A High Throughput Substrate Binding Assay Reveals Hexachlorophene as an Inhibitor of the ER-resident HSP70 Chaperone GRP78.

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Abstract:

Glucose regulated protein 78 (GRP78) is the ER resident 70 kDa heat shock protein 70 (HSP70) and has been hypothesized to be a therapeutic target for various forms of cancer due to its role in mitigating proteotoxic stress in the ER, its elevated expression in some cancers, and the correlation between high levels for GRP78 and a poor prognosis. Herein we report the development and use of a high throughput fluorescence polarization-based peptide binding assay as an initial step toward the discovery and development of GRP78 inhibitors. This assay was used in a pilot screen to discover the anti-infective agent, hexachlorophene, as an inhibitor of GRP78. Through biochemical characterization we show that hexachlorophene is a competitive inhibitor of the GRP78-peptide interaction. Biological investigations showed that this molecule induces the unfolded protein response, induces autophagy, and leads to apoptosis in a colon carcinoma cell model, which is known to be sensitive to GRP78 inhibition.

The human heat shock protein of 70 kDa (HSP70) family consists of 13 highly homologous isoforms that vary in cellular and tissue localization as well as inducibility[1]. HSP70 proteins are made up of an N-terminal nucleotide binding domain (NBD) connected to a substrate binding domain (SBD) by a flexible linker. HSP70s function to facilitate protein folding by cycling through two conformations which are dependent on nucleotide state. In the ADP (SBD closed) state, HSP70s have a high affinity for unfolded polypeptide substrates. Whereas in the ATP (SBD open) state they have low affinity for these substrates[2]. The domains are allosterically regulated such that binding of a peptide to the SBD stimulates ATP hydrolysis in the NBD which, in turn, causes a closing of the SBD, drastically decreasing the substrate's off rate. By cycling through the open and closed states, HSP70s prevent non-specific hydrophobic interactions from forming between exposed unfolded regions in substrate proteins resulting in a decrease in aggregation of the unfolded protein and a thermodynamic shift toward the folded state of the substrate protein[3].

It has long been known that, HSP70s can contribute to the survival of cancer cells due to their role in alleviating proteotoxic stress[1,4,5]. Interestingly, many cancer types have divergent panels of HSP70 induction and repression[6,7]. Additionally, genetic data have shown that repression of HSP70s in certain cancer cell lines leads to sensitization to chemotherapy or even cell death[6]. Many groups have tried to discover inhibitors of HSP70s for use in cancer therapy, and although molecules have been discovered none are currently FDA approved or currently in clinical trials[8]. A recent computational study calculated five potential biding sites in HSPA1A, the major inducible HSP70. Of these, two were in the NBD, one was at the NBD-linker interface, and two were in the SBD. Of the SBD sites, one is the binding site of the HSP70 inhibitor pifithrin-μ and the other is the substrate binding site[9]. Most currently known HSP70 inhibitors bind to the NBD and, somewhat surprisingly, the substrate binding site is often avoided in drug discovery efforts due to its small size and hydrophobic nature[9,10]. However, given the fact that a minimum substrate peptide is 7 amino acids in length and that these peptides have been reported to bind

with as low as 100 nM affinity, we decided to develop an HTS assay and screen for competitive inhibitors of the GRP78-substrate interaction. We chose GRP78 as the initial HSP70 to target based on our recent success with the bis-steroidal compound, ritterostatin G_N1_N and the therapeutic potential of targeting GRP78 to treat cancers, but believe this strategy can be extrapolated to other HSP70s[4,11,12]. We now report the development and implementation of an FP-based high-throughput screening (HTS) platform that revealed hexachlorophene as a substrate-competitive inhibitor and the biochemical and cellular characterization of this compound.

With the goal of identifying an inhibitor of GRP78 that binds to the SBD, a previously described substrate binding assay was optimized and adapted for use in 384 well plates. The assay measures a fluorescein isothiocyanate (FITC)-labeled fluorescent peptide (FITC-NRLLLTG) binding to full-length GRP78 at the SBD in the closed (ADP bound) state using fluorescence polarization (Figure 1A)[13,14]. Upon binding, the polarization increases, but in the presence of an inhibitor, the low polarization state predominates. Using DMSO as a negative control and heat denatured GRP78 as a positive control, the assay conditions were optimized to give a Z-factor of 0.8 using 0.75 μM GRP78 and 100 nM FITC-NRLLLTG (Figure S1). After optimization, a pilot screen was carried out in singlicate using the Spectrum Collection of bioactive compounds and natural products. A scatter plot was then generated as a function of percent inhibition and compounds that were at least three standard deviations from the mean were selected (circled in red, Figure 1B), resulting in the identification of 13 putative hits (Figure 1B). Of these 13 hits, nine compounds were eliminated because they displayed fluorescent activity, leading to interference with the assay, and the rest were screened in triplicate. Purified powders of the remaining compounds, after triplicate screening, were purchased and measured in a 10-point dose response (Figure 1C). This resulted in one molecule that showed dose dependent inhibition, hexachlorophene (**1**), with an IC_{50} of 9.1 ± 1.4 μM (Figure 1D).

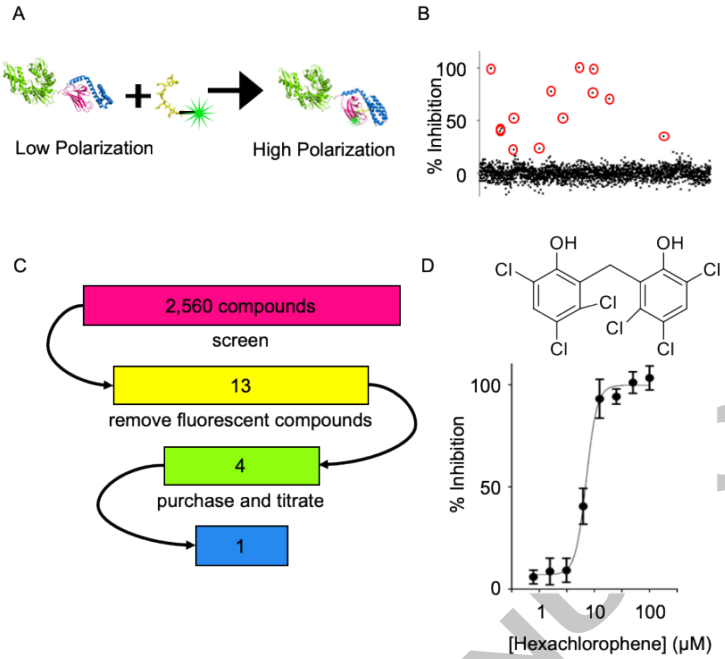

Figure 1. A 2560 compound screen of bioactive compounds reveals hexachlorophene as an inhibitor of GRP78. (A) A cartoon representation of the assay used. A minimal peptide sequence is labeled with a fluorophore and binding is measured using fluorescence polarization (FP). (B) A scatter plot of raw screening data. Each data point represents a unique compound. Compounds circled in red are at least three standard deviations from the mean and were cherry picked for confirmation of activity. (C) Flow chart describing confirmation and removal of false positives. (D) A 10-point dose response and the structure of hexachlorophene (**1**).

Although the presently reported assay measurers disruption of a binding event between a peptide and GRP78 in the SBD, this assay requires ADP to convert the SBD to the closed, high affinity substrate binding state. It is, therefore, possible to block peptide binding through inhibition of nucleotide binding either through direct competition or through some allosteric perturbation. To account for this possibility, experiments to probe the binding site of hexachlorophene were carried out. Direct competition with the substrate binding event predicts that this should be independent of ADP concentration. By varying the concentration of ADP in the dose response and comparing this to a known nucleotide competitive inhibitor, VER155008, hexachlorophene can be ruled out as a competitor for nucleotide binding[15]. At both 10 and 1000 μM ADP, hexachlorophene exhibits the same potency of 9.1 ± 1.4 μM. This contrasts with VER155008, which does not inhibit in the

presence of 1000 μM ADP but inhibits with an IC_{50} of 0.80 ± 0.68 μM in the presence of 10 μM ADP (Figure 2A and S2). These data strongly argue for hexachlorophene binding to the SBD, but formally, cannot rule out an ADP independent allosteric mechanism. To further support the hypothesis that hexachlorophene binds to the SBD, the isolated substrate binding domain of GRP78 (amino acids 419-633) was generated recombinantly and challenged with hexachlorophene in an 8-point dose response. Although there was a slight shift in the IC_{50} of hexachlorophene for the SBD construct, the IC_{50} of hexachlorophene did not have a statistically significant difference relative to that of the full-length protein, further supporting the hypothesis that hexachlorophene directly binds the SBD and likely competes with peptide binding (Figure 2B). Additionally, we probed the selectivity of hexachlorophene with the bacterial HSP70, DnaK. This was accomplished by repeating the fluorescence polarization assay with DnaK in the presence of hexachlorophene (Figure 2C). We observed a statistically significant ($p<0.05$) 5.5 fold loss of potency with DnaK (51.6 ± 1.33 μM) compared to GRP78 (9.1 ± 1.4 μM).

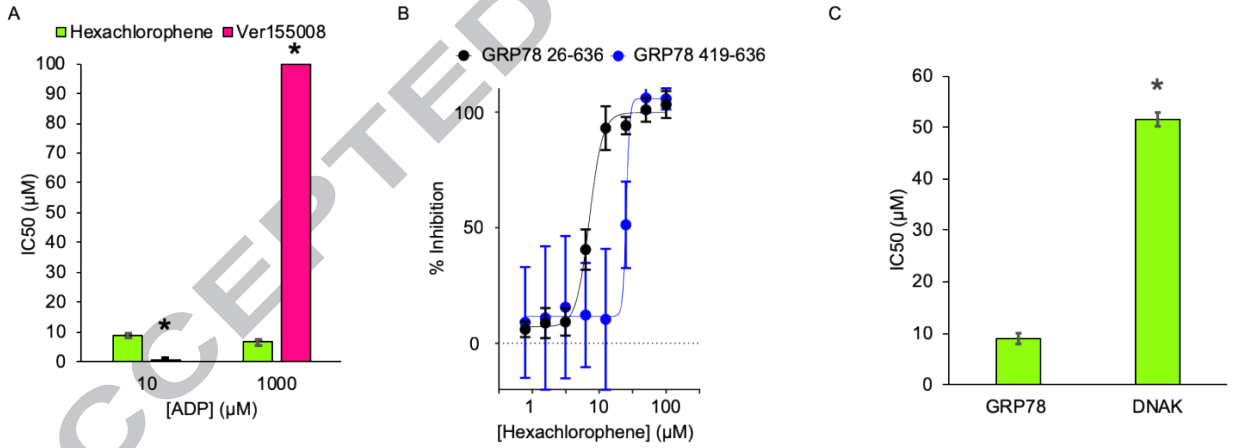

Figure 2. Hexachlorophene binds selectively to the substrate binding domain of GRP78. (A) Hexachlorophene does not compete with ADP for inhibition of GRP78 as indicated by the green bars. In contrast, the known nucleotide competitive GRP78 inhibitor, VER155008 (pink bars), shows inhibition at low ADP concentration but this is lost when ADP is increased. The asterisk indicates $p<0.05$ in an independent t-test. (B) Hexachlorophene inhibits full-length GRP78 26-636 and the isolated SBD, GRP78 419-633 with equal potency. The large error bars in the SBD measurement were caused by the relatively low molecular weight of the construct resulting in a

reduced signal to noise ratio in the assay. (C) Hexachlorophene is a significantly more potent inhibitor of GRP78 than it is of DnaK (* indicates $p<0.05$).

To probe the structure activity relationship of hexachlorophene, 9 derivatives were purchased and tested for GRP78 inhibition (Figure 3 and S3). Based on these data, a sulfur atom is tolerated at the position between the two aromatic rings (compare compounds **1** and **2**) and the chlorines meta to the hydroxyls are important for binding (compare compounds **1** and **2** to **3** and **4**). However, removal of the ortho and meta chloro substituents or all chloro substituents gave inactive compounds (**5** and **7**) none of the derivatives were more potent than hexachlorophene and very few changes were tolerated in its structure. Alkyl substitutions were not tolerated (**8** and **10**), an asymmetric compound with an oxygen linker was not tolerated although the direct ether analog of **1** is not available (**6**), and an amide linker was not tolerated (**9**). It is clear that a much larger swath of chemical space must be explored to develop a full SAR map.

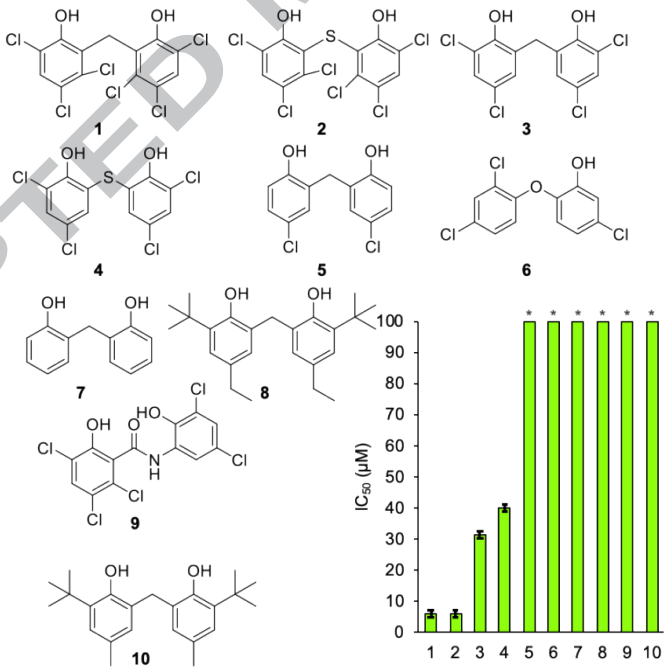

Figure 3. SAR of hexachlorophene derivatives. 10 molecules with scaffolds resembling the diphenylmethane backbone of hexachlorophene were purchased and measured in the peptide binding assay in 8-point dose responses. Only minimal structural changes are tolerated to preserve activity. Asterisk indicates an IC_{50} of greater than 100 μM.

It has previously been shown that hexachlorophene exhibits toxicity in colon cancer cell lines, so we decided to see if this toxicity could be related to GRP78 inhibition using the HCT116 colon carcinoma cell line as a model[16]. To set the treatment dose, we first determined the CC_{50} (concentration cytotoxic to 50% of cells) by treating cells with 10 concentrations of hexachlorophene for 48 hours ranging from 100 μM to 195 nM yielding a CC_{50} of 3.43 ±1.42 μM, in good agreement with the biochemical IC_{50} (Figure S4). We were able to show that HCT116 cells treated with three different concentrations of hexachlorophene for 16 hours followed by immunoblot analysis showed a dose dependent induction of markers involved in the unfolded protein response (UPR), including XBP1s in the IRE1α pathway, ATF4 in the PERK pathway, and ultimately CHOP, which leads to apoptosis if UPR is not resolved (Figure 4). Because GRP78 is a primary gatekeeper of ER protein quality control, enhanced UPR is an expected result of GRP78 inhibition and has been confirmed in genetic studies and in our mode of action studies of ritterostatin G_N1_N [11,12].

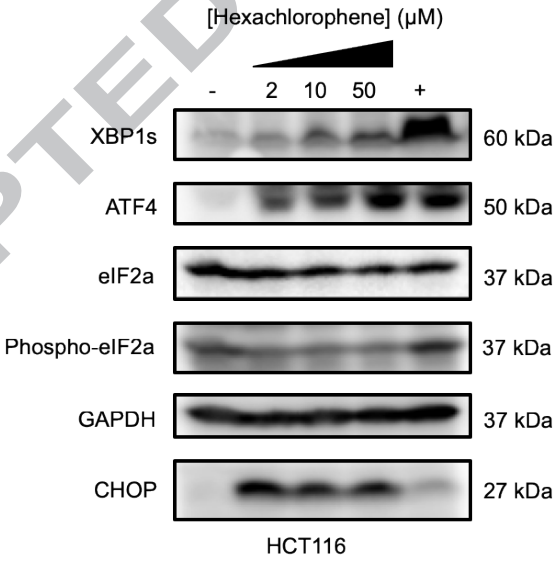

Figure 4. Hexachlorophene induces the unfolded protein response. HCT116 cells were treated with increasing concentrations of hexachlorophene for 16 hours and prominent markers of the UPR were detected by immunoblot. DMSO was used as a negative control and 20 μg/ml tunicamycin was used as a positive control.

It has been previously demonstrated that all three branches of the UPR pathway lead to an induction of autophagy, so autophagy levels upon hexachlorophene treatment were investigated [12,17,18]. HCT116 cells were treated with three concentrations of hexachlorophene, 2, 10, and 50 μM, for 16 hours and p62 and LC3-I and II, markers of autophagy, were measured through immunoblotting. These studies showed that treatment with hexachlorophene decreased the levels of p62 and LC3-I while increasing the levels of LC3-II relative to the negative, DMSO, control (Figure 5). Positive controls in these experiments were: starvation using serum free Hawk's balanced salt solution (HBSS), which activates autophagic flux and bafilomycin (Baf) which inhibits acidification of autolysosomes and thus inhabits autophagic flux. Because p62 is a substrate of autophagy, decreased levels are indicative of increased autophagic flux. Additionally, a shift in the LC3-I/II ratio toward LC3-II also suggests that autophagic flux is upregulated. Starvation with HBSS is known to rapidly induce a high rate of autophagic flux. We think this is why the data between HBSS and hexachlorophene look different. At this time point, the HBSS treated cells have already cleared LC3-II. However, we do not see an accumulation of LC3-I nor p62 as observed for Baf treatment and therefore do not think we are seeing blocked autophagy.

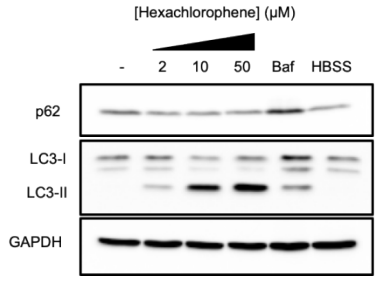

Figure 5. Hexachlorophene induces autophagy. HCT116 cells treated with hexachlorophene show a reduction in LC3-I and p62 and an increase in LC3-II indicating an increase in autophagic flux. DMSO was used as a negative control, bafilomycin was used as an autophagy inhibition control, and starvation (HBSS) was used as a positive control.

To determine the mechanism of death induced by hexachlorophene, HCT116 cells were treated with 5, 10, or 25 μM hexachlorophene for 24 hours and were stained with annexin V and

propidium iodide (PI) and analyzed by flow cytometry (Figure S5). Annexin V measures phosphatidyl serine on the cell surface indicating an apoptotic mechanism. PI stains DNA, but can only penetrate cells through a compromised cell membrane indicating cell death. DMSO was used as a negative control and Taxol, which induces apoptosis, was used as a positive control. Hexachlorophene led to apoptotic cell death in a dose (Figure 6A) and time (Figure 6B) dependent manner. Treatment with 10 μM hexachlorophene for 24 hours showed the same percentage of apoptotic cells (annexin V staining only) as the Taxol control and 25 μM hexachlorophene showed a larger percentage of apoptotic cells than the Taxol control (Figure 6A). We also measured early (annexin V positive only) and late apoptosis (annexin V and PI positive) cells, which showed increased early apoptosis events for hexachlorophene relative to Taxol, but both showed similar late apoptosis events (Figure 6B). Induction of UPR is designed to handle ER stress, if proteins remain misfolded in the ER they are subject to ER associated protein degradation (ERAD), and when this is insufficient apoptotic cell death programs are initiated. It is, therefore anticipated that hexachlorophene mediated inhibition of GRP78 would lead to apoptosis, consistent with the observed data[17,18].

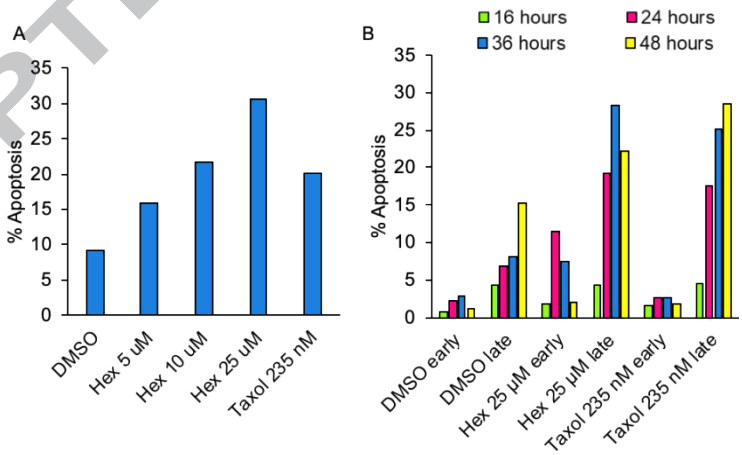

Figure 6. Hexachlorophene (hex) induces apoptosis in a time and dose dependent manner. (A) HCT116 cells treated with hexachlorophene for 24 hours were stained with annexin V and propidium iodide and subject to FACS demonstrating a dose dependent increase in total apoptosis (all annexin V positive cells). (B) Hexachlorophene treated cells peak in early apoptosis

(annexin V positive and PI negative) at about 24 hours and steadily decrease. Conversely, hexachlorophene treatment causes a peak in late stage apoptosis (Annexin V and PI positive) at 36 hours.

While just a pilot screen, we have reported the first successful use of an HTS fluorescence polarization-based substrate binding assay to detect GRP78 inhibitors. Using the assay in 384-well plates, we identified and validated the antibiotic hexachlorophene to be a GRP78 inhibitor (Figure 1). This assay is robust, has an excellent Z-factor, is readily scalable to 1536 well plates, and can be used to probe both nucleotide competitive and substrate competitive inhibitors (Figure 2A). Taken together, our results show that hexachlorophene inhibits substrate binding independently of the nucleotide state by binding directly to the SBD (Figure 2B). In addition, we do not believe this to be a non-specific interaction, as the compound showed selectivity for GRP78 relative to the highly conserved bacterial HSP70, DnaK (Figure 2C). We also studied the effects of hexachlorophene in HCT116 cells to show it induces the unfolded protein response (Figure 4), autophagy (Figure 5), and ultimately leads to cell death through apoptosis (Figure 6), all of which are predicted, based on genetic and chemical biology data, to be a consequence of the inhibition of GRP78 [11,12,17,18]. Although hexachlorophene itself has some known therapeutic liabilities and off target effects, our results provide evidence that non-peptidic inhibition of the SBD of HSP70 isoforms is a viable strategy for selectively targeting these enzymes. We also carried out a small-scale structure activity relationship (Figure 3), which indicated there is some potential to optimize hexachlorophene. This will be reported at a later date.

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