Targeting non-canonical pathways as a strategy to modulate the sodium iodide symporter

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Targeting non-canonical pathways as a strategy to modulate the sodium iodide symporter

Graphical abstract

Highlights
- YFP biosensor identifies FDA-approved drugs that increase intracellular iodide
- Proteostasis pathways central to control of NIS symporter activity are identified
- A 13-gene risk score classifier predictive of thyroid cancer recurrence is devised
- A model for targetable steps of intracellular processing of NIS is proposed

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In brief
Read et al. investigate the key druggable non-canonical pathways to recover function of the sodium iodide symporter (NIS). They identify mechanisms in NIS intracellular processing that could be exploited therapeutically for patients treated with radioiodide who typically have poorer clinical outcomes.

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Targeting non-canonical pathways as a strategy to modulate the sodium iodide symporter

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SUMMARY

The sodium iodide symporter (NIS) functions to transport iodide and is critical for successful radioiodide ablation of cancer cells. Approaches to bolster NIS function and diminish recurrence post-radioiodide therapy are impeded by oncogenic pathways that suppress NIS, as well as the inherent complexity of NIS regulation. Here, we utilize NIS in high-throughput drug screening and undertake rigorous evaluation of lead compounds to identify and target key processes underpinning NIS function. We find that multiple proteostasis pathways, including proteasomal degradation and autophagy, are central to the cellular processing of NIS. Utilizing inhibitors targeting distinct molecular processes, we pinpoint combinatorial drug strategies giving robust >5-fold increases in radioiodide uptake. We also reveal significant dysregulation of core proteostasis genes in human tumors, identifying a 13-gene risk score classifier as an independent predictor of recurrence in radioiodide-treated patients. We thus propose and discuss a model for targetable steps of intracellular processing of NIS function.

INTRODUCTION

For ~80 years, radioiodide (RAI) has been the central post-surgical treatment for patients with differentiated thyroid cancer (DTC). However, at least 25% of DTC patients have radioiodide-refractory thyroid cancer, being unable to uptake adequate radioiodide for effective therapeutic ablation (Schlumberger et al., 2014). This is especially problematic for DTC patients with metastatic disease who have a life expectancy of 2.5–3.5 years (Lirov et al., 2017). New therapeutic strategies are urgently required to address this clear unmet medical need with >43,000 lives lost worldwide to thyroid cancer per annum, which is estimated to rise to 74,733 by 2040 (Ferlay et al., 2019).

Sodium iodide symporter (NIS) is the sole transporter responsible for specific cellular iodide uptake (Dai et al., 1996); exploitation of its function permits specific targeting of high-energy β-emitting 131I to destroy remaining thyroid cells post-surgery, and target metastases. The central mechanisms that underlie radioiodide refractoriness are decreased levels of NIS expression and/or its diminished targeting to the plasma membrane (PM) (Spitzweg et al., 2001). The regulation of NIS is principally accomplished via transcription factors, histone acetylation, post-translational modifications, hormonal signaling, and by iodide itself (Arriagada et al., 2015; Passon et al., 2012; Ravera et al., 2017; Zhang et al., 2014). These levels of control are generally disrupted in cancer by a plethora of mechanisms, including altered histone acetylation and methylation of the NIS promoter (Passon et al., 2012; Zhang et al., 2014), distorted miRNA expression (Cancer Genome Atlas Research, 2014; Riesco-Eizaguirre et al., 2015), increased oxidative stress (Azouzi et al., 2017), and changed growth factor signaling (Knauf et al., 2011; Riesco-Eizaguirre et al., 2009). Thus, in addressing repressed NIS function in thyroid cancer, this inherent multiplicity of regulation adds significantly to the complexity of potential therapeutic strategies.

Extensive pre-clinical and clinical studies have attempted to enhance NIS expression and function, focusing mainly on “re-differentiation agents,” which stimulate the expression of thyroid-specific genes including NIS. An integral component is the canonical mitogen-activated protein kinase (MAPK) signaling
Figure 1. YFP-based biosensor strategy to identify drugs that increase intracellular iodide

(A) Schematic of high-throughput screening approach used to identify compounds that increase intracellular iodide. Created with BioRender.com. See also Figure S1A.

(B) Validation of YFP-iodide assay in TPC-1-NIS-YFP cells using increasing NaI (0–68 mM).

(C) TPC-1-NIS-YFP cells exposed to PBS or 60mM NaI for 300s and 1800s.

(D) Prestwick library of 1200 drugs: Increased NIS activity 50 drugs (ΔYFP > 2) 99 drugs (ΔYFP > 1.5) Decreased NIS activity 56 drugs (ΔYFP < 2) 96 drugs (ΔYFP < 1.5)

(F) Drug response curves for Demecarium, Clotrimazole, Terfenadine, and Niflumic acid.

(G) TPC-1-NIS cells treated with various concentrations of drugs and analyzed for iodide uptake.

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pathway which has a pivotal role in DTC, with ~70% of thyroid carcinomas (THCA{s}) driven by mutations that directly stimulate this pathway (Zaballos and Santisteban, 2017). Because constitutive activation of the MAPK pathway in thyroid cancer frequently represses NIS expression and function and is associated with decreased radioiodide uptake and poor patient prognosis, many studies have focused on MAPK inhibitors (Dunn et al., 2019; Ho et al., 2013). However, sustained inhibition of the MAPK pathway will be needed to maximize responses to radioiodide therapy and help overcome the comparatively ineffective suppression of oncogene-driven signaling by RAF and MEK inhibitors (Nagarajah et al., 2016). Other valuable approaches have addressed transcriptional and epigenetic alterations (Kita-zono et al., 2001; Mancikova et al., 2014), including the use of histone deacetylase (HDAC) inhibitors (Pugliese et al., 2013; Wachter et al., 2018). However, while some drugs have enhanced NIS expression in a subset of patients, new approaches are required to address the intricacy of altered NIS regulation in thyroid neoplasia.

Here, we utilized high-throughput screening (HTS) with the aim of identifying key targetable processes underlying NIS function. HTS of NIS function has been reported, but primarily to identify pollutants that disrupt NIS activity (Buckalew et al., 2020; Hallinger et al., 2017). Our study is therefore—to our knowledge—the first to directly screen and identify FDA-approved drugs capable of enhancing radioiodide uptake on a large pharmacological scale. Importantly, our findings reveal cellular processes that modify radioiodide uptake outside of the canonical pathways of NIS processing, leading to an improved mechanistic understanding of endogenous NIS function, which is subverted in cancer, as well as facilitating the identification of key target genes to construct an independent predictive risk model of recurrent thyroid cancer.

RESULTS

Drug screening identifies novel compounds that alter surrogate NIS activity

We first adapted and validated yellow fluorescent protein (YFP)-iodide sensing to an HTS of 1,200 drugs (Prestwick Chemical Library; 95% FDA approved) as outlined (Figures 1A–1C and S1A). Primary screening in TPC-1-NIS-YFP cells revealed that ~8% of drugs (99/1,200; ΔYFP > 1.5) quenched YFP fluorescence (Figures 1D and 1E) with good overall cell viability (70% viability for >90% of drugs; Figure S1B; Table S1). For 73 leading drugs we performed secondary screening at 10 different drug doses and compared YFP fluorescence in control TPC-1-YFP cells, as well as cell viability (Figures 1F and S1C). Overall, adjusting for cell viability and YFP-effects only, we ranked the top drugs that specifically dimmed YFP fluorescence as a marker of increased cellular iodide uptake (Table S2).

Impact of drugs on thyroid cancer and human primary cells

We tested 40 of our top drugs in radioiodide uptake assays in TPC-1 cells stably expressing NIS; in total 17 of these gave significant dose-dependent increases in 125I uptake (Figures 1G and S1D; Table 1). To investigate potential mechanisms, we initially assessed the impact of seven of these drugs on NIS protein expression in total cell lysates; three drugs (formoterol, pravastatin, and disulfiram) significantly increased NIS protein levels both in TPC-1 and 8505C cells stably expressing NIS (Figures 2A–2D). Importantly, increased NIS protein matched greater radioiodide uptake induced by these three drugs in 8505C-NIS cells (Figure 2E). Formoterol and pravastatin further induced NIS mRNA expression in TPC-1-NIS cells (Figure 2F). Thus, whereas formoterol and pravastatin act transcriptionally, disulfiram exerts posttranscriptional effects on NIS expression. In contrast, imatinib, niflumic acid, or vatalanib did not significantly alter NIS mRNA or protein expression, and their well-characterized inhibitory effects on ERK/MAPK signaling (Luo et al., 2015; Montor et al., 2018) may instead contribute toward enhancing radioiodide uptake.

TPC-1 and 8505C thyroid cancer cells have inherently dysregulated cellular signaling (Landa et al., 2019). We therefore examined whether drugs identified from our HTS would modulate endogenous NIS function in non-transformed human primary thyroid cells. Significant induction of radioiodide uptake (>1.5-fold) was apparent for 15 drugs, including chloroquine, disulfiram, formoterol, niflumic acid, and pravastatin (Figures 2G and S1E; Table 1). Of these, 13 drugs induced significant 125I uptake in thyroid cancer cells stably expressing NIS as well as in human primary thyrocytes, implicating targeting of pathways which control normal physiological NIS function (Table 1). Half-maximal effective concentration (EC50) values were broadly comparable between TPC-1-NIS cells and human primary thyrocytes (Figure 2H).

Categorization of drugs revealed a high proportion known to modulate proteostasis (20/50 drugs), including key endoplasmic reticulum-associated protein degradation (ERAD) processes (Figure 2; Table S1). To predict in more detail the pathways of NIS processing that might be targeted by our drug approaches, we next used the Connectivity Map hierarchical clustering from available drug data, which identified a large cluster strongly associated with genes implicated in the proteasomal pathway and unfolded protein response (Figure 3A; cluster 5). Strikingly, most drugs (Figure 3A; cluster 5) were associated with loss of function of valosin-containing protein (VCP), recently reported as a critical component of ERAD that increased NIS proteolysis, permitting less NIS to be trafficked to the PM (Fletcher et al., 2020). In keeping with this, seven drugs validated for enhancing 125I uptake in TPC-1-NIS cells (Table 1) were linked with inhibiting VCP, including ebastine and astemizole.
In addition, we established using NanoBiT that increasing doses of the VCP inhibitor CB5083 impacted directly on the stringency of the NIS: VCP interaction (Figure 3B).

Mechanistic interaction between VCP inhibitors and SAHA increases radioiodide uptake

Targeting HDAC activity along with VCP to maximize radioiodide uptake has not been investigated. Suberoylanilide hydroxamic acid (SAHA) is a well-characterized FDA-approved HDAC inhibitor, induces robust NIS mRNA expression in thyroid cells (Cheng et al., 2016; Puppin et al., 2005; Wachter et al., 2018), and was shown to improve radioiodide uptake in one of three patients enrolled in a phase 1 trial (Kelly et al., 2005). To better understand the mechanistic impact of putative VCP inhibitors, we next addressed the hypothesis that SAHA treatment of thyroid cancer cells would induce NIS mRNA expression, and that increased NIS protein might then “benefit” from inhibition of VCP via repressed ERAD to enhance radioiodide uptake. As expected, SAHA induced large dose-dependent increases in NIS protein in stable TPC-1 and 8505C cells, accompanied by marked increases in radioiodide uptake (Figures S1F–S1I). Importantly, co-treatment with SAHA and the VCP inhibitors ebastine (EBT), astemizole (AST), or clotrimazole (CLOT) (Segura-Cabrera et al., 2017) led to significant increases in radioiodide uptake compared with SAHA alone (Figures 3C and 3D). This was supported by findings for the established VCP inhibitors

<table>
<thead>
<tr>
<th>Table 1. Comparison of drug efficacy on radioiodide uptake</th>
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<tr>
<td><strong>A</strong> Drug (Mean value)</td>
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**B** Drug combinations

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Human thyrocytes</th>
<th>TPC-1-NIS</th>
<th>8505C-NIS</th>
<th>Parental TPC-1</th>
<th>Parental 8505C</th>
<th>Parental MDA-MB-231</th>
<th>Mean value</th>
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<td>NFA SAHA DSF NFA SAHA</td>
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<td>3.5</td>
<td>2.4</td>
<td>1.1</td>
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<tr>
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<td>N/A</td>
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<td>5.6</td>
<td>2.3</td>
<td>3.0</td>
<td>3.9</td>
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<td></td>
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<td>5.2</td>
<td>6.7</td>
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<td>2.8</td>
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</table>

A, Drugs ranked on mean radioiodide uptake in TPC-1-NIS cells and human primary thyrocytes at maximal biological effect.

B, Mean radioiodide uptake in multiple cell types using drug combinations as indicated. Drugs included: niflumic acid (NFA), chloroquine (CQ), disulfiram (DSF), imatinib (IMAT), ebastine (EBT), carebastine (CBT), astemizole (AST), clotrimazole (CLOT), selumetinib (SEL), and suberoylanilide hydroxamic acid (SAHA).

N/A, not applicable; NS, not significant.

aNumber of biological replicates performed for each drug treatment. Each biological replicate typically consisted of three to four technical replicates.

bDrug dose to achieve maximal radioiodide uptake. Astemizole data is compiled from treating human primary thyrocytes with two different doses (0.5 and 0.75 µM).

cMean fold increase in 125I uptake ± SEM for each drug relative to 0.1% DMSO as vehicle control, except for chloroquine (0.5% PBS), formoterol (0.2% DMSO), pravastatin (0.4% DMSO), and phenformin (0.25% ETOH).

dp values determined using the unpaired two-tailed t test.

eCells treated for 24 h before assaying 125I uptake, except for chloroquine in which cells were treated for 8 h.

(Table S3). In addition, we established using NanoBIT that increasing doses of the VCP inhibitor CB5083 impacted directly on the stringency of the NIS: VCP interaction (Figure 3B).
Figure 2. Targeting multiple processes that regulate NIS to enhance radioiodide uptake

(A) Western blot analysis of NIS expression levels in TPC-1-NIS cells treated with the indicated drugs for 24 h. Doses (μM) are indicated. (B) Quantification of NIS protein levels in (A).

(C and D) Same as (A) and (B) but NIS expression levels in 8505C-NIS cells.

(E) Radioiodide uptake of 8505C-NIS cells treated with the indicated drugs for 24 h.

(F) Relative NIS mRNA expression levels in TPC-1-NIS cells treated with the indicated drug for 24 h.

(legend continued on next page)
eyarestatin-1 (ES-1) and NMS-873, which reflected increases in NIS protein mediated via SAHA but also by the VCP inhibitors themselves (Figures S2A and S2B).

The ability of carebastine (CBT), the active metabolite of EBT (Fujii et al., 1997), to modulate NIS function has not been reported. Here, we found a dose-dependent effect of CBT on 125I uptake in TPC-1 stable cells (Figure 3E), with similar efficacy to its parent compound. To test whether the impact of CBT was simply a thyroid-specific phenomenon, we utilized breast cancer MDA-MB-231 cells lentivirally expressing NIS. CBT was similarly potent in stimulating radioiodide uptake, confirming that it targets a generic mechanism of NIS processing (Figure S2C).

Critically, CBT worked additively with SAHA to induce 125I uptake in parental TPC-1 cells which have low NIS expression typical of DTC cells (Figure 3F). Significantly greater radioiodide uptake was accompanied by the induction of NIS mRNA and protein by SAHA but not CBT (Figures 3G and S2D). Protein levels of VCP, a highly abundant cellular protein (Meyer and Weihl, 2014), were unaltered by either treatment (Figure S2E). In contrast, CBT worked additively with SAHA to induce radioiodide uptake in parental breast cancer MDA-MB-231 and MCF7 cells without affecting NIS mRNA or VCP protein (Figures S2F–S2K). Importantly, both SAHA and SAHA + CBT induced NIS protein in parental MCF7 cells (Figure S2L).

To determine to what extent the action of CBT and SAHA were dependent upon VCP, we next depleted VCP in TPC-1-NIS cells. Both VCP siRNA and SAHA increased 125I uptake, with a maximal effect when both were combined (Figure 3H). However, CBT was unable to induce 125I uptake when VCP was depleted, whereas SAHA retained its induction of NIS activity (Figures 3I and S3I), with no significant change in VCP protein levels (Figures 3I and S3I). In contrast, NIS mRNA levels were not altered by VCP siRNA or CBT but were altered by SAHA (Figure 3J). In control experiments, CBT did not affect siRNA ablation of VCP mRNA (Figure 3K), and VCP depletion similarly abrogated the CBT effect on NIS function in breast MDA-MB-231-NIS cells (Figures S2M and S2N). Furthermore, in contrast to the well-established autophagy inhibitor bafA1, CBT did not affect LC3B-II or p62 levels (Figures 3L, S3A, and S3B), suggesting that it acts independently of autophagy.

Collectively, these data support the hypothesis that the additive effects on radioiodide uptake arise from SAHA’s induction of NIS expression, and VCP inhibitor blockade of VCP-dependent proteasomal pathways, which control the subsequent protein processing of NIS.

**Autophagy inhibitors enhance radioiodide uptake in multiple cell types**

We next sought to investigate the mechanism(s) underlying the significant impact of chloroquine and disulfiram on radioiodide uptake. Both drugs have been associated with modulating autophagy in cancer settings (Chude and Amaravadi, 2017; Zhang et al., 2019). First, we demonstrated that the autophagy inhibitor bafA1 enhanced NIS expression and 125I uptake in thyroid cells (Figures S3C–S3E), as well as increasing expression of autophagy markers (Figures 3L, S3A and S3B). Similarly, chloroquine induced LC3B-II, accompanied by a time-dependent increase in NIS protein in total cell lysates (Figure 4A), with radioiodide uptake peaking at 8 h post-treatment (Figures 4B and 4C). In contrast, maximal 125I uptake and NIS protein levels occurred later with disulfiram at 24 h (Figures S3F and S3G). Disulfiram also did not impact LC3B-II (Figure S3H), but increased p62 expression, which may be indicative of its ability to inhibit the VCP/proteasomal machinery (Lovborg et al., 2006; Skrott et al., 2017). Disulfiram also failed to impact the stringency of the NIS/VCP interaction using NanoBiT (Figure S3I) and retained the ability to enhance NIS function in VCP-ablated 8505C-NIS cells (Figures S3J and S3K), confirming that its effect on NIS is via targeting of VCP-independent proteasomal pathways.

We next progressed to determine whether combining drugs with distinct modes of action on NIS processing, such as chloroquine and disulfiram with SAHA, might give a robust increase in radioiodide uptake. Importantly, chloroquine doubled the impact of SAHA on 125I uptake in TPC-1-NIS cells (~8.3-fold; Figure 4D), without additional upregulation of NIS mRNA expression (Figure 4E). Virtually identical data were apparent in 8505C cells (Figure 4F). As a control, we inhibited BRAFV600E activity via vemurafenib, which approximately doubled radioiodide uptake in BRAFV600E-mutant 8505C cells (Figure 4G). Chloroquine did not enhance the action of vemurafenib further, whereas co-treatment of chloroquine and SAHA resulted in a much more potent 5.6-fold effect. Again, the predominant mechanistic impact was induction of NIS protein expression (Figure 4H).

The impact of SAHA alone was relatively less effective in human primary thyrocytes (~1.6-fold increase) than in TPC-1 cells (Figure 4I), which likely reflects reduced epigenetic repression of NIS mRNA in non-transformed thyrocytes compared with transformed thyroid cells (Passon et al., 2012; Zhang et al., 2014). An important finding from our combination strategies, however, was that chloroquine and SAHA retained a robust and additive 4.3-fold effect on radioiodide uptake in primary cells, which was greater than chloroquine alone (Figure 4J). By comparison, there was no additive effect from combining disulfiram with SAHA or chloroquine (Figure 4K). The highest fold induction of radioiodide uptake from combinational drug screening in non-transformed primary thyroid cells came from chloroquine plus niflumic acid (7.3-fold; Figure 4L).

Full drug combination data (Table 1) revealed that the best overall strategies that enhanced radioiodide uptake across at least four different cell types were chloroquine + SAHA (5.3-fold), CBT + SAHA (9.6-fold), and EBT + SAHA (6.4-fold). The majority of drugs acted in cooperation with SAHA (7/8, 87.5%; p < 0.05).
Figure 3. Carebastine inhibits VCP to enhance NIS function and radioiodide uptake

(A) Connectivity Map (L1000 assay) hierarchical cluster analysis comparing the transcriptional signatures of 47 drug treatments with 45 genetic perturbations. Blue, major clusters; green, validated drugs.

(B) NanoBiT evaluation of protein:protein interaction between VCP and NIS (left). Normalized NanoBiT assay results at 10 min after addition of Nano-Glo live cell assay solution to HeLa cells treated with VCP inhibitor CB5083 for 24 h (right, n = 4).

(C and D) Radioiodide uptake (C) and relative NIS protein levels (D) in TPC-1-NIS cells treated with VCP inhibitors (ebastine [EBT], clotrimazole [CLOT], and astemizole [AST]), either alone or in combination with SAHA.

(E) Radioiodide uptake of TPC-1-NIS cells treated with carebastine (CBT) at indicated doses versus EBT. See also Figure S2 C.
versus SAHA alone), with most sharing a common target in modulating NIS processing (i.e., ERAD/ubiquitin-proteasome and autophagy-lysosome pathways). Control sodium perchlorate treatments clearly demonstrated that radiiodide uptake was NIS specific in drug-treated TPC-1-NIS cells (Figure 4M) and primary thyrocytes (Figure 4N).

**Clinically relevant proteostasis drug targets are associated with recurrence in RAI-treated patients**

Having identified drug strategies that robustly enhance 125I uptake with stasis genes significantly correlated with an increased risk of recurrence and/or RAI treatment are both highly indicative of significantly more advanced PTC (Figure 5I).

All 13 core proteostasis genes were associated with a significant reduction in disease-free survival following stratification of RAI-treated PTC into subgroups of high versus low tumoral expression with optimal cutoffs determined by receiver operating characteristic analysis (Figures 5D and S5A–S5C). Stratification of PTC using percentile cutoff values further validated the influence of each proteostasis gene on recurrence, especially with higher VCP expression (Figures 5E and S5D). Univariate Cox regression analysis (Figure S5B) indicated that all 13 proteostasis genes significantly correlated with an increased risk of recurrence in RAI-treated BRAF-like PTC (p < 0.05; Figure 5F; Table S5) and were therefore all considered to construct the prognosis risk model.

Subsequently risk scores were calculated using multivariate Cox regression coefficient and fragments per kilobase transcript per million mapped reads expression values (Figure S6A). Importantly, a higher area under the curve (AUC) of 0.842 (Figure S5G) indicated a greater prediction effect for the 13-gene-based risk score compared with individual genes (AUC = 0.652–0.726; Figure S5A). In agreement, stratification into risk groups demonstrated that patients at high risk had a significantly worse prognosis (hazard ratio = 35.862; 95% confidence interval: 4.81–267.405; Figures S5H and S6B) at significance levels several orders of magnitude greater than individual genes (Figure S5C). By comparison, there was no difference in the prognosis of non-RAI-treated patients stratified into risk groups (Figure 5).

After controlling for age, gender, disease stage, tumor stage, and node status, multivariate analysis showed that the 13-gene risk score remained the sole independent predictive factor for the entire group of THCA patients, as well as for the RAI-treated, BRAF-like and RAI-treated cohorts (Tables S6 and S7). The performance of the 13-gene risk score classifier was also superior to VCP, which did not retain its predictive value in other THCA cohorts (Table S7). Consistent with TCGA, there was extensive dysregulation of proteostasis genes in PTC with BRAF or RAS mutations in the independent GEO dataset GSE27155 (Figures S6C–S6F), as well as the primary anaplastic GSE76039 dataset (Figure S7A). Critically, the 13 proteostasis gene set lacked any strong association with aggressive tumor types with only heat shock 70 kDa protein 5 (HSPA5) common in 3 datasets (Figures S7A–S7C), further supporting that its predictive value would be defined predominately by molecular influences affecting RAI therapy. Subsequent validation also showed a greater predictive value in RAI-treated PTC against seven biomarkers of thyroid cancer recurrence (Cheng et al., 2011; Khoo et al., 2002) (Figures S7D and S7E).

Collectively, we have identified core proteostasis genes that are significantly dysregulated in PTC and associated with poorer survival characteristics, especially in patients treated with radiiodide. In support of this, our drug screening data particularly highlight mechanisms of proteostasis as being critical to NIS function. We therefore hypothesize that dysregulation of specific proteostasis genes in PTC leads to abrogation of NIS localization at the PM, as demonstrated recently for VCP and ADP-ribosylation factor 4 (Fletcher et al., 2020). Based on our collective drug screening, experimental manipulations, and clinical gene expression analyses, we therefore propose a model for the key targetable steps of intracellular processing of NIS expression and function in PTC (Figure 6).

**DISCUSSION**

New approaches to better understand and identify key targetable processes that govern NIS function are urgently needed.
Figure 4. Drug combinations targeting non-canonical pathways augment NIS function
(A) Western blot analysis of LC3B-I, LC3B-II, p62, and NIS protein levels in TPC-1-NIS or 8505C-NIS cells treated with chloroquine (CQ).
(B and C) Radioiodide uptake in TPC-1-NIS (B) or 8505C-NIS (C) cells treated with CQ at different time points.
(D) 8505C-NIS or TPC-1-NIS cells treated with different combinations of drugs and their effects on radioiodide uptake
(F and G) Comparison of LC3B-II and NIS protein levels in 8505C-NIS cells treated with different drugs
(H) Beta-actin levels in 8505C-NIS cells treated with different combinations of drugs
(I) Radioiodide uptake in human primary thyrocytes treated with different concentrations of SAHA
(J and K) Radioiodide uptake in human primary thyrocytes treated with different combinations of drugs and SAHA
(M) TPC-1-NIS or 8505C-NIS cells treated with different concentrations of SAHA and NaCl0, and their effects on radioiodide uptake
(legend continued on next page)
to improve the efficacy of radioiodide therapy and diminish recurrence of thyroid cancer. HTS of NIS function has been reported before, but primarily for toxicology purposes. One previous study used HTS of the NIS promoter to detect drugs that increased NIS expression (Oh et al., 2018). However, our study directly “drugged” NIS function. Of particular significance, we illustrate that pathways entirely separate to the canonical MAPK regulation of NIS exist, which can be drugged to enhance radioiodide uptake.

A significant advantage of HTS was that it enabled the objective evaluation of drugs targeting multiple cellular processes that regulate NIS. For example, we identified that formoterol and pravastatin directly increase NIS mRNA expression, while carebastine, a second-generation antihistamine, inhibited VCP to enhance radioiodide uptake. The importance of VCP and other critical components of proteostasis pathways was further highlighted by the abundance of putative proteasomal inhibitors identified by HTS that were subsequently validated to enhance radioiodide uptake. For instance, disulfiram—a prominent drug in our study—when combined with VCP to enhance the proteasome (Lovborg et al., 2006) and has been associated with the accumulation of ubiquitylated proteins in esophageal squamous cell carcinoma (Jivan et al., 2018). We thus propose that VCP and related proteasomal pathways represent key targetable processes to modulate NIS function in thyroid cancer. In agreement, the appraisal of TCGA and GEO datasets revealed profound dysregulation of core proteostasis genes, including those involved in proteasomal degradation. Together, these findings support our hypothesis that increased expression of VCP and multiple components of the proteasome in PTC enhance the degradation of NIS, leading to reduced radioiodide uptake.

Autophagy has been associated with NIS function (Chai et al., 2019), although the precise mechanism of how this might regulate NIS remains obscure. In thyroid cancers, markers of increased autophagy correlate with higher levels of plasma memranous NIS, and better clinical outcome (Plantinga et al., 2016). Our data clearly show that well-established inhibitors of autophagy, such as chloroquine and batimontacin, induce significant increases in iodide uptake in transformed and untransformed thyroid cells, accompanied by increased classical autophagy markers, suggesting that autophagy is critical to the central processing of NIS. VCP has been reported to be essential for autophagosome maturation (Tresse et al., 2010), and hence it is possible that autophagosome maturation or processes downstream of it, rather than increased autophagy per se, are the more pertinent pathways to NIS drugability.

An alternative pathway might involve endosomes recycling NIS away from the PM, a process described previously by ourselves and others (Fletcher et al., 2020; Smith et al., 2019, 2013). Endosomes are known to fuse with autophagosomes as part of lysosomal degradation. Autophagy selectively degrades targets and contributes to intracellular homeostasis (Kawabata and Yoshimori, 2018). Drugs that interfere with autophagosome initiation, maturation, or fusion with lysosomes, such as chloroquine, might all potentially result in NIS-bearing endosomes not being degraded, and hence subsequently increased intracellular NIS protein levels. The ability of autophagy inhibition to modulate trafficking of NIS-containing endosomes and the possibility of NIS recycling to the PM to expedite iodide uptake warrants further investigation.

New drug strategies, such as combining BRAF or MEK inhibitors with pan-PI3K inhibitors, are beginning to show pre-clinical promise in thyroid cancer (Nagarajah et al., 2016). However, we propose that drug combinations targeting pathways outside those canonical signaling pathways may be capable of augmenting NIS expression, processing, and trafficking to the PM to boost the efficacy of radioiodide therapy. We identified at least three drug combinations (i.e., ebastine + SAHA, carebastine + SAHA, or chloroquine + SAHA) which target non-canonical pathways and represent realistic drug strategies in thyroid cancer with favorable side effect profiles. Effective drug doses for ebastine, carebastine, and SAHA to promote radioiodide uptake were comparable with mean plasma concentrations that can be safely achieved in patients (Dickson et al., 2011; Yamaguchi et al., 1994). The maximal effective concentration of chloroquine in cells was relatively high at 50 μM, with an EC50 of 10.14 μM in human primary thyroctyes. Given that the concentration of chloroquine in tissues can be many times greater than in plasma due to accumulation in cell structures (Adelusi and Salako, 1982), it will be important to determine if sufficient distribution of chloroquine in thyroid tissue can be achieved using standard doses. Further studies will be needed to investigate the in vivo efficacy of these drugs in combination to determine the precise timing and order of drug administration to maximize recovery of radioiodide uptake. In agreement with our findings with carebastine in MDA-MB-231 and MCF7 cells, these drug strategies also have potential for broader application to boost NIS function in non-thyroidal settings, including the radioiodide treatment for breast cancer in which NIS expression is typically induced in ~70%-80% of cases (Tazebay et al., 2000).

Despite extensive studies, there are no molecular biomarkers currently in clinical use for predicting recurrence. Here, an important clinical observation was the striking correlation with recurrence in PTC and the dysregulation of core proteostasis genes, including those involved in proteasomal degradation and autophagy. Via bioinformatic analyses we identified the most clinically relevant core proteostasis genes associated with a poor radioiodide response in recurrent thyroid cancer and...
Figure 5. A 13 proteostasis gene risk score classifier is predictive of thyroid cancer recurrence

(A) Volcano plot comparing log₂FC with q value (–log base 10) for 142 core proteostasis genes in the THCA cohort (C versus N). Gene categories indicated by colored spots. (Right) Stacked bar chart indicates frequency of altered expression; Fisher’s exact test (NS, not significant; *p < 0.05, **p < 0.01).

B) THCA (n = 501): Proteostasis genes

C) THCA: BRAF-like, RAI-treated REC (n = 21) v NON-REC (n = 116)

D) THCA (BRAF-like, high v low expression)

E) THCA (BRAF-like, Non-RAI treated)

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constructed a 13-gene predictive risk model. Critically, the 13-gene risk score classifier yielded higher specificity and sensitivity than single gene biomarkers, as well as being the sole independent predictive factor for recurrence. Based on these observations, we envisage that the 13 proteostasis gene classifier represents a promising biomarker for predicting thyroid cancer recurrence, especially in more aggressive thyroid cancers requiring radioiodide. Further validation will now be required using larger independent clinical datasets, which are not yet available in public data repositories.

Overall, we report a large-scale screen aimed at enhancing NIS activity and identifying cellular processes that impact radioiodide uptake. Our findings demonstrate that cellular processing of NIS involves VCP and suggest that the proteasome is an integral regulatory pathway of NIS activity given the marked dysregulation of proteostasis genes in PTC. Notably, combinatorial drug strategies targeting proteostasis networks and HDAC revealed that significant induction of NIS function is routinely possible in vitro, with translatable potential to address the lack of therapeutic options for patients treated with radioiodide who typically have poorer clinical outcomes.

SIGNIFICANCE

The sodium iodide symporter (NIS) is the sole conduit for cellular iodide uptake. Dysregulation of NIS function is common in thyroid cancer, significantly impacting therapeutic radioiodide ablation of tumor and metastatic cells. Here, we summarize our findings on the use of NIS as a drug target to both understand the full gamut of mechanisms that underpin NIS function and to transform the efficacy of radioiodide treatment. Our study yielded critical insights into cellular processes central to NIS regulation, including proteasomal degradation and autophagy. Dose-dependent increases in drug effects were apparent across multiple cancer cell models, as well as human primary thyrocytes, implying that proteostasis pathways are central to the innate control of NIS function. Exploiting these mechanistic insights, several drugs—which target pathways entirely separate to the canonical MAPK regulation of NIS—gave robust increases in radioiodide uptake when combined. There are currently no molecular biomarkers in clinical use for predicting thyroid cancer recurrence. We also show significant perturbation of proteostasis genes in thyroid cancer and identify a 13-proteostasis gene risk score classifier as an independent predictor of recurrence in radioiodide-treated patients. Collectively, we propose a model for the targetable steps of intracellular processing of NIS function, with translatable potential to address the current lack of clinical options for those with aggressive thyroid cancer. This work will be of relevance to a broad readership in academia, medicine, and the pharmaceutical industry given the potential application of our findings across a wide-ranging disease spectrum.

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at [https://doi.org/10.1016/j.chembiol.2021.07.016](https://doi.org/10.1016/j.chembiol.2021.07.016).

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**AUTHOR CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


Selumetinib-enhanced radioiodine uptake in advanced thyroid cancer.


tyrosine kinase inhibitor (K905-0266) excavated by high-throughput NIS (sodium iodide symporter) enhancer screening platform using dual reporter gene system. Oncotarget 9, 7075–7087.


**STAR METHODS**

**KEY RESOURCES TABLE**

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### Reagents or Resources

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### Deposited Data

- **The Connectivity Map (CMAP)**
  - Subramaniam et al., 2017
  - https://clue.io/touchstone

- **GSE27155**
  - Giordano et al., 2005

- **GSE76039**
  - Landa et al., 2016

- **TCGA GDAC Firehose standard data – Thyroid Carcinoma (THCA)**
  - Broad Institute of MIT and Harvard
  - Firehose stddata__2016_01_28 run.
  - https://doi.org/10.7908/C11G0KM9

- **TCGA THCA**
  - Grossman et al., 2016

### Experimental Models: Cell Lines

- **TPC-1**
  - Rebecca Schwegpée’s lab
  - N/A

- **TPC-1-YFP**
  - This paper
  - N/A

- **TPC-1-NIS-YFP**
  - This paper
  - N/A

- **8505C**
  - DSMZ
  - Cat# ACC-219, RRID:CVCL_1054

- **8505C-YFP**
  - This paper
  - N/A

- **8505C-NIS-YFP**
  - This paper
  - N/A

- **MDA-MB-231**
  - ECACC
  - Cat# 92020424, RRID:CVCL_0062

- **MCF7**
  - ECACC
  - Cat# 86012803, RRID:CVCL_0031

- **MDA-MB-231-NIS**
  - Fletcher et al., 2020
  - N/A

- **HeLa**
  - ECACC
  - Cat# 93021013, RRID:CVCL_0030

### Oligonucleotides

- **MISSION VCP siRNA**
  - Sigma-Aldrich
  - Cat# NM_007126

- **LgBiT (Forward)5′-GGCGGTACCACCACCATGCCAG-3′**
  - Sigma-Aldrich
  - Custom synthesis

- **LgBiT (Reverse)5′-CCGCAGCTTGGCTGTGGTCAATCT-3′**
  - Sigma-Aldrich
  - Custom synthesis

- **VCP (Forward)5′-CCCGAGCTTGGACAGACCTCT-3′**
  - Sigma-Aldrich
  - Custom synthesis

- **VCP (Reverse)5′-CCCGAGCTACCTGGCTGTGGTCAATCT-3′**
  - Sigma-Aldrich
  - Custom synthesis

- **NIS-SmBiT (Forward)5′-GACGGCACTCAATATCT-3′**
  - Sigma-Aldrich
  - Custom synthesis

- **NIS-SmBiT (Reverse)5′-GGCGGTACCATGGGTACTGTTG-3′**
  - Sigma-Aldrich
  - Custom synthesis

### Recombinant DNA

- **pcDNA3.1-EYFP-H148Q/I152L/F46L**
  - Alan Verkman’s lab
  - N/A

- **pcDNA3.1-NIS**
  - Smith et al., 2009
  - N/A

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the lead contact, Christopher McCabe (mccabcjz@bham.ac.uk).

Materials availability
There are restrictions to the availability of cell lines (TPC-1, 8505C) and recombinant DNA (pcDNA3.1-EYFP-H148Q/I152L/F46L) due to material transfer agreement terms.

Data and code availability
The datasets generated in this study have been deposited at Mendeley Data and are publicly available as of the date of publication. DOI is listed in the key resources table. This paper also analyzes existing, publicly available data. Identifiers for these datasets are listed in the key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

REAGENT or RESOURCE SOURCE IDENTIFIER
Halo-tagSmBiT (negative control) Caroline Gorvin’s lab N/A
LgBiT-PRKAR2A (positive control) Caroline Gorvin’s lab N/A
SmBiT-PRKACA (positive control) Caroline Gorvin’s lab N/A
pcDNA3.1-LgBiT-VCP This paper N/A
pcDNA3.1-NIS-SmBiT This paper N/A

Software and Algorithms

GraphPad Prism Version 9 Graphpad Software Inc https://www.graphpad.com/scientificsoftware/prism/
BioRender BioRender https://biorender.com/
EC50 Calculator AAT Bioquest https://www.aatbio.com/tools/ec50-calculator
GEO2R National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/geo/geo2r/
DynaVenn Chair for Clinical Bioinformatics at Saarland University https://ccb-compute.cs.uni-saarland.de/dynavenn

Other

Opera Phenix High-Content Screening System Perkin Elmer Cat# HH14000000
PHERAspec FS microplate reader BMG Labtech https://www.bmglabtech.com/pheraspec-fs/
POLARstar Omega microplate reader BMG Labtech https://www.bmglabtech.com/polarstar-omega/
7500 Real-Time PCR system ThermoFisher Scientific Cat# 4351105
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
Sources of cell lines
TPC-1 cells were kindly provided by Dr Rebecca Schweppe (University of Colorado, Denver, USA). Other cell lines were obtained from DSMZ (Brunswick, Germany) and ECACC (Salisbury, UK). Human thyroid carcinoma cells TPC-1 and 8505C, and human breast cells MDA-MB-231 and MCF7 were cultured in RPMI-1640 (Life Technologies), and HeLa cells were cultured in DMEM Medium (Life Technologies), supplemented with penicillin (105 U/L), streptomycin (100 mg/L) and 10% FBS (Life Technologies). All cell lines were maintained at low passage, authenticated by short tandem repeat analysis (NorthGene), and tested for mycoplasma contamination (EZ-PCR; Geneflow).

Generation of reporter cell lines
Thyroidal reporter cell lines were made using EYFP-H148Q/I152L/F46L subcloned into pcDNA3.1 (Invitrogen, Hygromycin B-resistant; provided by Peter Haggie and Alan Verkman, UCSF) or full-length human NIS cDNA subcloned into pcDNA3.1 (Invitrogen, Geneticin-resistant; described in Smith et al., 2009). Stable TPC-1-YFP and 8505C-YFP cells were generated by transfection of parental cells with EYFP-H148Q/I152L/F46L/pcDNA3.1. Then Hygromycin B (Invitrogen, 150 μg/ml) was added to the media as a selection marker after 48 hours of transfection. Hygromycin B-resistant monoclonal colonies (i.e., 1 cell per well) were expanded following FACS single cell sorting (University of Birmingham Flow Cytometry Facility), and colonies with YFP fluorescence resolved by flow cytometry. Stable TPC-1-NIS-YFP and 8505C-NIS-YFP cells were generated by transfection of TPC-1-YFP or 8505C-YFP cells with NIS/pcDNA3.1. Geneticin (TPC-1-YFP cells, 300 μg/ml; 8505C-YFP cells, 500 μg/ml) was added to the media as a selection marker after 48 hours of transfection. Geneticin-resistant monoclonal colonies were expanded, and qPCR used to confirm NIS mRNA expression. Modified characteristics of stable cell lines were further verified by radioiodide (125I) uptake assays and Opera Phenix™ live cell imaging (EvoTec, Germany). Generation of stable NIS-expressing Breast MDA-MB-231 cell lines by lentiviral transduction has been described in detail previously (Fletcher et al., 2020).

Human primary thyroid tissue
The collection of normal human thyroid tissue was approved by the Local Research Ethics Committee (Birmingham Clinical Research Office, Birmingham, UK), and subjects gave informed written consent. No age/gender information was available as human subjects were anonymised and tissue collected as excess to surgery as part of our ethics agreement. To prepare primary thyrocyte cultures, thyroid tissue was homogenised and incubated overnight at RT with end-over-end-rotation in 0.2% collagenase (Worthington Biochemical Corporation) in HBSS (Sigma-Aldrich). Digested thyroid tissue was pelleted at 300 g for 10 minutes, which was washed 8x in HBSS by centrifugation at 300 g for 3 minutes. The pellet was re-suspended in Coon’s F-12 modified liquid medium (with 2.5 g/l NaHCO3, without L-glutamine) (Biochrom; Cambridge, UK) supplemented with 4% FBS, 1% L-glutamine (200 mM), penicillin (105 U/L), streptomycin (100 mg/L), thyroid-stimulating hormone (TSH) (0.3 U/L) (Sigma-Aldrich) and insulin (300 μg/L) (Sigma-Aldrich). Cells were then plated and maintained at 37 °C and 5% CO2 within a humidified environment. After at least 48 hr, FBS was omitted and cells cultured for approximately 6 days.

As a marker of thyroid function, the TSH-responsiveness of each batch of primary thyrocytes was verified. In brief, 3 days after cell plating, Coon’s F-12 medium was replaced with FBS-free and TSH-free Coon’s F-12 medium. After a further 3 days, medium was changed again to FBS-free Coon’s F-12 medium containing TSH at 0 U/L, 0.3 U/L, 1 U/L or 10 U/L. On day 11, radioiodide uptake assays were performed to measure NIS function. Thyroid samples that responded to 0.3 U/L TSH were used for downstream analyses.

METHOD DETAILS

Drug screening strategy
High-throughput drug screening was performed via an automated biochemical screening platform using the Microlab STAR Liquid Handling Robotic System (Hamilton) integrated with a PHeraStar FS microplate reader (BMG Labtech; 510 nm-550 nm optic module). In brief, TPC-1 cells expressing NIS and halide-sensitive YFP were seeded into 96-well tissue culture plates at a density of 15,000 cells/well (black-walled, clear-bottom), using plates pre-loaded with test compounds. ~1200 FDA-approved compounds (Prestwick Chemical Library) were used for the primary high throughpout screen at a single dose (10 μM; 1.25% DMSO). All plates contained positive (34 mM NaI) and negative (PBS) controls. The assay was performed using the PHeraStar FS microplate reader at 37 °C with orbital averaging, 20 flashes/well and focal height kept constant throughout. The initial YFP fluorescence intensity was recorded prior to injection of sodium iodide (NaI) at a final concentration of 4 mM. YFP fluorescence measurements were taken by the PHeraStar FS microplate reader every 3 minutes over 4 cycles and ΔYFP values calculated. A secondary multi-dose screen was performed using the same protocol to validate 73 drugs at 10 different doses (0.1 – 50 μM) in two YFP-expressing cell lines (i.e., TPC-1-NIS-YFP and TPC-1-YFP).

The Z’ factor was used to validate the suitability of the YFP-iodide assay for high throughpout drug screening. For this, the YFP fluorescence of TPC-1-NIS-YFP cells was quenched with different NaI concentrations (0-68 mM) and Z’ factor values calculated using the following equation: $Z' = 1 - \frac{3\text{SD}_{\text{p}} + \text{SD}_{\text{n}}}{\text{Mean}_{\text{p}} - \text{Mean}_{\text{n}}}$. Where Meanp is the mean of the positive control, Meann is the mean of the negative control, SDp is the standard deviation of the positive control, and SDn is the standard deviation of the negative control.
We used ΔYFP, area under the curve (AUC), EC50 and radioiodide \(^{125}\text{I}\) uptake values to rank the efficacy of drugs to increase intracellular iodide levels. AUC values were determined (log\(_{10}\) scale; GraphPad Prism) for positive peaks only. AUC values for drug-treated TPC-1-YFP cells were subtracted from corresponding values in TPC-1-NIS-YFP cells to determine ΔAUC values. EC50 values were calculated using EC50 calculator [AAT Bioquest, Inc. (2020, April 08)].

### Inhibitors/ drugs used

Drugs used to treat cells in radioiodide uptake assays are listed in the key resources table. Chloroquine diphosphate was resuspended in PBS without calcium/magnesium (Thermo Fisher); Phenformin HCL in 100% ethanol and all other drugs in dimethyl sulfoxide (DMSO; Sigma-Aldrich), before being diluted in RPMI-1640 medium (Life Technologies) and added to cells at 1:100 dilution. Drugs reported to enhance NIS function and used as positive controls were DBeQ (Sigma-Aldrich), Dynasore (Sigma-Aldrich), Eeyar-estatin-1 (Cayman Chemicals), NMS-873 (SelleckChem), Selumetinib (SelleckChem) and Vemurafenib (SelleckChem).

### Cell viability assay

Cells seeded in 96 well plates were incubated with alamarBlue (0.02%; resazurin) solution for 1 hour and absorbance measured using the POLARstar Omega plate reader (BMG Labtech; 572-583 nm optic module) according to manufacturer’s protocols.

### Determination of ΔYFP values

ΔYFP values signify changes in intracellular iodide (i.e., positive ΔYFP values denote increased intracellular iodide) and were derived relative to standard deviation (SD) of YFP fluorescence of DMSO-treated TPC-1-NIS-YFP cells (n = 120 wells). Candidate drugs were considered top hits in the primary high throughput screen at ΔYFP values > 1.5-2. Raw YFP fluorescence measurements were normalised using an interquartile mean well-based method (IQM\(_m\)) (Mangat et al., 2014), according to the following equations:

\[
%\text{YFP} = \frac{\text{YFP}_0 - \text{YFP}_4}{\text{YFP}_0} \times 100
\]

(Equation 1)

\[
S_{\text{IQM}} = \frac{%\text{YFP}}{h_Q}
\]

(Equation 2)

\[
S_{\text{IQMW}} = \frac{S_{\text{IQM}}}{h_{\text{IQW}}}
\]

(Equation 3)

\[
\Delta\text{YFP} = \frac{S_{\text{IQMW}}(\text{DRUG}_x) - S_{\text{IQMW}}(\text{DMSO}_x)}{SD_{\text{IQMW}}(\text{DMSO}_n)}
\]

(Equation 4)

[\(\Delta\text{YFP} = \text{Fold-change in YFP fluorescence for drug treatment wells relative to SD of YFP fluorescence in DMSO control wells; } S_{\text{IQMW}}(\text{DRUG}_x) = \text{Sample value after IQM normalization for each drug treatment well; } S_{\text{IQMW}}(\text{DMSO}_x) = \text{Sample value after IQM normalization for plate matched DMSO control wells; } SD_{\text{IQMW}}(\text{DMSO}_n) = \text{SD of sample values after IQM normalization for DMSO control wells across the entire screen, n = 120}.\)]

### Radioiodide uptake assay

Cells were seeded in 24-well plates with at least three biological replicates per condition, and then treated with drugs and/or transfected with siRNA. Cells were then incubated for 1 hour with 0.05 μCi iodine-125 \(^{125}\text{I}\) (Hartmann Analytic; Folkestone, UK) at a final concentration of 10\(^{-7}\) moles/l NaI. Following incubation, cells were washed twice in HBSS (Sigma-Aldrich) to remove unincorporated \(^{125}\text{I}\) and lysed in 100 μl 2% SDS. Radioiodide uptake was determined using the Berthold Gamma Counter (Berthold) to measure gamma radiation (counts per minute). Protein concentrations were determined using the Pierce\textsuperscript{TM} BCA colorimetric assay (ThermoFisher Scientific). \(^{125}\text{I}\) uptake relative to protein concentration was calculated in picomoles of \(^{125}\text{I}\)/ μg protein, according to the following equation:

\[
\text{Picomoles of } ^{125}\text{I}/ \mu\text{g protein} = \frac{\text{5000 + (Counts/Total Protein)}}{12000}
\]
In control experiments, cells were treated with 100 μM sodium perchlorate for 1 hour at 37°C and 5% CO₂ prior to the assay to inhibit NIS-mediated radiodiode uptake.

**Immunobots**

For western blotting, primary antibodies were used against LC3B (1:1000; Cell Signalling Technology, cat# 2775), NIS (1:1000; Proteintech, cat# 24324-1-AP), p62/SQSTM1 (1:4000; Proteintech, cat# 18420-1-AP), VCP (1:1000; Proteintech, cat# 10736-1-AP), HA (1: 1000; BioLegend, cat# 901501), and β-actin (1:2000; Sigma-Aldrich, cat# A1978). An HRP-conjugated secondary antibody (Agilent Technologies) against either mouse or rabbit IgG was used at 1:10000 dilution.

Cells were washed with phosphate buffered saline (PBS) and then lysed in high salt buffer (50 mM Tris, pH 7.4, 400 mM NaCl, 1% NP-40 1% v/v Igepal CA-630) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Protein concentrations were determined using the Pierce™ BCA colorimetric assay (ThermoFisher Scientific). Equal amounts of protein were resolved on SDS-PAGE and subsequently transferred onto polyvinylidene difluoride (PVDF) transfer membrane (ThermoFisher Scientific).

The membranes were blocked in 5% w/v skimmed milk powder (Marvel) in Tris-buffered saline containing tween (TBS-T, 20 mM Tris pH 7.6, 137 mM NaCl, Sigma-Aldrich), and then incubated with primary antibodies in 5% skimmed milk in TBS-T overnight at 4°C with gently shaking. After washing, membranes were incubated for 1 hour with horseradish peroxidase (HRP)-conjugated polyclonal goat anti-rabbit or rabbit anti-mouse secondary antibodies (Agilent Technologies) in 5% skimmed milk in TBS-T. The membrane was then washed three times in TBS-T prior to incubation with the ECL plus Western blotting substrate (ThermoFisher Scientific). Membranes were exposed to x-ray film (Scientific Laboratory Supplies) for antigen detection. Protein expression was quantified by scanning densitometry using ImageJ (National Institutes of Health).

**siRNA transfections**

A pool of three siRNAs (MISSION; Sigma-Aldrich) targeted to VCP were used which had been designed using an algorithm from Rosetta Inpharmatics LLC (Washington, USA) and targeted the VCP gene at nucleotides 768 (Hs01_00118726), 1908 (Hs02_00343009) and 2548 (Hs01_00118728). In brief, MISSION VCP siRNA or a negative control scrambled siRNA (ThermoFisher Scientific) was diluted in Opti-MEM® I reduced serum medium (Life Technologies) at a final concentration of 100 nM. Lipofectamine RNAiMAX (ThermoFisher Scientific) at RT was briefly vortexed and added to the diluted siRNA at 6 μl/ml Opti-MEM® I reduced serum medium prior to vortexing and incubating at RT for 25 minutes to facilitate siRNA-lipid complex formation. Medium from cells was replaced with the transfection mix at 1 ml/well in 6-well plates and 250 μl/well in 24-well plates and gently mixed. 6 hours later, transfection mix was replaced with complete medium. Downstream analyses were performed at least 48 hours post-transfection.

**qPCR**

Cells were lysed directly in 12-well plates using TRI reagent (Sigma-Aldrich) and RNA was isolated using the RNeasy Micro Kit (Qiagen) following manufacturers protocol. cDNA was synthesized from RNA using the Reverse Transcription System (Promega) following manufacturers protocols. qPCR was performed using TaqMan® Gene Expression Master Mix (Applied Biosystems) on an Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific). Real-time qPCR expression assays used are listed in the key resources table. RNA expression was determined using the 2-ΔΔCt method.

**Plasmid transfections**

To construct plasmids for NanoBiT assays VCP cDNA was cloned into pcDNA3.1 with an added N-terminal LgBiT tag, whereas a SmBiT tag was added to the C-Terminal of NIS cDNA. Control plasmids included Halo-tagSmBiT (negative control vector) and LgBiT-PRKAR2A/SmBiT-PRKACA (positive control vectors), kindly provided by Dr Caroline Gorvin (University of Birmingham). Plasmid DNA was transfected into cells using TransIT®-LT1 (Mirus Bio) at a 3:1 ratio (lipid: DNA) following the manufacturer’s protocol.

**NanoBiT cell-based assays**

HeLa cells were seeded in 6-well plates at a density of 3.5 × 10⁵ cells per well and transfected with 1 μg plasmid DNA. 24 hours post-transfection, cells were detached using trypsin, resuspended in phenol-red-free DMEM (Life Technologies), re-plated into white 96-well plates and incubated for five hours at 37°C to ensure sufficient attachment. Cells were then treated with disulfiram (Sigma-Aldrich) or CB-5083 (Cayman Chemical) for 24 hours. 25 μl Nano-Glo live cell assay solution (Promega) was added to each well and luminescence measured at 120 second intervals for 40 minutes using the PHERAgstar FS microplate reader (BMG Labtech) preheated to 37°C.

**TCGA and GEO datasets**

Normalized gene expression data and clinical information for papillary thyroid cancer (PTC) were downloaded from TCGA via cBioPortal (cbioportal.org/), FireBrowse (firebrowse.org) and NCI Genomic Data Commons (GDC; portal.gdc.cancer.gov/) (Cerami et al., 2012; Gao et al., 2013; Grossman et al., 2016). Gene expression values were transformed as X= log₂(X+1) where X represents the normalized fragments per kilobase transcript per million mapped reads (FPKM) values. In total, RNA-seq data for 59 normal thyroid and 501 PTC TCGA samples were analyzed (Broad GDAC Firehose, https://doi.org/10.7908/C11G0KM9).
Differential gene expression analysis was also performed using the GEO2R interactive web tool in GEO (Barrett et al., 2013) to investigate proteostasis genes in thyroid cancer. Thyroid cancer GEO datasets used were GSE27155 (Giordano et al., 2005) and GSE76039 (Landa et al., 2016). Significance of gene overlap between datasets was determined using the DynaVenn algorithm (see key resources table).

**Connectivity map analysis**

The Connectivity Map (CMAP) resource (https://clue.io/touchstone) was used to probe the transcriptional relationship between top drug hits from the primary high-throughput screen (47 available in CMAP) and loss of function of a panel of 45 proteostasis genes (Lamb et al., 2006; Subramanian et al., 2017). Hierarchical cluster analysis was used to identify patterns of drugs associated with genes. This study used the current CMAP (L1000) dataset which relies on perturbational data generated with the L1000 assay. The previous Affymetrix-based CMAP dataset is referred to as (build 02).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Details regarding statistical tests are reported in the legends to Figures 1, 2, 3, 4, and 5, Table 1 and the supplemental information (Figures S1–S7, and Tables S2, S5, S6, and S7). Statistical analyses were performed using IBM SPSS Statistics (Version 27), GraphPad Prism (Version 9) and Microsoft Excel. All results were obtained from triplicate biological experiments unless otherwise indicated. For comparison between two groups, data were subjected to the Student’s t-test, and for multiple comparisons one-way ANOVA was used with either Dunnett’s or Tukey’s post-hoc test. p < 0.05 was considered significant. All p-values reported from statistical tests were two-sided.

Kolmogorov-Smirnov, Kruskal-Wallis, and Spearman’s correlation tests were performed on non-parametric TCGA data. P-values were adjusted using the Benjamini-Hochberg FDR correction procedure to correct for multiple comparisons. Dunn’s multiple comparison post-hoc testing was used after Kruskal-Wallis tests to determine significance between datasets in groups of 3 or more. Fisher’s exact test was used to determine the significance of nonrandom associations between two categorical variables.