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TITLE:	Development of Molecular Chaperone Inhibitors for (Re)Sensitization of CRPC to Enzalutamide and Abiraterone and to Synergize with Metabolic Inhibitors
PRINCIPAL INVESTIGATOR:	Dr. Leonard Neckers
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PREPARED FOR:	U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012
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W81XWH-19-1-0716

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Leonard Neckers

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Geneva Foundation
917 Pacific Ave. # 600
Tacoma, WA 98402

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Objective: (1) Improve the solubility and pharmacodynamic properties of previously identified Hsp40 & Hsp70 inhibitors in preparation for Phase I clinical evaluation in CRPC patients. (2) Determine whether these chaperone inhibitors sensitize resistant CRPC to Enz and/or Abi and, if so, by what mechanism(s). (3) Increase our understanding of the role of these chaperones in regulating AR-driven metabolic deregulation, with the goal of identifying novel synergistic combinatorial approaches to target CRPC.

Impact: By identifying a chaperone-based approach to inhibit or reverse CRPC resistance to Enz and/or Abi, the current research proposal addresses the dual 2018 PCRP Overarching Challenges of (1) developing treatments that improve the outcomes for men with lethal prostate cancer and (2) better defining the biology of lethal prostate cancer to reduce death. Further, consistent with the mandate of the PCRP Impact Award, a key goal of our research strategy is to position the program for first-in-human clinical evaluation of one or more of these chaperone inhibitors within 5 years after completion of this Award.

15. SUBJECT TERMS
Cancer, Prostate Cancer, Oncology

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a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	21	19b. TELEPHONE NUMBER

Table of Contents

1. INTRODUCTION: Androgen receptor (AR) signaling remains important in CRPC. Accordingly, two potent second line anti-androgen agents, abiraterone and enzalutamide, were developed. Abiraterone is a CYP17A1 inhibitor (blocking both 17-alpha-hydroxylase and 17,20 desmolase enzymatic activities) which causes a marked reduction of androgen in serum and in CRPC osseous metastases. Enzalutamide is a competitive antagonist of AR, which binds the ligand binding domain (LBD), preventing nuclear translocation and AR-dependent gene transcription. Unfortunately, most patients that initially respond to these drugs develop resistance, concomitant with reactivated AR signaling. The emergence of ADT-resistant CRPC is frequently associated with expression of a number of AR splice variants (ARv), including but not limited to ARv7, which lack a carboxy-terminal LBD. Consequently, these ARv are insensitive to anti-androgens or androgen ablation and are constitutively active. Notably, ARv7 expression has been associated with poor prognosis, shorter overall survival and resistance to standard of care treatments in CRPC patients. In addition to ARv expression, another key mechanism underlying resistance to enzalutamide is metabolic dysregulation through enhanced dependence on glucose metabolism. PCa is characterized by dependence on glycolysis and altered fatty acid and glutamine metabolism, and this metabolic reprogramming is regulated, in part, by the transcriptional activity of full-length AR. Expression of ARv (in particular, ARv7) has been shown to further increase dependence of CRPC on glutaminolysis and reductive carboxylation. Thus, alternative approaches to disrupt the AR and ARv7 signaling axis and its effects on metabolic disrregulation and ADT resistance in CRPC are of great clinical importance and remain a critical unmet need. Such a strategy would be expected to provide efficacy in CRPC and may also (re-)sensitize ADT-resistant CRPC to LBD targeted therapy (e.g., enzalutamide and/or abiraterone). The research plan described in the current proposal builds on the current team's multi-year ongoing and successful collaboration, funded by the 2015 PCRP Idea Development Award, that identified Hsp40 and Hsp70 inhibitors as novel therapeutic agents with *in vitro* and *in vivo* activity toward enzalutamide- and abiraterone-resistant CRPC expressing AR splice variants, including ARv7. The current research proposal addresses the dual 2018 PCRP Overarching Challenges of (1) developing treatments that improve the outcomes for men with lethal prostate cancer and (2) defining the biology of lethal prostate cancer to reduce death. Further, consistent with the mandate of the PCRP Impact Award, a key goal of our research strategy is to position the program for first-in-human clinical evaluation of one or more of these chaperone inhibitors within 5 years after completion of this Award.

2. KEYWORDS: castration-resistant prostate cancer, androgen, androgen receptor, ARv7, chaperone inhibitors, Hsp70, Hsp40, metabolism, glycolysis, oxidative phosphorylation

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

SA1/Major Task 1: Synthesis and characterization of sufficient amounts of C86, JG-98, JG-231 and other analogs.

Milestones	target dates	actual dates	%completion

n/a	1-36 months	1-12 months	25%

SA1/Major Task 2: Lead optimization of JG-231 analogs. From this Task, we will produce 1-2 optimized candidates for testing in efficacy models.

Milestones	target dates	actual dates	%completion
n/a	1-18 months	1-12 months	30%

SA1/Major Task 3: Hit-to-lead optimization of C86 and analogs. From this Task, we will identify the binding site of C86 and understand whether this site can accommodate drug-like molecules.

Milestones	target dates	actual dates	%completion
n/a	12-36 months	1-12 months	0%

SA2/Major Task 4: Assess combinatorial activity of chaperone inhibitors in 3 resistant CRPC models (22Rv1, VCaP, C4-2).

Milestones	target dates	actual dates	%completion
Validation of ability of Hsp40/Hsp70 inhibitors to sensitize resistant CRPC cells and tumors to enzalutamide and/or abiraterone	1-18 months	1-12 months	33%

SA2/Major Task 5: Determine mechanistic basis of combinatorial activity underlying chaperone inhibitor-mediated sensitization to enzalutamide and/or abiraterone.

Milestones	target dates	actual dates	%completion
Identification of one or more mechanisms by which Hsp40 and/or Hsp70 inhibitors sensitize resistant CRPC cells to enzalutamide and/or abiraterone.	6-18 months	1-12 months	40%

SA3/Major Task 6: Assess combinatorial activity of Hsp40 and Hsp70 inhibitors with inhibitors of glycolysis, pentose phosphate pathway, glutaminolysis and fatty acid synthesis.

Milestones	target dates	actual dates	%completion
Identification of synergy between chaperone inhibitors and selected metabolic inhibitors; identification of metabolic pathways, whose inhibition (alone or combined with chaperone inhibition) sensitizes resistant CRPC to enzalutamide and/or abiraterone; validation of non-invasive MRSI to predict treatment efficacy *in vivo*.	12-36 months	1-12 months	30%

Specific Aims:

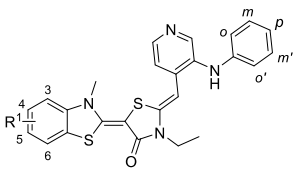
1. Initiate a pre-clinical development program to improve solubility and pharmacokinetic

properties of Hsp40 & Hsp70 inhibitors to maximize in vivo safety, efficacy and bioavailability.

2. Examine whether current Hsp40 and/or Hsp70 inhibitors sensitize CRPC to Enz and/or Abi in vitro and in vivo, and by what mechanism(s).
3. Determine whether additive/synergistic activity is observed in CRPC when Hsp40/Hsp70 inhibition is combined with inhibitors of distinct metabolic pathways deregulated in CRPC.

What was accomplished under these goals?

Specific Aim 1: The goal of Specific Aim 1 is to advance the development of clinical candidates targeting Hsp70 and Hsp40. In the most recent funding cycle, we have pursued this objective through two, parallel set of experiments. In both approaches, the major technical hurdle with the current Hsp70 and Hsp40 inhibitors is their unoptimized physicochemical properties, so the experiments were designed to overcome these specific problems. In the first approach, we designed and synthesized 23 analogs of the allosteric Hsp70 inhibitor, JG-231 (see Table). JG-231 emerged from a medicinal chemistry campaign (Shao et al. 2018 J. Med. Chem.) and it showed promising activity in cellular and animal models of CRPC (Moses et al. 2018 Cancer Res). However, JG-231 contains a cationic pyridinium, which gives rise to unfavorable spectral properties. In an attempt to solve this issue, we recently explored alternative, neutral isosteric replacements of the pyridinium. Briefly, we found that replacement of the pyridinium with an aniline retained much of the anti-CRPC activity of JG-231, as measured by anti-proliferative activity in 22Rv1 cells (see Table). We are currently testing the metabolic stability and permeability of these analogs to understand whether they are superior to JG-231.



Compd	R_1	R_2	MCF-7 IC_{50}/ μM	22RV1 IC_{50}/μM	PC3 IC_{50}/μM
17a	H	*o,m'*-Me	1.7 ± 0.05	1.8 ± 0.43	2.0 ± 0.60
17b	H	*o, m'*-F	1.5 ± 0.49	1.1 ± 0.55	1.2 ± 0.23
17c	H	*o*-Me	0.99 ± 0.01	0.47 ± 0.20	1.2 ± 0.11
17d	H	*o*-Cl	0.75 ± 0.11	0.46 ± 0.25	0.78 ± 0.07
17e	H	*o*-F	0.33 ± 0.06	0.30 ± 0.04	0.36 ± 0.02
17f	4-Me	*o*-Me	0.40 ± 0.10	0.48 ± 0.15	0.33 ± 0.02
17g	4-Me	*o*-Cl	0.65 ± 0.01	0.35 ± 0.05	0.50 ± 0.10
17h (JG2-38)	4-Me	*o*-F	0.10 ± 0.01	0.15 ± 0.02	0.07 ± 0.01
17i	4-OCH_3	*o*-Me	0.42 ± 0.03	0.41 ± 0.08	0.37 ± 0.03
17j	4-OCH_3	*o*-Cl	0.71 ± 0.30	0.50 ± 0.08	0.76 ± 0.01
17k	4-OCH_3	*o*-F	0.13 ± 0.01	0.19 ± 0.02	0.10 ± 0.01
17l	5-F	*o*-Me	1.3 ± 0.35	2.3 ± 0.94	2.3 ± 0.31
17m	5-F	*o*-Cl	1.3 ± 0.15	0.49 ± 0.03	1.2 ± 0.31
17n	5-F	*o*-F	0.23 ± 0.04	0.15 ± 0.07	0.26 ± 0.06
17o	5-ethyl	*o*-F	1.2 ± 0.02	1.2 ± 0.10	2.2 ± 0.01

In the second, complementary approach, we have initiated a computationally-directed "scaffold hopping" campaign. Through that effort, we hope to identify alternative scaffolds to replace the core rhodocyanine and pyridinium of JG-231 with equivalent cores, with the goal of improving solubility and reducing toxicity. Briefly, we developed a pharmacophore model and performed a screen of the 20M+ Zinc collection in Smallworld. This effort was performed in consultation with Brian Schoicet (UCSF). Then, the top 20 analogs were ordered from a commercial vendor. To date, we have received 12 of these

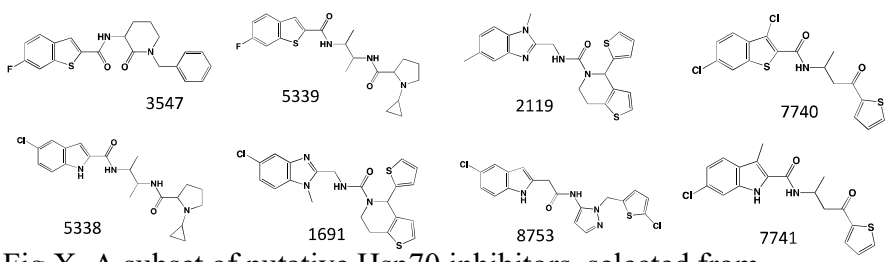
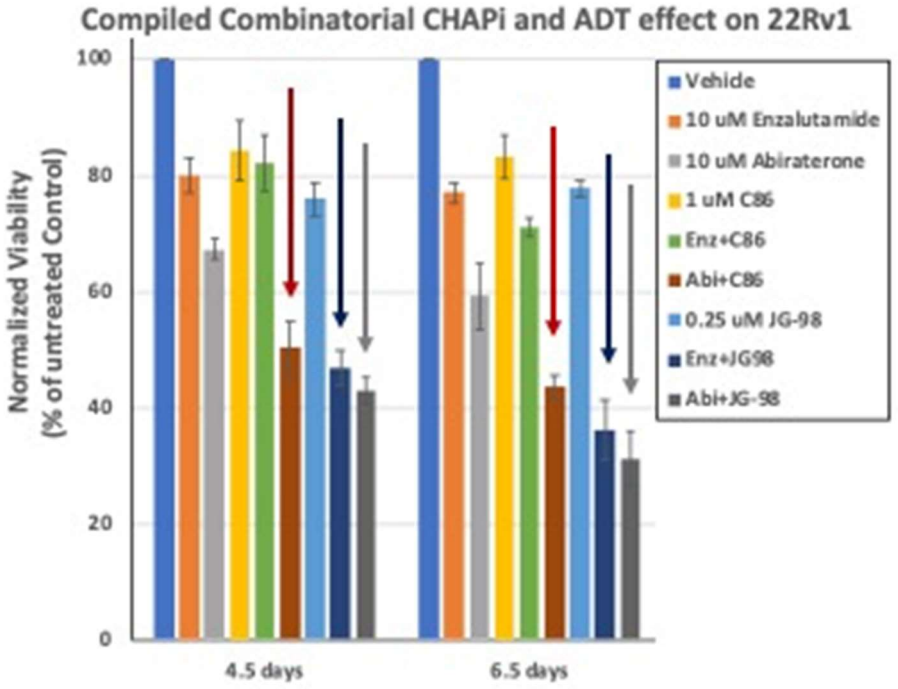


Fig X. A subset of putative Hsp70 inhibitors, selected from pharmacophore computational scaffold hopping in SmallWorld.

molecules and are in the process of testing them in a battery of standard Hsp70 biochemical assays (e.g. ATP turnover, luciferase refolding). If any of the molecules have EC50 values less than 20 μM, we will proceed with analoging efforts to probe structure-activity relationships.

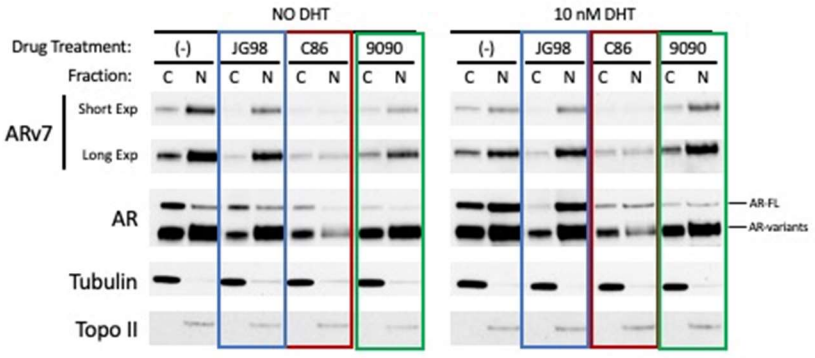
Specific Aim 2: The goal of Specific Aim 2 (and a Major Task under this aim) is to determine whether Hsp40 and/or Hsp70 inhibitors sensitize resistant CRPC to enzalutamide and/or abiraterone. In a previous publication (Moses MA, et al. Cancer Res. 2018 Jul 15;78(14):4022-4035) we demonstrated that the Hsp40 inhibitor C86 and the Hsp70 inhibitor JG-98 each had single agent activity toward 22Rv1 CRPC cells in vitro and in vivo. The data provided in the figure below show that both agents, at defined concentrations, do re-sensitize 22Rv1 cells to enzalutamide and abiraterone. The data show that more than an additive response is obtained by combining C86 (1uM) with abiraterone (10 uM) for 4.5 and 6.5 days. JG-98 (0.25 uM) re-sensitizes these cells to both abiraterone and enzalutamide (both at 10 uM) when cells are exposed for either 4.5 days or 6.5 days. Thus, we have partially achieved the milestone of this Major Task (validation of ability of Hsp40 and/or Hsp70 inhibitors to sensitize resistant CRPC cells and tumors to enzalutamide and/or abiraterone). The planned in



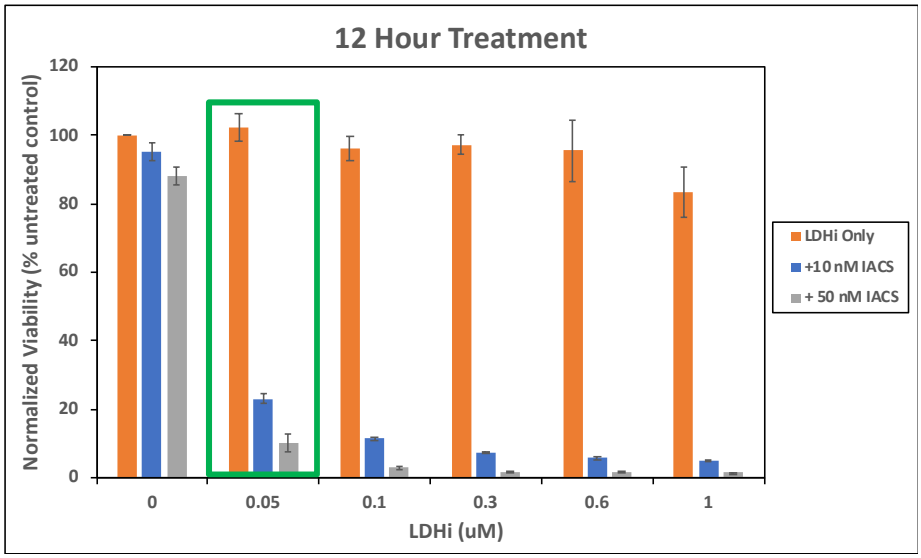
vivo experiments to corroborate the in vitro data were halted by the Covid-19 pandemic. We hope that we will be able to complete these experiments in year 2 of this award.

Specific Aim 2 also includes the Major Task of determining the mechanistic basis of the combinatorial activity underlying chaperone inhibitor-mediated sensitization to enzalutamide and/or abiraterone. During year 1, we have begun to make inroads in addressing this question (see Figure below). After treating 22Rv1 cells with either C86 or JG-98, we subfractionated the cells into nuclear and cytosolic fractions. We found that ARv7, the spliced variant of the androgen receptor lacking the ligand binding domain is primarily (although not entirely) located in the nuclear fraction. As expected, its subcellular location does not respond to

dihydrotestosterone (DHT). However, its cytosolic component is particularly sensitive to JG-98 (see below). In contrast, the Hsp40 inhibitor C86 depletes ARv7 protein in both nuclear and cytosolic compartments in the presence or absence of DHT. In the case of full-length AR, C86 is most effective at depleting the nuclear component of the full-length receptor, while the Hsp70 inhibitor JG-98 preferentially depletes the cytosolic portion of the receptor. Further studies will determine whether either inhibitor affects nuclear import or export of both the full-length receptor and the variant ARv7. The Hsp90 inhibitor, 9090, is included to show that, while it depletes both cell fractions of full-length AR protein, it is completely ineffective against ARv7 (Hsp90 binds to the LBD which is lacking in ARv7).

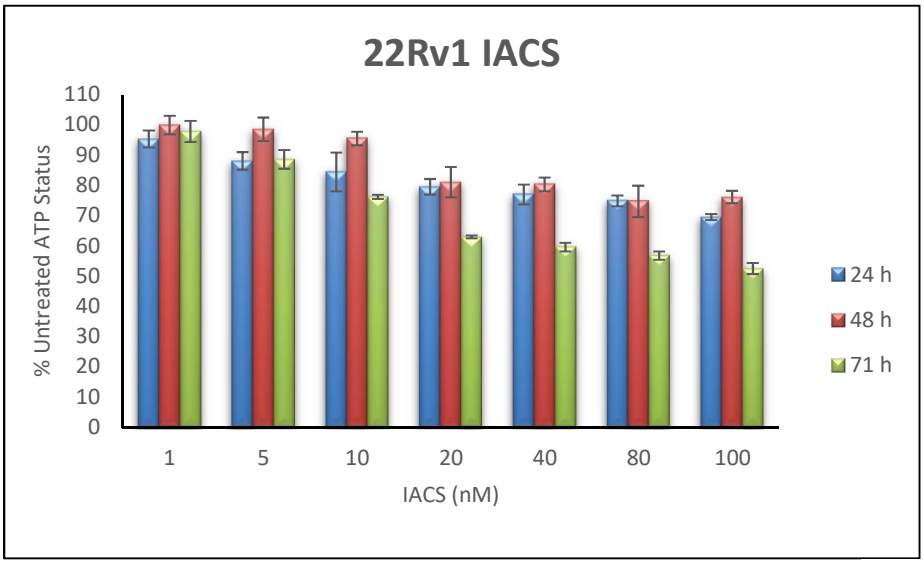


Specific Aim 3: The goal of Specific Aim 3 is to determine whether combinatorial activity can be demonstrated between Hsp40/Hsp70 inhibitors and specifically targeted metabolic inhibitors. Further, the ability of certain metabolic inhibitors to synergize with or restore sensitivity to enzalutamide and/or abiraterone is a sub-task of SA3. We have obtained preliminary in vitro evidence that certain metabolic inhibitors have significant growth inhibitory activity in 22Rv1 cells that are constitutively resistant to enzalutamide and abiraterone. Further, we have obtained preliminary evidence of reversal of resistance. IACS-010759 (IACS) is a synthetically derived inhibitor of mitochondrial complex I. Complex I oxidizes NADH supplied by glycolysis and the TCA cycle to help establish a proton gradient across the inner mitochondrial membrane while generating electrons which pass along the mitochondrial electron transport chain (ETC), comprised of complexes 1-4, resulting in oxidative phosphorylation and ATP synthesis. 22Rv1 CRPC cells are growth inhibited by low concentrations of IACS. These cells are primarily OxPhos driven and do not depend on glycolysis to any great extent under normal conditions. Thus, they are resistant to lactate dehydrogenase inhibition (LDHi), which depletes NAD+ from the cytoplasm (while inhibition of complex 1 inhibits NADH oxidation in mitochondria. Unexpectedly, we found that combination of LDHi and IACS concentrations that are themselves ineffective lead to rapid reduction in cellular ATP, while inducing a state of reductive stress (decreased NAD+/NADH ratio) in the cells (see Figure below for cellular ATP effects).

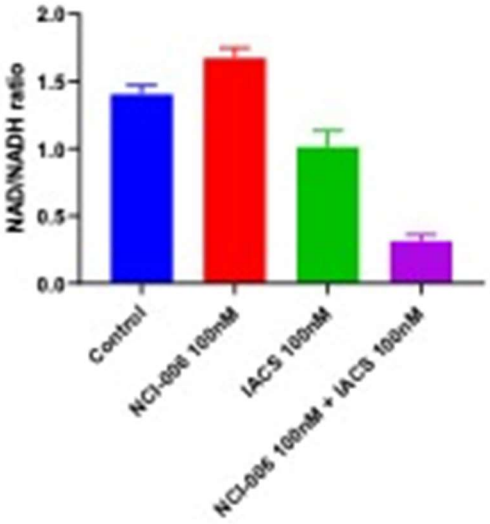


22Rv1 cells are unresponsive to LDHi alone (orange bars), as well being unresponsive to 10 or 50 nM IACS alone (blue and grey bars, respectively). Viability is assessed by Cell Titer Glo (measures ATP). However, combination of as little as 50 nM LDHi with 10 or 50 nM IACS causes marked cytotoxicity within 12 h (see bars within green rectangle).

In contrast to the results shown above, single agent IACS (up to 100 nM) has only moderate inhibitory activity toward 22Rv1 cells in vitro (see Figure below).



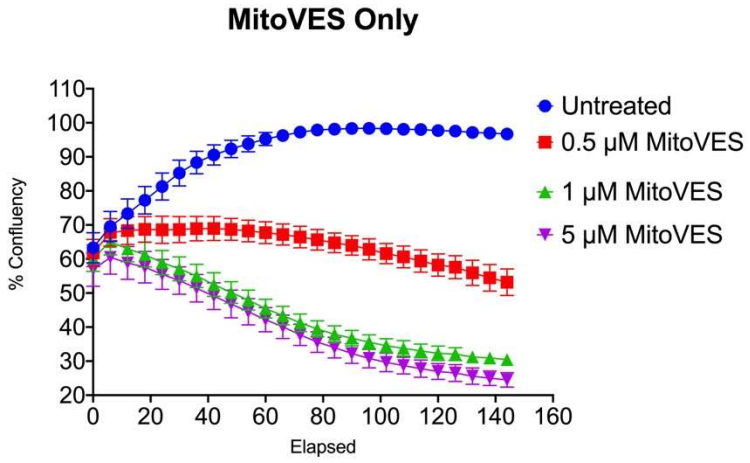
The relatively weak single agent activity of this complex I inhibitor, compared to the profound synergy obtained by combining low doses of IACS with low doses of LDH inhibitor, which itself is not active toward 22Rv1 cells as a single agent, prompted us to explore potential mechanisms underlying the synergy of this particular drug combination. Since mitochondrial complex I oxidizes NADH to NAD+ and cytosolic LDH catalyzes the reduction of pyruvate by NADH to form lactate and NAD+, we decided to determine the NAD+/NADH ratio in 22Rv1 cells treated with either complex I inhbitior or LDH inhibitor alone, or in combination. The data are presented in the figure to the right.



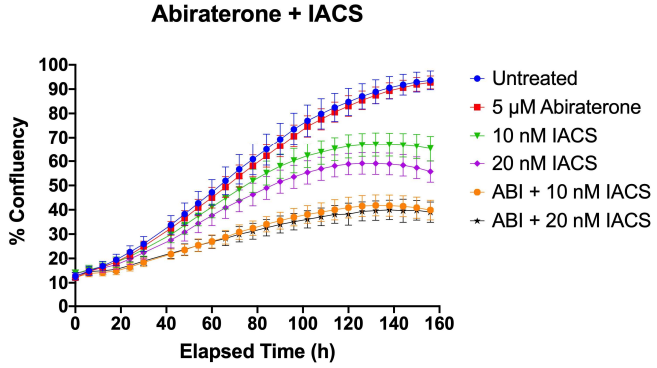
As these data show, LDHi (NCI-006, 100 nM/24 h) alone has no effect on the NAD^+/NADH ratio. IACS alone (100 nM/24 h) modestly reduces theNAD$^+$/NADH ratio. However, combination of both agents (purple bar) greatly decreases the NAD^+/NADH ratio, causing cellular rreductive stress. These results are consistent with the combinatorial

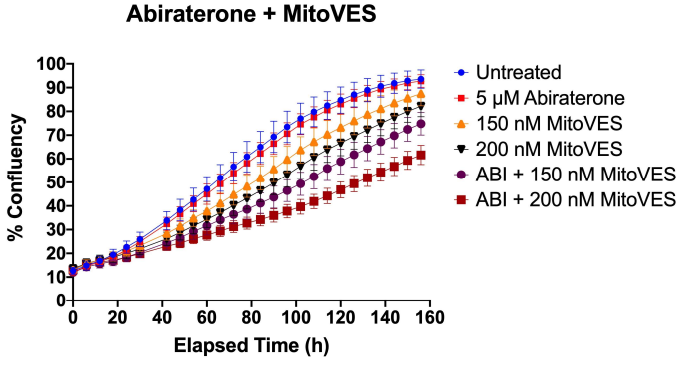
impact of these agents on cellular ATP levels, shown earlier. The data further suggest that 22Rv1 cells are highly sensitive to loss of NAD+.

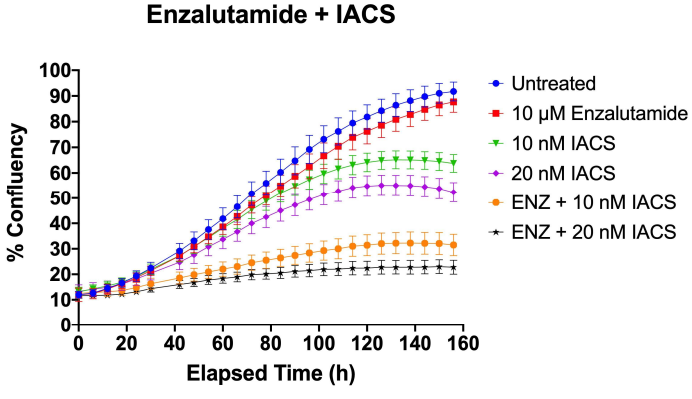
The mitochondrial ETC is comprised of four multi-subunit complexes. Like the complex I inhibitor IACS, the complex II inhibitor, MitoVES, displays dose-dependent, single-agent growth inhibition of 22Rv1 cells as visualized by Incucyte assay. However, single agent MitoVES causes 22Rv1 cell death at concecntrations of 1 μM and above (see Figure below).

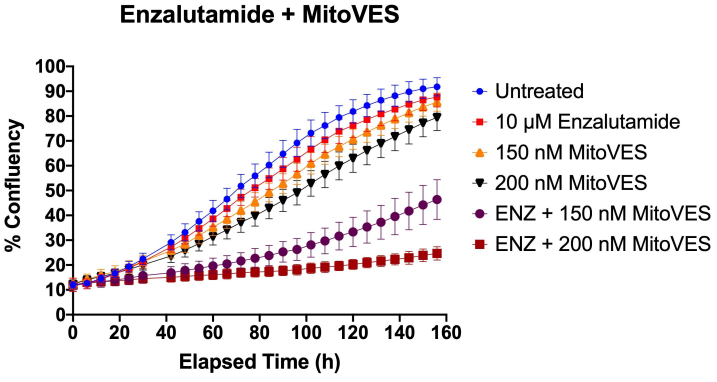


Further, we have obtained preliminary data showing that both the mitochondrial complex I inhibitor IACS and the mitochondrial complex II inhibitor MitoVES partially re-sensitize 22Rv1 cells to enzalutamide and abiraterone in vitro (see figures below). In these experiments, data were obtained by Incucyte assay. Elapsed culture time is shown on the x-axis (h) and percent confluency is shown on the y-axis. 22Rv1 cells are fully resistant to abiraterone (5 μM) and enzalutamide (10 μM) alone. However, IACS (10-20 nM) is synergistic with abiraterone (Incucyte data), as is MitoVES (200 nM). The two metabolic inhibitors provide even greater synergy with enzalutamide (see below). These data strongly suggest that enzalutamide, and abiraterone to some extent, are strongly dependent on proper function of the mitochondrial electron transport chain. Since MitoVES does not affect the NAD+/NADH ratio, we are currently exploring additional possible mechanisms underlying the synergy of complex II inhibition with both enzalutamide and abiraterone. Although preliminary, these data make clear that sensitivity of CRPC cells in vitro to both enzalutamide and abiraterone can be restored by targeting mitochondrial function.

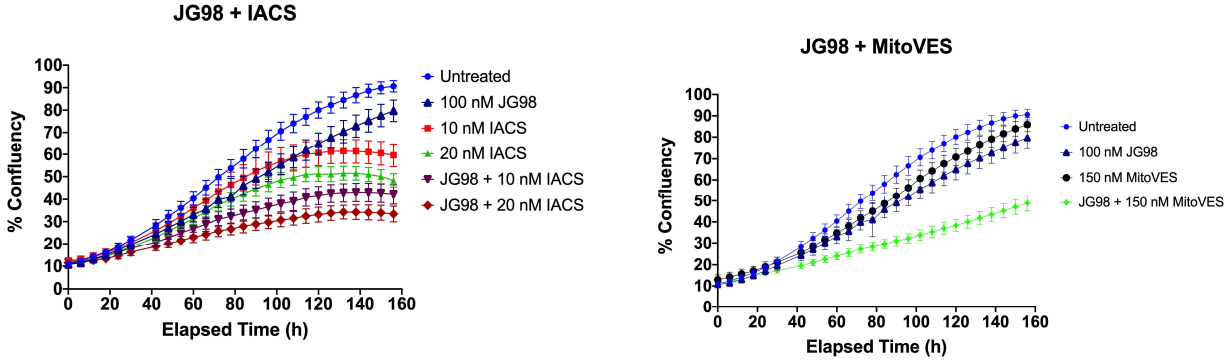








Another Major Task under Specific Aim 3 is to determine whether Hsp40 or Hsp70 inhibitors can synergized with metabolic inhibitors in inhibiting the growth of CRPC cells in vitro and in vivo. To date, we have begun to explore these combinations in vitro. We hope in year 2 to begin exploring some of these combinations in vivo as well. As demonstrated in the 2 panels of the figure below, the complex I inhibitor IACS and the Hsp70 inhibitor JG-98 display combinatorial activity (left panel). The same is true for the combination of JG98 and the complex II inhibitor MitoVES (right panel). The concentration of JG-98 used in these Incucyte experiments is only 100 nM, while IACS is used at 10 and 20 nM, and MitoVES is used at 150 nM.



What opportunities for training and professional development did the project provide?
How were the results disseminated to communities of interest? Krista Reynolds joined the project as a new Post-Bac Fellow in the Neckers lab. Her previous experience in college was in animal science, where she obtained proficiency in several useful molecular and cellular biology techniques. In the current project, she is being mentored by a Senior Research Fellow to develop further skills related to the studies described in the SOW, Specific Aims 2 and 3. She is also able to take relevant courses at the FAES Graduate School, which are offered to her at no cost. She will be able to attend seminars, workshops and conferences related to her work. As a participant in this award, Krista will obtain increased knowledge and skills relating to translational biology of CRPC.

How were the results disseminated to communities of interest?
Publication and virtual seminar

What do you plan to do during the next reporting period to accomplish the goals and objectives?
Work on Specific Aim 1 was and continues to be severely impacted by the Covid-19 pandemic in California. In year 2, Dr. Gestwicki will make every effort to complete the sub-tasks in SA1 in as timely a manner as possible. We will continue working on uncompleted sub-tasks in SA2 and SA3, with a particular effort put forth to begin animal studies, which were delayed to to impact of Covid-19. Conditions permitting, we hope to be on track with in vivo experiments by the end of year 2. In vitro experiments will continue as planned.

4. IMPACT: D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

The N-terminal domain (NTD) of the androgen receptor has become appreciated as a novel drug target that is retained in all androgen receptor splice variants with constitutive transcriptional activity. This provides a domain of the receptor shared by full-length and variant forms as a novel drug target. The importance of targeting metabolic dysregulation of CRPC (and particularly targeting their mitochondria) as a potential Achilles heel has also been validated as a novel therapeutic strategy.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS:

The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change: Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them: Work on SA1 and in vivo studies in SA2 and SA3 were significantly impacted by Covid-19. We hope to get back on schedule in year 2, but this will depend on the status of the pandemic in California and Maryland.

Changes that had a significant impact on expenditures
Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: Animal studies were delayed because of impact of Covid-19.

Significant changes in use or care of human subjects: Nothing to Report

Significant changes in use or care of vertebrate animals: Animal experiments have been delayed due to the Covid-19 pandemic, but these should resume within the next 3-4 months, conditions permitting.

Significant changes in use of biohazards and/or select agents: Nothing to Report

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications: **S**hao and Gestwicki (2020) Bioorgan. Med. Chem. Lett. 30:126954. PMC7205417.

Books or other non-periodical, one-time publications:
Nothing to Report

Other publications, conference papers, and presentations: **Presentation:** Neckers L. Targeting Castration-resistant prostate cancer with inhibitors of molecular chaperones Hsp40 and Hsp70. [to be presented on 11/18/2020 as part of the Proteostasis Consortium Weekly Seminar Series (virtual).

Website(s) or other Internet site(s): Nothing to Report

Technologies or techniques: Nothing to Report

Inventions, patent applications, and/or licenses: Nothing to Report

Other Products: Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	Jason Gestwicki
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
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Differential scanning fluorimetry (DSF) screen to identify inhibitors of Hsp60 protein–protein interactions†

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There are relatively few methods available for discovering inhibitors of the protein–protein interactions (PPIs) that hold together homo-oligomers. We envisioned that Differential Scanning Fluorimetry (DSF) might be a versatile way to discover this type of inhibitor because oligomers are often more thermally stable than monomers. Using the homo-heptameric chaperonin, Hsp60, as a model, we screened ~5000 diverse compounds in 384-well plates by DSF, revealing molecules that partially inhibited oligomerization. Because DSF does not require protein labeling or structural information, we propose that it could be a versatile way to uncover PPI inhibitors.

Introduction

There is great interest in finding small molecules that disrupt protein–protein interactions (PPIs).[1–4] Accordingly, many high throughput screening (HTS) methods have been developed for this purpose, such as fluorescence polarization (FP),[5] fluorescence resonance energy transfer (FRET),[6] nanoLuc complementation,[7] AlphaLISA[8] and others.[9] Together, these methods have proven to be powerful engines for the discovery of PPI inhibitors, with chemical probes for more than 200 + targets now reported.[10–12] However, despite this progress, specific subcategories of PPIs have remained relatively difficult to inhibit.[12] Here, we focus on one of these classes: the PPIs that occur within large, homo-oligomeric complexes, such as tetramers, hexamers, heptamers, *etc*. The PPIs within these complexes can be especially difficult to inhibit, because they involve interactions between multiple copies of the same protomer, making it challenging to deploy HTS methods that involve labelling of individual subunits. For example, FRET and AlphaLISA work best when the target is a hetero-dimer in which both partners can be individually labelled.

We wondered whether differential scanning fluorimetry (DSF, also called Thermofluor or thermal shift assay) might be a possible platform for identifying inhibitors of this difficult class of PPIs. Briefly, DSF is a rapid and inexpensive way to measure the melting temperature (T_m) of a protein sample.[13] In a DSF experiment, a sample is heated and its unfolding is estimated *via* binding to a fluorescent, solvatochromic dye, such as SYPRO Orange. This method has recently been shown to be suitable for low volume, high throughput applications.[14,15] However, DSF has not been widely applied to the identification of PPI inhibitors. Because oligomers are often more thermally stable than monomers, we envisioned that DSF might be adapted for the discovery of molecules that perturb oligomerization state.

As a model system, we focused on the homo-heptamer: heat shock protein 60 (Hsp60). Hsp60 is a barrel-shaped, molecular chaperone that promotes protein folding in the mitochondria[16] and it has been implicated as a potential target in cancer and neurodegenerative diseases.[17–19] Early efforts towards Hsp60 inhibitors have focused on its ATPase activity or ability to refold damaged proteins.[20–25] However, Hsp60 is only active when it assembles into oligomers, so inhibiting PPIs is another possible way to interrupt its function. Each Hsp60 monomer is composed of an apical, intermediate and equatorial domain. Inter-protomer contacts involving the apical and equatorial domains hold together the homo-heptameric rings. This architecture allows unfolded proteins to enter the heptamer's central cavity, where they are folded in the interior chamber.[26] Importantly, the assembly and function of Hsp60 is further regulated by ATP and the co-chaperone, Hsp10.[27] Specifically Hsp10 forms a "cap" on top of the heptamer to promote folding, while ATPase activity in each Hsp60 monomer drives progression through conformational cycles.[28] Because oligomer formation is required for Hsp60's

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chaperone functions, one way to interrupt its function would be to block PPIs between monomers.

Here, we report the development of a robust, DSF-based method for rapidly discerning between oligomeric and monomeric Hsp60. We then use this platform to discover inhibitors of Hsp60 assembly. Because DSF does not require labeling or structural knowledge, we anticipate that it might be a versatile platform for finding inhibitors of difficult PPIs.

Results and discussion

Using a previously reported method,[29] we expressed human Hsp60 in *E. coli* and purified it as monomer. The purified monomer was then partially reconstituted into oligomers through incubation with ATP. After purification by size-exclusion chromatography (SEC, ESI Fig. 1†), this process yielded samples that were largely oligomeric or monomeric, as judged by native gels (Fig. 1a).

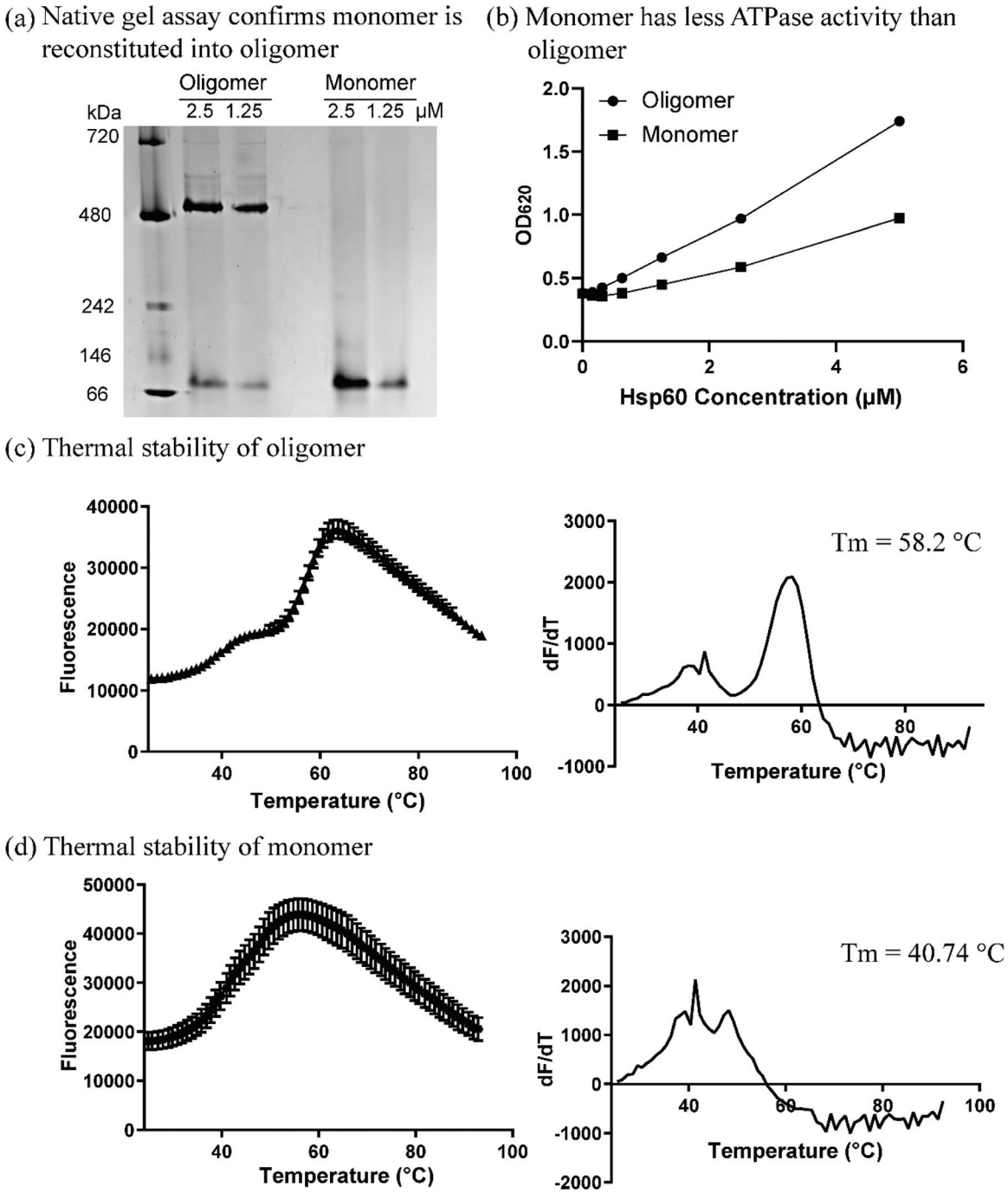

Fig. 1 Design rationale of DSF assay for screening Hsp60 oligomers. (a): Hsp60 monomers can be reconsituted into oligomers, as confirmed by native gel assays; (b): Monomer has decreased ATPase activity compared with Hsp60 oligomer; (c) and (d): comparison of the melting curves and first derivatives of Hsp60 oligomer and monomer samples. Results are the average of experiments performed in triplicate. Error bars represent SD.

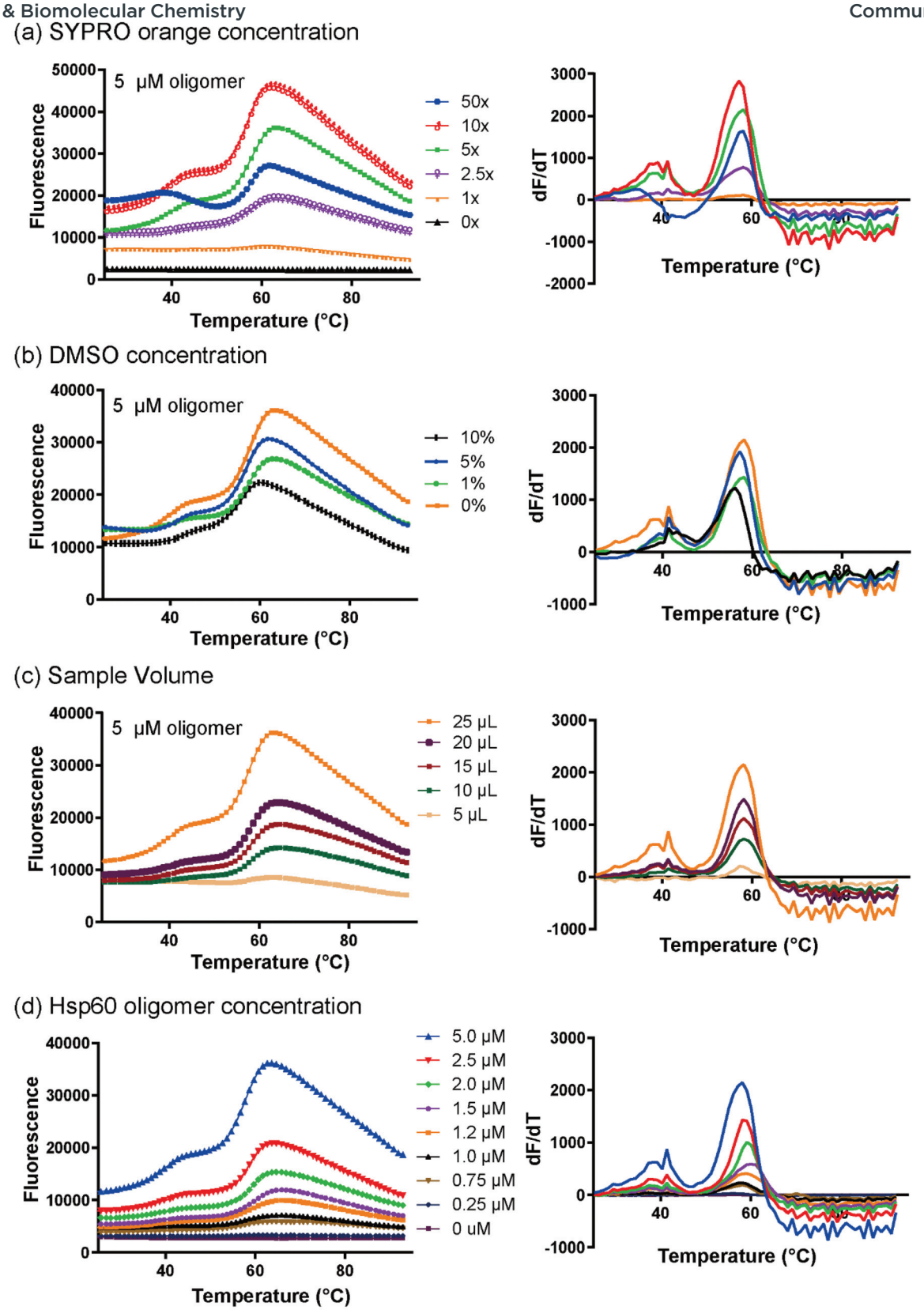

Fig. 2 Optimization of a DSF assay in 96 well plates. Optimization efforts focused on investigating: (a) SYPRO Orange concentration; (b) DMSO concentration; (c) sample volume; and (d) Hsp60 oligomer concentration. Results shown here are from one replicate for clarity. Experiments performed in triplicate with error bars representing SD are shown ESI Fig. 2.†

Consistent with the literature,[30] the Hsp60 oligomers had significantly greater, steady-state ATPase activity, compared to the monomers (Fig. 1b).

With these samples in-hand, we wondered whether Hsp60 oligomers might have a substantially higher T_m value than the monomers. Indeed, using DSF, we found that the T_m value of the Hsp60 oligomer was 58.2 ± 0.4 °C (Fig. 1c), while the T_m for the Hsp60 monomer was 40.7 ± 0.5 °C (Fig. 1d). Upon examination of the DSF curve for the oligomer, we noted a minor population with a lower melting temperature (~40 °C), which is likely the sub-population of Hsp60 that remains as a monomer in this sample (see Fig. 1a). This observation was encouraging to us because it suggested that DSF might be sensitive enough to discriminate between the oligomer and monomer populations. Guided by this idea, we moved to optimize the DSF assay for high throughput screening.

There have been few reported DSF screens; therefore we will report the highlights of the optimization procedure, with additional information provided in the ESI.† As a first step, we examined the role of SYPRO Orange concentration. Using a solution of Hsp60 oligomers (5 μM), we varied the dye level and found that the fluorescence signal increased up to 10X (Fig. 2a). At 50X, the signal decreased, likely due to aggregation. Based on these observation, we selected 5X SYPRO Orange for further assay development. Using a similar, systematic workflow, we optimized the concentration of DMSO, assay volume and the concentration of Hsp60 oligomers (Fig. 2b–d). Based on these results, we selected 2.5 μM Hsp60, 25 μL volume and 4% DMSO as the final parameters (see ESI† for details). In pilot screens, we tested 88 kinase inhibitors (20 μM; single concentration) in 96-well plates and identified 2 compounds that reduced the T_m value by at least 3 SD (Fig. 3). Kinase inhibitors were selected for this pilot because Hsp60 contains a well-defined, ATP-binding cleft, although the topology of this site is significantly different than kinases, so the relatively low hit rate was expected.

The success of the pilot screen in 96-well plates motivated us to further miniaturize the assay into 384-well format. During this miniaturization process, we found that decreasing the volume below 25 μL gave rise to poor signal : noise

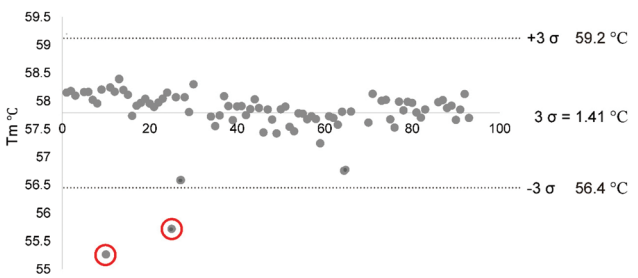

Fig. 3 Summary of the DSF pilot screen. 88 kinase inhibitors were screened at 20 μM. Molecules that decreased the T_m value of Hsp60 oligomer (>3SD) are shown in red circles.

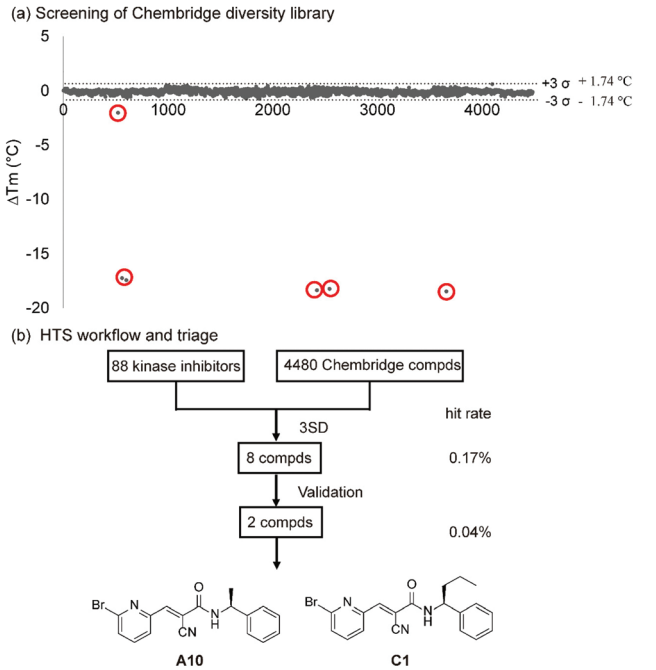

Fig. 4 Summary of the DSF screen. (a) 4480 compounds from the Chembridge diversity library were screened. Molecules that decreased the T_m value of Hsp60 oligomer (>3SD) are shown in red circles; (b) The chemical structures of 2 compounds are shown, which were confirmed as hits from the screening campain.

(ESI Fig. 3†), so we finalized the screening conditions as: 2.5 μM protein, 25 μL volume and 5X SYPRO Orange. Next, using Hsp60 monomer as a positive control and DMSO (4%) as a negative control, we determined that the 384-well assay had an excellent Z' = 0.93 (T_m oligomer = 57.4 ± 0.2 °C, T_m monomer = 40.5 ± 0.2 °C). Then, we screened 4840 compounds from the ChemBridge diversity library at 20 μM. The assay performance was excellent, with low signal : noise and minimal plate variation or drift (Fig. 4a). In total, the two screens yielded 8 active compounds (3SD; 0.17% hit rate). Two of the active molecules showed high initial starting fluorescence, which suggested artifacts, so they were removed. All the remaining molecules were re-tested at 20 μM in the DSF assay. Four molecules were found to be false-positives, while two of them validated (Fig. 4b), giving a final, confirmed hit rate of 0.04%.

One of the active molecules, A10, was re-purchased and its identity confirmed by mass spectrometry (ESI Fig. 4†). Then, the dose responsive activity of A10 was measured in the DSF assay, showing that it caused an increase in the low melting, monomer peak (~40 °C) (Fig. 5a). Native gels confirmed that A10 indeed increased the relative amount of monomer (Fig. 5b and ESI Fig. 6†), with an EC_{50} around 5.0 μM. Notably, A10 did not completely block oligomer formation; significant amounts of heptamer remained at even the highest A10 concentration. The binding site(s) of A10 are not clear, so the reasons for this mechanisms for this activity are not known. Regardless, we next tested the hypothesis that an

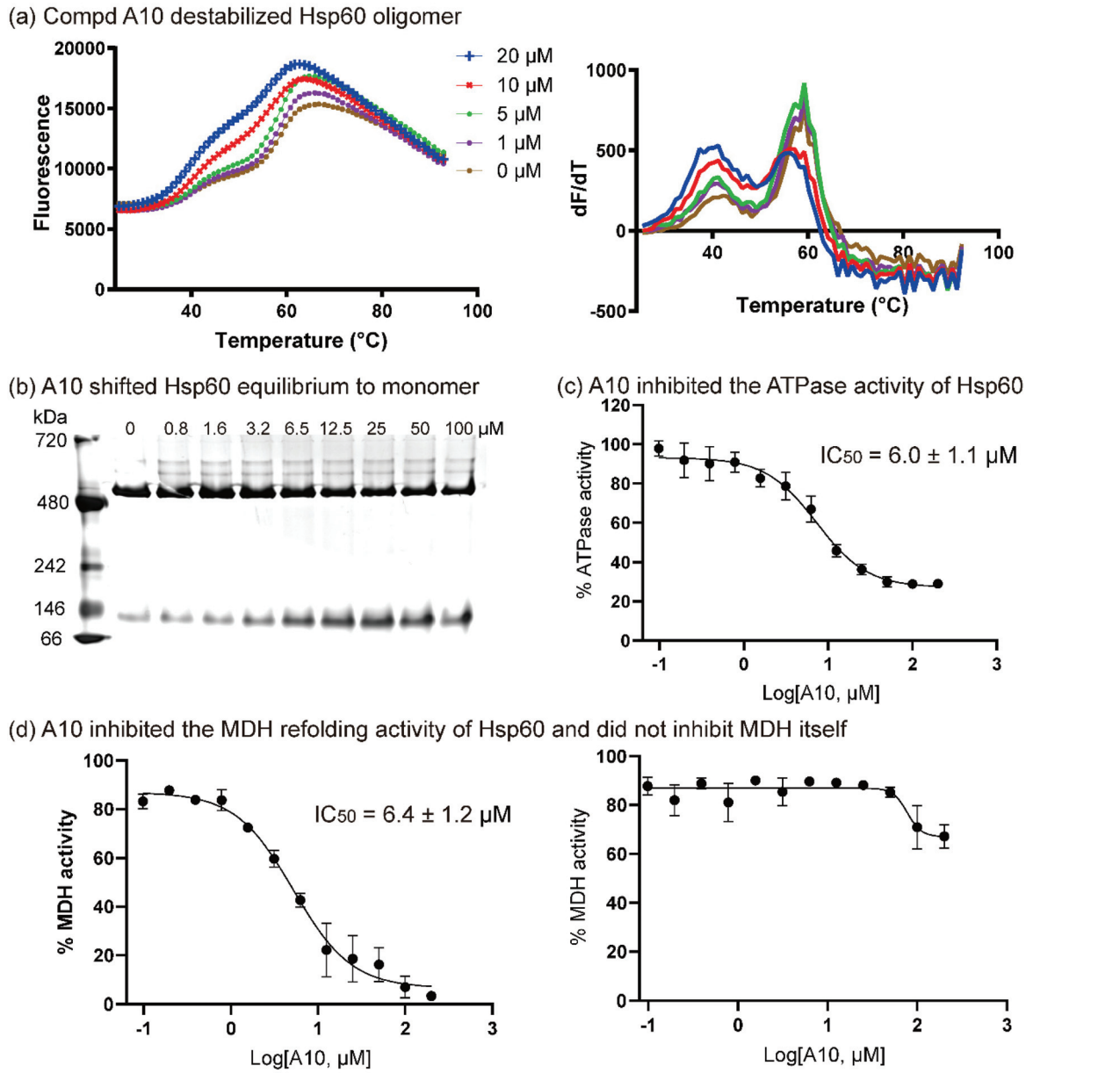

Fig. 5 Validation of hit compound A10 as a putative Hsp60 oligomer destabilizer. (a) Compound A10 enhanced the levels of Hsp60 monomer in a dose-dependent manner. Melting curves of Hsp60 oligomer treated with various concentrations of compound A10 are shown, along with the first derivatives. The curves shown here are from one replicate for clarity. Experiments performed in triplicate with error bars representing SD are shown in ESI Fig. 5†; (b) Compound A10 partially disassociated Hsp60 oligomer into monomer in a native gel assay; (c) Compound A10 inhibited Hsp10-mediated ATP turnover; (d) A10 inhibited the MDH refolding activity of Hsp60 in a dose-dependent manner and only mildly inhibited MDH itself at high concentrations.

inhibitor of Hsp60's PPIs might interrupt its function. Because Hsp60 monomer has lower ATPase activity than oligomer (see Fig. 1b), we hypothesized that A10 should reduce overall nucleotide activity. Consistent with this idea, treatment inhibited ATPase activity with an IC_{50} value ~6 μM (Fig. 5c). Then, we investigated whether A10 might inhibit Hsp60's ability to refold denatured proteins.[26] We first confirmed that Hsp60 could refold denatured malate dehydrogenase (MDH) into an enzymatically active state, in an Hsp10- and ATP-dependent manner (ESI Fig. 7†). Using this protocol, we found that A10 could block Hsp60's refolding activity, with an IC_{50} value that is consistent with the other assays (~6 μM; Fig. 5d). To exclude direct inhibitory effects of A10 on MDH in this assay, we showed that the enzymatic activity of native MDH was largely unaffected by A10 in the absence of Hsp60 (Fig. 5d). Together, these proof-of-concept results suggest that DSF can indeed be used to identify inhibitors of a difficult PPI.

Conclusions

High throughput assays for identifying inhibitors of the most difficult PPIs are still needed. Using the homo-heptamer, Hsp60, as a model, we showed that DSF can be used to discriminate between the oligomeric and monomeric states and, further, that it can be used to screen for inhibitors in 384-well plate format. In pilot screens, we identified compound A10 as being capable of partially disrupting Hsp60 PPIs. Further work will be needed to determine A10's binding site and improve its potency, but this scaffold might be optimized to create chemical probes for this enigmatic target.[31]

These results suggest that DSF could be a powerful addition to the methods available for discovering PPI inhibitors, especially those that are homo-oligomeric. This goal is important because an analysis of structures available in the Protein Database (PDB) showed that proteins self-interact as homo-oligomers more commonly than expected by chance,[32] involving up to 70% of eukaryotic proteins.[33] Indeed, many potential drug targets are active as homo-oligomers, including cell surface receptors[34] and their ligands, such as chemokines.[35] Yet, the PPIs within these homo-oligomers are largely unexplored surfaces for drug discovery, and, as mentioned above, most HTS methods for finding inhibitors, such as FRET or nanoLuc complementation, are not readily adapted to systems larger than dimers (*e.g.* trimer, heptamers, *etc.*). Additionally, it is not always advantageous to inhibit PPIs; sometimes, the goal may be to stabilize them.[36,37] Here, DSF might also be a useful platform because both the oligomer and monomer T_m values can be resolved and quantified, perhaps allowing for the identification of molecules that promote, rather than inhibit, oligomer formation. For example, trimer-disrupting mutations in the tumor suppressor, Smad4, are linked to cancer,[38] so a translational goal might be to identify small molecules that stabilize the native state. Thus, in the discovery of both inhibitors and activators, we envision that DSF could be a powerful addition to the methods available for screening homo-oligomeric protein systems.

Conflicts of interest

There are no conflicts of interest to declare.

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