilution, Cell Signaling Technology, Danvers, MA, USA, or anti-mouse antibody at 1:10 000 dilution, Santa Cruz Biotechnology). An ECL assay (Thermo Scientific, Rockford, IL, USA) was used to detect proteins.

**In vivo mouse studies**

A metastatic ATC mouse model was utilized for all in vivo studies of carfilzomib treatment (Zhang et al. 2014). Stably transduced 8505C cells, containing a linearized pGL4.51[luc2/CMV/Neo] vector luciferase reporter (8505C/Luc cells), were used. NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice received injections of 8505C-Luc2 cells (30 000 cells/mouse) via the tail vein. Metastases were confirmed at 1 week after tumor cell implantation, using bioluminescence imaging and the Xenogen in vivo imaging system. The images were analyzed using the IVIS Living Image Software (Caliper Life Sciences, Inc., Hopkinton, MA, USA). The mice were maintained according to the guidelines of the National Cancer Institute’s Animal Research Advisory Committee.

Carfilzomib (Onyx Pharmaceuticals, San Francisco, CA, USA) was reconstituted with 10% Captisol (Ligand Technology, La Jolla, CA, USA), according to the manufacturer’s instructions. On day 7 after tumor cell injection, mice were stratified into carfilzomib and vehicle treatment groups. Carfilzomib (6 mg/kg) or vehicle was administered intraperitoneally, three times a week. Treatment was continued until death or upon reaching humane criteria endpoints.

To assess the effects of carfilzomib on tumor metastasis, pretreatment in vivo mouse studies were also completed. NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice first received intraperitoneal injections of either carfilzomib (6 mg/kg) or vehicle (10% Captisol) for 2 consecutive days. The following day, the mice received injections of 8505C-Luc2 cells (30 000 cells/mouse) via the tail vein. On day 7 after tumor cell injection, carfilzomib (6 mg/kg) or vehicle was administered intraperitoneally, three times a week.

**Statistical analyses**

To determine compound activity in the qHTS assay, the titration-response data for each sample were plotted and modeled by a four-parameter logistic fit, yielding IC₅₀ (concentration of half-maximal inhibition) and efficacy (maximal response) values. Raw plate reads for each titration point were first normalized relative to positive control-only (tetraoctylammonium bromide, 100% inhibition) and negative control-only (DMSO, 0% inhibition) wells. Curve fits were then classified by criteria described previously (Inglese et al. 2006). Based on the quality of curve fit and efficacy, screened compounds were then classified into three categories: active, inactive, and inconclusive (Zhang et al. 2012).

To assess drug activity on the basis of the mechanism of action of the drug, we performed enrichment analysis by therapeutic category. The enrichment score is the ratio of the number of active drugs to the total number of tested drugs in the same therapeutic category based on the mechanism of action of the drug. To further assess active compounds, we also completed pharmacokinetic analyses. The candidate compound was selected for validation based on a demonstrated antiproliferative efficacy of over 80%, an IC₅₀ less than 1 μM, high maximum achievable plasma concentration (C₀), and a wide therapeutic window (defined as having an IC₅₀ less than the C₀).

Statistical analyses were performed using the GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Data were analyzed using a two-tailed t-test and the Mann–Whitney U test. Differences in survival for in vivo studies were assessed using Kaplan–Meier survival curves. A P value <0.05 was considered statistically significant. Data are expressed as mean ± S.D. or mean ± S.E.M.
Results

qHTS of drug library

To identify compounds with potential anticancer activity against ATC, we screened the NPC library using qHTS. This strategy used concentration–response curve information to identify 100 compounds that were active in all three tested ATC cell lines (8505C, C-643, and SW-1736). Enrichment analysis of active agents in each drug category revealed that proteasome inhibitors were among the most active drug classes screened. Among the proteasome inhibitors, carfilzomib, a second-generation proteasome inhibitor, was one of the most active agents, with a low IC₅₀ and highest efficacy in ATC cells and a high achievable serum concentration in humans (Table 1). Carfilzomib not only had an efficacy ≥89% in all three screened cell lines, but also had a high Cₘₐₓ (5.9 μM) and high potency, as reflected by an IC₅₀ in the nanomolar range. Most importantly, the IC₅₀ was found to be well below the Cₘₐₓ, indicating that carfilzomib has a wide therapeutic window. We then compared the activity of carfilzomib with drugs used in patients with ATC, such as doxorubicin and docetaxel (Smallridge et al. 2012). The activity of carfilzomib was better than those of doxorubicin and docetaxel, with a lower IC₅₀ and higher efficacy in all three ATC cell lines (Fig. 1A).

![Figure 1](image)

**Figure 1**

(A) Comparison of dose–response curves for carfilzomib, doxorubicin, and docetaxel in three ATC cell lines. Carfilzomib has a greater potency and a lower IC₅₀ in all three ATC cell lines. (B) Carfilzomib inhibits cellular proliferation and causes cell death. The effect of carfilzomib was determined using three ATC cell lines: 8505C, C-643, and SW-1736, and the CyQUANT assay. Cells were treated at concentrations (2.96–88.8 nM) within the clinically achievable range. *P < 0.05 for all concentrations compared with vehicle control.

### Table 1  Pharmacokinetic analysis of proteasome inhibitors screened using qHTS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cₘₐₓ (ng/ml)</th>
<th>Curve class</th>
<th>IC₅₀ (μM)</th>
<th>Maximum attainable response (%)</th>
<th>Curve class</th>
<th>IC₅₀ (μM)</th>
<th>Maximum attainable response (%)</th>
<th>Curve class</th>
<th>IC₅₀ (μM)</th>
<th>Maximum attainable response (%)</th>
<th>Activity category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carfilzomib</td>
<td>4232</td>
<td>−1.1</td>
<td>0.004</td>
<td>−121</td>
<td>−1.1</td>
<td>0.012</td>
<td>−92</td>
<td>−1.1</td>
<td>0.03</td>
<td>−89</td>
<td>Active</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>112</td>
<td>−1.1</td>
<td>0.013</td>
<td>−107</td>
<td>−1.1</td>
<td>0.004</td>
<td>−106</td>
<td>−1.1</td>
<td>0.017</td>
<td>−84</td>
<td>Active</td>
</tr>
<tr>
<td>Ixazomib</td>
<td>NA</td>
<td>−1.1</td>
<td>0.042</td>
<td>−108</td>
<td>−1.1</td>
<td>0.013</td>
<td>−93</td>
<td>−1.1</td>
<td>0.13</td>
<td>−108</td>
<td>Active</td>
</tr>
</tbody>
</table>

NA, not available. Cₘₐₓ, maximum achievable serum concentration.
Carfilzomib inhibits cell proliferation and induces cell death

To confirm carfilzomib’s growth-inhibitory effect in ATC cells, we validated our qHTS results. Treatment with carfilzomib inhibited cellular proliferation and induced cell death (Fig. 1B). This antiproliferative effect was observed at concentrations up to 1000-fold lower than the drug’s $C_{\text{max}}$, the concentration at which most of the drug’s side effects are likely to occur. Furthermore, at concentrations as low as 8.88 nM, carfilzomib not only inhibited cancer cell growth, but also induced over 90% cell death within 48 h of treatment.

Carfilzomib induces cell cycle arrest and upregulates p27

Given the antiproliferative and cytotoxic effects of carfilzomib in proliferation assays, we performed cell cycle analyses to explore the underlying mechanisms of carfilzomib’s activity in ATC cells. According to our flow cytometry analysis, carfilzomib treatment resulted in ATC cells accumulating in the G2/M phase within 18 h of treatment (Fig. 2). The relative sensitivity of the ATC cell lines to carfilzomib was associated with the accumulation of cells in G2/M. In order to further clarify the mechanism behind carfilzomib-induced cell cycle arrest, we examined the drug’s effect on several important cell cycle regulatory proteins: p21 (CDKN1A), p27 (CDKN1B), and p53 (TP53). Following treatment with carfilzomib, we observed an increase in p27 protein expression in all three ATC cell lines (Fig. 2), but no effect on p53 and p21 protein expression (not shown).

Carfilzomib induces caspase-dependent apoptosis

As carfilzomib also induced cell death, we then determined whether this effect was mediated through apoptosis, as has been observed with other proteasome inhibitors. Carfilzomib treatment of ATC cells resulted in apoptosis according to APO-BrdU flow cytometry analysis (Fig. 3). We also measured caspase activity in the ATC cell lines after 24 h, with and without carfilzomib treatment, and found a three- to eightfold increase in caspase activity in the treated cells (Fig. 3).

To further characterize the effect of carfilzomib on dysregulated apoptosis-regulatory proteins in ATC, we evaluated the expression of ATF4, an anti-apoptotic transcription factor known to regulate the prosurvival autophagy pathway, as well as to mediate chemoresistance to proteasome inhibitors such as bortezomib (Milani et al. 2009, Rzymski et al. 2009). Following treatment with carfilzomib, we observed a decrease in the expression of ATF4 in all three ATC cell lines (Fig. 3).

Carfilzomib inhibits tumor growth and prolongs survival in vivo

We further validated the antitumor activity of carfilzomib in an in vivo metastasis mouse model, a more clinically relevant model for ATC. Following 3 weeks of treatment, there was less metastatic tumor burden and, more
Carfilzomib induces apoptosis and increases caspase 3/7 activity and downregulates expression of anti-apoptotic ATF4. Left panels show fold increase in apoptotic cells with carfilzomib treatment. Middle panels show apoptosis with increased caspase 3/7 activity in carfilzomib-treated cells. Right panels show western blots for ATF4 detection. The charts below show the band densitometry measurement. Apoptosis data shown represent treatment for 24 h with carfilzomib or vehicle. For corresponding western blots, subconfluent 8505C (A), C-643 (B), and SW-1736 (C) ATC cells were treated with carfilzomib or vehicle for 24 h.

Figure 3
importantly, a statistically significant increase in overall survival in mice with heavy tumor burden (Fig. 4A, B and C). Interestingly, mice treated with carfilzomib also had a significantly lower rate of distant metastasis (fewer sites of bone and liver metastasis) beyond the lung tumor site compared with the vehicle group (Fig. 4B). These results indicate that carfilzomib not only exhibits antitumor activity \textit{in vivo}, but may also directly inhibit metastasis of tumor cells. A statistically significant decrease in weight of untreated mice was observed compared with the treated mice beginning in week 2, probably due to a greater tumor burden (Fig. 4D). Mice treated with carfilzomib developed transient gastrointestinal distress, with diarrhea and loss of appetite, following the first week of treatment (beginning on day 9). Symptoms were effectively managed with fluid repletion and resolved by day 13.

**Carfilzomib inhibits distant metastasis \textit{in vivo} and reduces N-cadherin expression**

ATC metastasis and local invasion, and recurrence after complete resection, are common, thus an effective therapy for ATC could be an agent that inhibits cancer progression (Ain 1998, Besic \& Gazic 2013). Given the lower rate of progressive metastases we observed in our \textit{in vivo} study in mice that already carry a heavy tumor burden, we further investigated whether carfilzomib could inhibit metastases \textit{in vivo}, using a pretreatment model. We found that pretreatment with carfilzomib dramatically inhibited ATC progression (Fig. 5A and B). Furthermore, mice pretreated with carfilzomib also had profound inhibition of established lung tumor growth (Fig. 5), as well as prolonged survival compared with the controls (Fig. 5C). A statistically significant decrease in weight of untreated mice was observed, relative to the pretreated mice (Fig. 5D). Pretreated mice also developed transient gastrointestinal distress following the first week of treatment, which again resolved with fluid repletion.

Loss of epithelial markers and acquisition of mesenchymal markers, mediated by the epithelial-mesenchymal transition (EMT) process, are known to increase the metastatic ability of cancer cells (Chaffer \& Weinberg 2011). Given the significantly lower rate of metastasis we observed in the \textit{in vivo} pretreatment experiments, we examined whether carfilzomib activity in ATC also inhibits the expression of EMT mediators such as N-cadherin and vimentin. Carfilzomib treatment decreased the expression of N-cadherin in ATC cells (Fig. 5E), but no changes were observed in the vimentin expression level (not shown).

**Discussion**

This study has five major findings. First, we have shown the utility of qHTS of clinical drug libraries as an efficient approach for rapidly screening drug activity in multiple ATC cell lines. Secondly, we have demonstrated that carfilzomib, a second-generation proteasome inhibitor, has growth-inhibitory and cytotoxic effects in ATC cells. Thirdly, carfilzomib’s antiproliferative activity in
ATC is associated with induction of G2/M phase arrest and increased p27 expression, and caspase-dependent apoptosis. Fourthly, carfilzomib showed potent antitumor activities in vivo, including tumor growth inhibition and prolonged survival in ATC metastasis mouse models. Finally, carfilzomib treatment also inhibited cancer progression in vivo, with reduced expression of N-cadherin.

To identify carfilzomib from the NPC chemical compound libraries, we conducted a qHTS using three ATC cell lines (8505C, C-643, and SW-1736). Proteasome inhibitors were among the most active drug categories. The ubiquitin proteasome pathway (UPP) is responsible for the degradation of eukaryotic cellular proteins and is an important regulator of cancer initiation and progression (Voorhees et al. 2003, Adams 2004). Bortezomib, the first inhibitor of the UPP to be used in clinical studies, has been extensively studied for both hematological and solid cancers, including thyroid cancer (Hideshima et al. 2001, Papandreou et al. 2004, Mitsiades et al. 2006, Altmann et al. 2012, Wunderlich et al. 2012). Results of recent preclinical studies have also indicated that bortezomib inhibits ATC cell proliferation and induces caspase-dependent apoptosis in vitro, with potent antitumor effects in vivo as well (Mitsiades et al. 2006, Conticello et al. 2007, Altmann et al. 2012, Wunderlich et al. 2012). The clinical efficacy of bortezomib, however, is challenged by the rapid development of drug resistance, as it is not an irreversible proteasome inhibitor like carfilzomib, and also by the dose-limiting neurotoxicity associated with bortezomib treatment (Ruckrich et al. 2009, Jain et al. 2011). Given these limitations, carfilzomib represents...
a potentially more attractive systemic agent against ATC. It is an irreversible inhibitor of the 20S proteasome, hence carfilzomib causes a more sustained inhibition of cellular proteasome activity, requiring new protein synthesis for functional recovery (Grolli et al. 2006, Demo et al. 2007). Consequently, carfilzomib has also been found to be far more cytotoxic, compared with bortezomib, in a variety of human tumor cell lines, with less toxicity to normal or nontransformed cells (Demo et al. 2007, Parlati et al. 2009). Furthermore, carfilzomib shows minimal activity against targets other than the chymotrypsin-like activity of the 20S proteasome, and is therefore better tolerated than comparable first-generation proteasome inhibitors that have significant cytotoxicity in normal cells at high concentrations (Demo et al. 2007, O’Connor et al. 2009). Our own pharmacokinetic analysis of carfilzomib further highlighted its advantages over bortezomib, including greater potency and/or efficacy and a wider therapeutic window. Furthermore, screening data comparing the antiproliferative effects of carfilzomib with ATC chemotherapeutic agents, such as doxorubicin and docetaxel, indicated that carfilzomib had greater efficacy and potency.

We found that carfilzomib has potent anticancer activities against ATC, inducing dose- and time-dependent growth inhibition that is associated with increased G2/M arrest. The cyclin-dependent-kinase inhibitor p27 is an important cell cycle regulator that plays an essential role in the induction and maintenance of G2/M phase arrest in response to anticancer drugs (Cuadrado et al. 2009). Results of recent studies have indicated that there is reduced expression of p27 in a variety of human cancer cell lines (Catzavelos et al. 1997, Yasui et al. 1997). Moreover, this decreased expression is believed to be mediated by increased proteasome degradation and not altered gene expression (Loda et al. 1997). Given these findings, it is not surprising that we found upregulation of p27 with carfilzomib treatment. This indicates that increased p27 expression probably promotes the observed arrest of ATC cells in G2/M phase but may not be the only cell-cycle regulatory protein that mediates this effect.

Induction of p27 expression and cell cycle arrest has also been demonstrated to play an important role in caspase-dependent apoptosis, though details of this mechanism remain unclear (Wang et al. 1997, Kudo et al. 2000). As expected, we observed an increase in caspase 3/7 activity in ATC cells following treatment with carfilzomib. Analysis of apoptosis-related protein expression revealed decreased expression of an anti-apoptotic protein, ATF4, with carfilzomib treatment. ATF4 belongs to a large family of basic region/leucine zipper transcription factors, and mediates the cellular hypoxic response to the unfolded protein response (Ameri & Harris 2008). The protective role of ATF4 during cellular stress has been well documented, activating genes that promote restoration of normal ER function and prosurvival pathways, such as autophagy (Ameri & Harris 2008, Rzymski et al. 2009, Jiang et al. 2013). Given that we observed a decrease in ATF4 expression with carfilzomib treatment, this indicates that carfilzomib promotes cell death by overcoming ATF4-mediated autophagy. Recent evidence has indicated that induction of ATF4 also plays a critical role in resistance to many chemotherapeutic agents, including bortezomib. By activating pathways like autophagy, ATF4 helps to relieve the protein overload in bortezomib-treated cells (Milani et al. 2009, Rzymski et al. 2009). By down-regulating ATF4 expression, carfilzomib appears to also overcome this ATF4-mediated chemoresistance.

When Cg-Ptkd<sup>cre-lcl</sup>Il2rg<sup>em1Wjl/SzJ</sup> mice with established ATC metastases were treated with carfilzomib (6 mg/kg), we saw a trend of tumor growth inhibition during a 3-week treatment period. Furthermore, treatment resulted in a statistically significant increase in overall survival. The natural history of ATC is defined by its propensity to invade and metasatize widely (Ain 1998, Besic & Gazic 2013). Consequently, we also examined the effect of carfilzomib on ATC progression using an <i>in vivo</i> pretreatment model. While the initial lung tumor burden on day 7 following inoculation was not significantly different between pretreated and vehicle groups, we did observe significantly fewer metastases over the subsequent 4-week treatment period. Finally, pretreated mice also displayed a significant increase in overall survival, probably a result of the decreased metastatic tumor burden associated with carfilzomib treatment.

Metastasis is associated with the loss of epithelial features and acquisition of mesenchymal properties, including increased invasiveness and resistance to apoptosis, through EMT (Chaffer & Weinberg 2011). Proteasome inhibition has been associated with both the induction and inhibition of EMT, depending on the specific human cancer cell line (Baritaki et al. 2009, Kim et al. 2011). However, to our knowledge, no study to date has examined the effect of proteasome inhibition on EMT in thyroid cancer. Given our <i>in vivo</i> pretreatment findings, we anticipated that proteasome inhibition would repress EMT. After treating ATC cells for 24 h with carfilzomib, we observed a dose-dependent decrease in expression of the EMT protein N-cadherin. These results may explain the decreased rate of distant...
metastasis observed in vivo following carfilzomib pre-treatment. On the basis of these findings, carfilzomib may also prove to be an effective therapy for ATC in the adjuvant setting.

In conclusion, we used qHTS to test 3282 different compounds against ATC and identified a second-generation proteasome inhibitor, carfilzomib, as one among the most active compounds. We have demonstrated that carfilzomib has strong antitumor effects in human ATC cells in vitro, inducing cycle arrest and caspase-dependent apoptosis. The antitumor activity of carfilzomib was further validated in vivo, using an ATC metastasis mouse model that recapitulates the clinical features of ATC. Thus, carfilzomib is a promising therapeutic agent for ATC.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

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