Title: Time Course of Changes in Sorafenib-Treated Hepatocellular Carcinoma (HCC) Cells Suggests Involvement of Phospho-Regulated Signaling in Ferroptosis Induction

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Abstract

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Ferroptosis is a form of regulated, non-apoptotic cell death characterized by excessive lipid peroxidation that can be triggered by inhibition of the cystine-glutamate antiporter, system X_c ⁻. Sorafenib, an FDA-approved multi-kinase inhibitor drug that is used for treatment of hepatocellular carcinoma (HCC), has been shown to induce ferroptosis. Protein phosphorylation changes upon sorafenib treatment have been previously reported in patient studies and in cell culture. However, early phosphorylation changes during induction of ferroptosis are not reported. This work highlights these changes through a time course from 7 to 60 min of sorafenib treatment in human (SKHep1) HCC cells. A total of 6,170 unique phosphosites from 2,381 phosphoproteins were quantified, and phosphorylation changes occurred after as little as 30 minutes of sorafenib treatment. By 60 minutes, notable changes included phosphosites significantly changing on p53 (P04637), CAD protein (P27708), and proteins important for iron homeostasis, such as FTH1 (P02794), HMOX1 (P09601), and PCBP1 (Q15365). Additional sites on proteins in key regulatory pathways were identified, including sites in ferroptosis-related proteins, indicating the likely involvement of phosphoregulated signaling during ferroptosis induction.

Statement of significance of the study

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Exploration of ferroptosis induction and inhibition can have potential applications in cancer therapeutics and degenerative diseases. Due to the discovery of the kinase inhibitor sorafenib's role in ferroptosis induction, the role of protein phosphorylation may regulate this cell death process. This study profiles the early protein phosphorylation events involved in ferroptosis induction following up to one hour of sorafenib treatment on human hepatocellular carcinoma cells. Through the use of quantitative phosphoproteomics, pathway annotation and data visualization techniques, this work provides a global map of phosphorylation sites potentially involved in ferroptosis that will aid diverse future studies.

Introduction

Induction of ferroptosis occurs following an iron-dependent accumulation of lipid peroxides in the cell ^[1, 2] with the selenoprotein glutathione peroxidase 4 (Gpx4) as the master regulator controlling lipid peroxide formation ^[3]. This regulated, non-apoptotic form of cell death requires abundant and accessible cellular iron and relies on tightly controlled molecular pathways. Ferroptosis induction can occur through inhibition of system X_c^- , a cystineglutamate antiporter composed of SLC7A11 (system X_c^-) and SLC3A2, which triggers ER stress ^[4] blocking cystine uptake ^[1] and has been suggested as a target in treatment of some sensitive cancers ^[5].

Sorafenib is a FDA-approved multikinase inhibitor drug used to treat advanced hepatocellular carcinoma $(HCC)^{[6]}$. It has been reported to induce ferroptosis in HCC cell lines ^[7, 8] inhibiting system X_c^- , in a manner similar to erastin, a known ferroptosis inducer ^[4]. Protein phosphorylation changes upon sorafenib treatment have been previously reported in patient studies ^[9] and in cell culture ^[10], with effects on downstream substrates of the (Raf-Mek-Erk)

MAPK pathway. However the early phosphorylation changes during induction of ferroptosis are not yet defined; indeed, ferroptosis is not currently associated with phosphorylation changes. Previous work suggested the Raf-Mek-Erk cascade is not an important target of sorafenib for ferroptosis induction in HCC cells, but instead that there is a distinct mode of action of sorafenib for induction of ferroptosis ^[7]. Since it has been suggested that sorafenib exerts the additional activity of inhibiting system X_c^- , compared with other similar kinase inhibitors ^[4], better understanding of the phosphorylation changes regulating system X_c^- activity by sorafenib would help to explain how a kinase inhibitor such as sorafenib can induce ferroptosis. Such understanding would enhance our understanding of ferroptosis, including its potential applications in therapeutics development.

Here, we profiled the early effects of sorafenib treatment on SKHep1 cells through a time course from 7 to 60 min of drug treatment. We found extensive modulation on protein phosphorylation in as early as 30 min and more drastically following 60 min of treatment. This study encompasses quantitative coverage on 6,170 unique phosphosites employing label-free quantitative (LFQ) phosphoproteomics to probe phosphorylation changes over time. Sites involved in pathways shown to regulate ferroptosis are highlighted, to gain insight into mechanisms of action of this ferroptosis inducer.

Materials and Methods

Materials

All chemicals were reagent grade. All solvents, including water used in both sample preparation and chromatography were Optima LC/MS grade (Thermo Scientific). Formic

acid and TFA were supplied in sealed 1 mL ampules (Thermo Scientific). Other chemicals are as specified below.

Cell collection and protein extraction

 $5x10^{6}$ SKHep1 cells per replicate were treated with 20uM sorafenib (LC Laboratories). Cells were collected at 0, 7, 15, 30, 60 min post treatment with n=4 replicates. For sample collection, plates with monolayer of cells were washed 3 times with 10 mL of cold PBS followed by the addition of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.3% SDS, 10 mM DTT) with phosphatase inhibitors sodium fluoride and sodium orthvanadate (New England Biolabs). Lysates were flash frozen and stored stored at -80°C until sample collection was complete. Next, samples were incubated for 30 min at 60°C followed by a methanol chloroform protein extraction as previously described ^[11]. Briefly, methanol and chloroform were added to the lysate in a 4:4:1 ratio of lysate:methanol:chloroform. Samples were performed to remove lipids and other potentially contaminating compounds prior to downstream processing.

Protein digestion

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Protein pellets were allowed to air dry followed by final resuspension in solubilization buffer (8M urea, 3 mM DTT, 100 mM ammonium bicarbonate in Optima water) and protein concentration was determined using a bicinchoninic acid assay kit (Pierce, Cat. #23225). 800 µg of each replicate were further reduced with additional DTT at a final total concentration of 5.5 mM at room temperature for 40 min. Proteins were alkylated with iodoacetamide at a concentration of 15 mM in the dark at room temperature for 30 min. Prior to overnight digestion, samples were diluted five-fold in 100 mM ammonium bicarbonate then digested

using sequencing grade trypsin (Promega V511) at a protease:protein ratio of 1:50 at room temperature for 16 h.

Solid-phase extraction

After digestion, samples were acidified to pH < 2 with TFA, kept on ice for 15 min to precipitate lipids, and centrifuged for 15 min at 3,000 x g. For desalting, 50 mg SepPak C18 desalting columns (Waters, Cat. #WAT051910) were conditioned using 5 mL of 100% acetonitrile (ACN) under vacuum followed by passing 1, 3, and finally 6 mL of 0.1% TFA in water to equilibrate the column. Then, acidified and cleared digests were loaded on the column, and the flow-through was passed through one additional time. Contaminating salts were washed off using increments of 1,3, and 6 mL of 0.1% TFA followed by 2 mL of 5% ACN in 0.1% TFA. Finally, eluent was collected with 6 mL of 40% ACN in 0.1% TFA and lyophilized to dryness.

Phosphopeptide enrichment

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From each sample 10 μ g was saved for whole proteome analysis and the remaining 790 μ g was subjected to TiO₂ phosphopeptide enrichment ^[12]. Briefly, pre-packed 3-mg TiO₂ tipcolumns (GL Sciences) were conditioned with 80% ACN, 1% TFA and samples were reconstituted in the same buffer conditions with the addition of 20% lactic acid to reduce non-specific binding during enrichment ^[13]. Samples were passed through the tip-based column and re-applied once followed by washing with 80% ACN, 1% TFA. Phosphorylated peptides were eluted from the tip-based column using 5% then 10% ammonium hydroxide in 20% ACN. Following enrichment, samples were again desalted using Nestgroup C18 Macrospin columns (Southborough, MA) as previously described ^[11] based on manufacturer Accepted Article

protocol and lyophilized to dryness before peptide quantitation via NanoDrop spectrophotometry (ThermoFisher Scientific) and LC-MS/MS acquisition.

LC-MS/MS acquisition

Following lyophilization, peptides were resuspended in 20 µL of 2% ACN, 0.1% formic acid. Peptides were loaded on a 75 µm ID x 2 cm Acclaim PepMap C18 trap column prior to separation at 5µL/min for 3 minutes. For both global protein analysis and phosphopeptide analysis, separations were performed with an Ultimate 3000 RSLCnano and a 75 µm ID x 50 cm Acclaim PepMap C18 column. Flow rate was 300 nL/min with an acetonitrile/formic acid gradient. Mobile phase A was 0.1% formic acid in water and mobile phase B was 20% water, 80% ACN and 0.1% formic acid. The column was held at 2% mobile phase B for 10 min followed by a linear gradient to 24% mobile phase B at 115 min. Following this, a linear gradient from 24%-40% mobile phase B to 135 min. After this, the column was washed with 95% mobile phase B for 5 min followed by 20 min of re-equilibration with 2% mobile phase B. Mass spectra were collected with a Q Exactive HF mass spectrometer (Thermo Scientific) in positive ion mode using data-dependent acquisition. All mass spectrometry files used in this work have been deposited to the Chorus Project (https://chorusproject.org) with the project identifier 3415 and at the MassIVE proteomics repository (https://massive.ucsd.edu/) with project identifier MSV000084140.

Data analysis

For the proteomic and phosphoproteomic datasets, 19 and 20 instrument raw data files, respectively, were imported into Rosetta Elucidator software (Ver. 4.0.0.2.31, Ceiba Solutions/ PerkinElmer). For the proteomic analysis, 19 raw files were used instead of 20 due to the exclusion of one of the four replicates from the 60 min time point due to low peptide concentration and inconsistent chromatography. For both datasets, LC-MS/MS runs were

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aligned based on accurate mass and retention time of detected features using the PeakTeller algorithm in Elucidator. Label-free quantitation was based on comparison of MS1 feature volume of aligned features across all runs. The proteome data contained 333,418 features. Of these 71,659 mapped to 17,042 peptides identified by database search. Similarly, the phosphoproteome data contained 415,444 features, and 77,566 of those mapped to 17,457 peptides identified by database search. As in many proteomic studies, significant numbers of features remain unmapped, and are even not necessarily of proteinaceous, biological or even chemical origin but may include electronics noise from the instrument. MS/MS data were searched with Mascot server (v2.5.1, Matrix Science Ltd., London, UK) against a database of human protein sequences (reviewed canonical sequences with isoforms) from UniProt/Swiss-Prot release 2019 02. The database was appended with common laboratory contaminants for a total of 42,460 forward and reverse protein sequences (total of 84,920 entries). Search parameters included fixed modification on Cys (carbamidomethyl) and mass accuracy limits of 10 ppm for MS and 0.02 Da for MS/MS. Variable modifications were Oxidation (Met), Gln->pyro-Glu (N-term Q), and Acetyl (Protein N-term). Additionally, a variable modification of phosphorylation on serine, threonine, or tyrosine were included in the phosphoproteome analysis. Identified features were annotated at a 0.5% false discovery rate (FDR) for the proteome dataset using the PeptideTeller algorithm in Elucidator. For the phosphoproteome dataset, identified features were annotated using a peptide Mascot Ion Score of 20. Additionally, phosphoproteomic data was analyzed in Elucidator using ModLoc to evaluate possible modification sites based on the AScore algorithm previously described by Beausoleil et al ^[14]. Only peptides containing phosphorylation were analyzed in the phosphoproteome dataset (Supplementary Table 1) and two or more peptides were required for protein identification in the proteome dataset (Supplementary Table 2). Downstream statistical processing was performed using Perseus v.1.6.2.3^[15] for Student's t-test analyses.

Raw intensity values were log-2 transformed followed by a two-sample t-test comparing each time point to the control with Benjamini-Hochberg (BH) FDR correction.

Data Visualization and Biological Inference

KEGG pathway annotation ^[16] and Gene Ontology terms ^[17] were used to infer biological significance of phosphosites of interest. For Gene Ontology terms and pathway enrichment analysis, a Fisher's Exact test using the PANTHER Overrepresentation Test (<u>http://pantherdb.org</u>) ^[18] was performed with a Bonferroni correction for multiple testing. Consensus sequence motifs were found using pLOGO ^[19].

SDS-PAGE and Western blotting

SKHep1 cells were lysed and total protein extracts (50 µg) were subjected to NuPage 4-20% SDS-PAGE and then transferred to nitrocellulose membranes using an iBlot 2 gel transfer device (Thermo Fisher). Antibodies and phospho-antibodies used were purchased from Cell Signaling Technology including CAD antibody (#11933), phospho-CAD (Ser1859) antibody (#12662), ERK1/2 (#9102), phospho-ERK1/2 (#9101), phospho-p53 (Ser392) antibody (#9281). The total p53 monoclonal antibody was provided (from Dr. Carol Prives). GADPH antibody (#5174) was used as a loading control. Phosphorylated sites were normalized to the total protein. Western blot densitometry was done in ImageJ.

Results

Quantitative coverage of the sorafenib-treated proteome and phosphoproteome in Hepatocellular Carcinoma (HCC) cells

A label-free quantitative approach identified and quantified proteins and phosphosites from whole cell lysates of 20 separate cultures (four biological replicates of 0, 7, 15, 30, 60 min Accepted Article sorafenib treatments). For the 20 phosphoproteome LC-MS/MS runs, principal component analysis revealed good reproducibility among biological replicates and a clear separation in phosphosite abundance patterns, especially for the 30 and 60 min time points, of sorafenib treatment (Figure 1A). As expected for a short time course experiment with a multi-kinase inhibitor, there was limited changes in protein abundance across time points. Only one protein at 15 min (RPL36AL) and one at 30 min (NLE1) were significantly altered in protein abundance, in contrast to the phosphoproteome, for which numerous phosphopeptides were differentially expressed after 30 and 60 min of treatment (Supplementary Tables 1 and 2).

In this study, 6,067 unique phosphopeptides containing 6,170 unique phosphosites were included in the phosphoproteome analysis and 2,201 proteins were included in the proteome analysis. For the phosphosites detected, 88.4% of the sites were on a Ser amino acid residue, 11.1% were on a Thr, and 0.5% were from a Tyr amino acid residue (Figure 1B). For comparison of the proteins identified in the proteome and phosphoproteome datasets, 681 proteins (17.5% of the 3,901 total proteins identified and 28.6% of phosphoproteins identified) had coverage for both phosphoproteome and proteome profile, while 1,520 and 1,700 proteins were unique to the proteome and phosphoproteome, respectively (Figure 1C).

At the phosphorylation level, cells required 30 min of incubation with 20 µM sorafenib treatment before sites showed significant modulation with this multikinase inhibitor shown to induce ferroptosis ^[8]. Overall, 41 phosphopeptides were decreasing in abundance after 30 minutes and 47 phosphopeptides were increasing, compared to one protein changing at the global level after 30 minutes (Figure 2A). Phosphosites involved in (Raf-Mek-Erk) MAPK pathway, including pTyr187 MAPK1 (P28482, FC=0.002 p=0.006), pTyr221 CRK (P46108, FC=0.08 p=0.05), and pSer527 on TAB2 (Q9NYJ8, FC=0.08 p=0.026), were among the sites

with the most prominent decreases after 30 minutes. Sites with the most prominent increases include pSer385 on BAG3 (O95817, FC=4.55, p=0.022) and pSer184 on TRAPPC12 (Q8WVT3, FC=4.27, p=0.032). By 60 minutes of sorafenib treatment, more phosphopeptides were decreasing (1,261) and increasing (123) compared to after 30 min with no proteins showing significant change at the global level (Figure 2B) suggesting that by one hour, numerous kinase signaling pathways were affected.

Phosphosite validation.

To validate some of the phosphoproteomic results, available commercial phosphosite specific antibodies against ferroptosis-related proteins were used for western blots. Antibodies specific for pTyr187 MAPK1 (P28482), pSer1859 CAD protein (P27708) and pSer392 p53 (P04637) were used and each site showed a decrease in phosphorylation following drug treatment, confirming trends identified in the mass spectrometry results (Figure 2C and 2D). pTyr187 on MAPK1 and pSer1859 on CAD protein had previously been shown to be affected by sorafenib treatment in tumor biopsies from HCC patients ^[9] and these results show how early the changes in phosphorylation occurs soon after sorafenib treatment. Interestingly, while p53 decreased more dramatically at pSer392 phosphorylation, the total protein also decreased. Not surprisingly, p53 has been previously shown to positively regulate ferroptosis by transcriptional inhibition of system $X_c^{-[1, 20, 21]}$. Total p53 was not detected in the LC-MS/MS study (Supplementary Table 2).

Gene Ontology and Pathway Coverage.

Gene ontology term enrichment analysis indicated that terms related to cell regulation and kinase activity were enriched in decreasing sites, among other terms (Supplementary Figure 1A, Supplemental Table S3). Due to the kinase-blocking activity of sorafenib treatment, this was expected and reflected in the pathways enriched in the decreasing sites such as (Raf-

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Mek-Erk) MAPK pathway (Supplementary Figure 1B, Supplemental Table S4). For terms enriched in the sites that were increasing following sorafenib treatment, "ER to Golgi transport vesicle membrane" and "regulation of organelle organization" were among them (Supplementary Figure 1C, Supplemental Table S3). Sorafenib-induced cell death has been previously linked to ER stress ^[22], brought on by the inhibition of system X_c^{-} ^[4], and is supported by this study. By 30 minutes, significant alterations to phospho-regulated signaling pathways appear. This becomes more pronounced by 60 minutes of sorafenib treatment, especially in kinase pathways such as mTOR, MAPK and PI3K-AKT, where 26, 25, and 36 sites were changing, respectively, (Figure 3, Supplementary Figure 2-4) in addition to ferroptosis-related proteins (Table 1, Figure 4).

Discussion

While the expected effect of phosphorylation changes on substrate targets of the (Raf-Mek-Erk) MAPK pathway were present, including pTyr187 on MAPK1 (P28482, FC=0.002 p=0.006), we focused on sites related to ferroptosis (Table 1 and Figure 4) and system X_c^- (Table 2) in this analysis. It has been suggested that there is a distinct mode of action of sorafenib for induction of ferroptosis ^[7] and the proteins highlighted are known to be associated with ferroptosis. The early phosphorylation changes in ferroptosis related proteins are detailed below.

Phosphosites on iron homeostasis-related proteins pSer229 on HMOX1 (P09601, FC=0.39, p=0.051) and pSer179 on FTH1 (P02794, FC=0.37, p=0.045) and an iron chaperone protein, pSer264 PCBP1 (Q15365, FC=0.01, p=0.006), all significantly decreased after 60 minutes of sorafenib treatment. HMOX1, heme oxygenase 1, is an essential enzyme in iron-dependent lipid peroxidation during ferroptotic cell death ^[23]. HMOX1 liberates iron from heme which

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allows Poly rC Binding-Protein 2 (PCBP2, Q15366) to capture the released iron ^[24]. Four phosphosites on PCBP2 were found in this work however none were significantly changing. Another protein in the PCBP family (PCBP1), however, was significantly changing on one of the four phosphosites identified in this work following 60 minutes of sorafenib treatment. PCBP1 binds cytosolic iron and delivers to proteins such as ferritin^[25] and phosphorylation at Ser264 was decreased one hundred-fold in this study (Table 1). FTH1, heavy chain ferritin, is an iron storage protein that functions as an iron buffer ^[26] which decreased by three-fold at pSer179 after 60 minutes of sorafenib treatment. In the context of ferroptosis induction, involvement of these sites were not previously known and how these phosphorylation events contribute to iron homeostasis in HCC remains to be tested.

An additional site of interest is pSer115 on VDAC2 (P45880, FC=14.49, p=.045), the only phosphosite involved in ferroptosis increasing after sorafenib treatment. Voltage-dependent anion-selective channel (VDAC) proteins form a channel through the mitochondrial outer membrane allowing for diffusion of small hydrophilic molecules across the mitochondrial outer membrane ^[27]. VDAC2 and VDAC3 (Q9Y277) are direct targets of the ferroptosis-inducer erastin ^[28] and previous work has shown that mitochondrial ferritin (FtMt) can inhibit erastin-induced elevation in VDAC2 and VDAC3 expression ^[29]. In this sorafenib study, pSer115 on VDAC2 was significantly increased by 14-fold after 60 minutes of treatment. The total protein abundance of VDAC1 (P21796), VDAC2, and VDAC3 were all unchanged (Supplemental Table 2) across the short time course, indicating the potential for a phosphorylation role in VDACs during the induction of ferroptosis.

The role of p53 (P04637) in ferroptosis was first reported in 2015 showing that p53 inhibits cystine uptake and sensitizes cells to ferroptosis by repressing expression of SLC7A11 ^[20]. Phosphorylation of p53 has been shown to increase sequence-specific DNA binding ^[30] and the mechanisms leading to p53 activation include DNA damage that promotes p53

phosphorylation and blocks MDM2-mediated degradation ^[31]. E3 ubiquitin-protein ligase, MDM2 (Q00987), is involved in p53 regulation and the phosphosite pSer166 on MDM2 (FC=0.16, p=0.022) has been reported as a crucial residue in this regulation ^[32]. In this work, pSer166 decreased 6-fold following 60 minutes of sorafenib treatment. Additionally, two sites on p53, pSer315 (FC=0.25, p=0.019) and pSer392 (FC=0.02, p=0.027), were significantly decreased by four-fold and fifty-fold, respectively.

Other proteins associated with ferroptosis induction were also identified in a previous study that identified binding partners of system $X_e^{-[33]}$. Sites on these proteins are of special interest since they have been experimentally identified in Gu et. al. ^[33] to be related to system X_e^{-} . To further explore our coverage on system X_e^{-} binding partners, we sought to compare the sites significantly changing after 60 minutes of treatment to the list of binding partners identified in literature. Table 2 shows the 35 phosphosites on 19 phosphoproteins considered potential binding partners to system X_e^{-} identified in our study. Phosphosites on potential binding partners include down-regulation of pSer1859 on CAD protein, pSer621 on C-Raf and pSer1261 on mTOR, in addition to sites on heat shock proteins, HSPA8 (pSer254) and HSP β 1 (pSer199), which are also involved in MAPK signaling. CAD protein catalyzes a rate-limiting step in pyrimidine synthesis ^[34] and protein phosphorylation at pSer1859 has previously been reported as sensitive to sorafenib treatment in HCC patients ^[9] and regulated by mTOR-S6K leading to enhanced pyrimidine synthesis ^[35].

Sorafenib has been shown to induce a wide array of effects not related to ferroptosis by targeting the RAF/MEK/ERK pathway ^[9, 36] and overall receptor tyrosine kinase activity. The focus of our analylsis was to highlight phosphorylation changes on proteins associated with ferroptosis following sorafenib treatment. This includes phosphosites on potential binding partners of cystine-glutamate antiporter system X_c^- , that were previously reported^[33] in addition to sites on proteins known to be associated with ferroptosis based on literature

review^[2] and KEGG pathway annotation^[16]. Based on our findings, numerous sites on ironhomeostasis proteins and other proteins involved in ferroptosis were significantly modulated after sorafenib treatment. These phosphorylated proteoforms are potentially significant in ferroptosis induction and can guide future work in pursuit of strategies to control ferroptosis through modulating some of these phosphorylation sites.

Associated Data

All mass spectrometry files used in this work have been deposited to the Chorus Project (<u>https://chorusproject.org</u>) with the project identifier 3415 and at the MassIVE proteomics repository (<u>https://massive.ucsd.edu/</u>) with project identifier MSV000084140.

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Conflict of interest statement

B.R.S. holds equity in and serves as a consultant to Inzen Therapeutics, and is an inventor on patents and patent applications related to ferroptosis.

Figure Captions:

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Figure 1: Overview of quantitative coverage and overlap in proteome analysis. Principal component analysis (PCA) for individual runs from the phosphoprotein (A.) analysis. Pie chart of phosphosite distribution on serine, threonine, and tyrosine amino acid residues quantified in this work (B.). Venn diagram of the overlap in quantitative coverage in the protein and phosphoprotein experiments (C.).

Figure 2: Significantly changing phosphosites after sorafenib treatment. Volcano plot showing log2-transformed fold changes against –log10p-values from 30 min to control (A.) and 60 min vs. control (B.) with their respective total protein volcano plot comparisons shown inset. Western blots for validation of three sites, pTyr187 on MAPK1 (ERK2), pSer1859 (CAD), and pSer392 (p53) found to be significantly changing after 60 min of sorafenib treatment (C.) and a densitometry bar chart for each (D.). CAD protein and p53 come from the same gel run and have the same GADPH image used as a loading control.

Figure 3: Heat Map and motif analysis of phosphosites changing after 60 min in mTOR (A.), MAPK (B.), and PI3K-AKT (C.) KEGG pathways. For the 26, 25, and 36 sites changing after 60 min of sorafenib treatment in the mTOR, MAPK, and PI3K-AKT signaling pathways, intensities were Z-score normalized and trends across the time course are represented by heat maps. For motif analysis, consensus motifs were plotted using pLOGO.

Figure 4: Ferroptosis KEGG pathway overlaid with quantitative coverage from this study. Phosphosite coverage is shown with red rectangles, proteome coverage with blue rectangles. Phosphosites differentially changing after 60 min are highlighted with ones decreasing in blue and ones increasing in red text.

Figures:



Fig. 2





■ p-ERK/ERK ■ p-CAD/CAD » p-p53/p53

Fig. 3





Fig. 4





Table 1. Sites on ferroptosis-associated proteins that are differentially changing after 60 min of Sorafenib treatment. All sites were corroborated in PTM databases PhosphositePlus (www.phosphosite.org) and iPTMnet (https://research.bioinformatics.udel.edu/iptmnet/)

Ferroptosis-	Description	Phosphosite (fold-change
associated Protein		after 60 min)
Uniprot ID (Gene		
Name)		
P04792 (HSPB1)	Heat shock protein beta-1	Ser199 (0.34)
P04637 (P53)	Cellular tumor antigen p53	Ser315 (0.25), Ser392
		(0.02)
P28482 (MAPK1,	Mitogen-activated protein	Tyr187 (0.002)
(ERK2))	kinase 1	
P27708 (CAD)	CAD protein	Ser1859 (0.45)
P02794 (FTH1)	Ferritin heavy chain	Ser179 (0.37)
Q15365 (PCBP1)	Poly(rC)-binding protein 1	Ser264 (0.01)
P09601 (HO-1)	Heme oxygenase 1	Ser229 (0.39)
P45880 (VDAC2)	Voltage-dependent anion-	Ser115 (14.49)
	selective channel protein 2	

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Table 2. Sites differentially changing after 60 min of Sorafenib treatment on proteins previously found to interact with system X_c⁻ based on Gu et. al. ^[33]. All sites were corroborated in PTM databases PhosphositePlus (<u>www.phosphosite.org</u>) and iPTMnet (<u>https://research.bioinformatics.udel.edu/iptmnet/</u>)

Protein	Description	Phosphosite (fold-	Median fold	P-value
Uniprot ID		change after 60 min)	enrichment	(BH-
(Gene Name)			(xCT/vector)	corrected
			(Adapted	n=4, from
			from Gu et	ttest)
			al.)	
P27708 (CAD)	CAD protein	Ser1859 (0.45)	863.0	0.027
	RAF proto-oncogene	Ser621 (0.32)	613.0	0.032
P04049 (RAF1)	serine/threonine-protein kinase			
P50402 (EMD)	Emerin	Ser171 (0.08)	590.6	0.008
Q9UNF1	Melanoma-associated antigen	Thr72 (0.31), Ser247	354.0	0.03, 0.04
(MAGED2)	D2	(0.42)		
	ATP-dependent 6-	Ser386 (0.40)	191.3	0.046
	phosphofructokinase, platelet			
Q01813 (PFKP)	type			
Q14204	Cytoplasmic dynein 1 heavy	Thr4369 (0.25)	171.5	0.028
(DYNC1H1)	chain 1			
	ATP synthase subunit beta,	Ser528 (0.38)	65.8	0.04
P06576 (ATP5B)	mitochondrial			
	Heat shock cognate 71 kDa	Ser254 (0.04)	48.6	0.032
P11142 (HSPA8)	protein			
P16070 (CD44)	CD44 antigen	Ser706 (0.41)	43.2	0.047
Q9UKV5	E3 ubiquitin-protein ligase	Ser523 (0.24)	32.3	0.041
(AMFR)	AMFR			
	DNA mismatch repair protein	Ser830 (0.40)	31.2	0.03
P52701 (MSH6)	Msh6			
P08238	Heat shock protein HSP 90-beta	Ser226 (0.43), Ser255	25.5	0.028, 0.04

(HSP90AB1)		(0.46)		
	Serine/threonine-protein kinase	Ser1261 (0.25)	23.6	0.019
P42345 (MTOR)	mTOR			
P04792 (HSPB1)	Heat shock protein beta-1	Ser199 (0.34)	20.2	0.028
P53814 (SMTN)	Smoothelin	Ser792 (0.09)	19.6	0.022
	26S proteasome non-ATPase	Ser16 (0.47)	12.9	0.047
Q13200 (PSMD2)	regulatory subunit 2			
		Ser93 (0.41), Ser135	15.6	0.037,
		(0.07), Ser177 (0.37),		0.015,
		Ser210 (2.07),		0.023,
		Ser216-Ser212 (6.76),		0.041,
		Ser511 (5.05), Ser559		0.04,
		(0), Ser3412 (11.49),		0.026,
		Thr4100 (0.05),		0.033,
		Thr4766 (0.03),		0.036,
		Ser5099 (6.13),		0.03,
		Ser5110 (0.13),		0.042,
		Thr5729 (0.18),		0.046,
		Ser5762 (0.21),		0.009,
Q09666	Neuroblast differentiation-	Ser5763-Ser5752-		0.024,
(AHNAK)	associated protein AHNAK	Ser5749 (19.61)		0.035 0.042
P34932 (HSPA4)	Heat shock 70 kDa protein 4	Ser76 (1,000)	23.7	0.048
015027	Protein transport protein	Ser2083 (3.72)	20.7	0.033
(SEC16A)	Sec16A			

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