Crystallization of Heat-Shock Protein 90 Bound with Bruceantin and NSC145366

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Introduction

Hsp90 (90 kDa heat-shock protein) is a central protein in a molecular Chaperone machine driven by ATP hydrolysis and is essential for activating, stabilizing, and regulating[1] over 200 client proteins[2], many of which fall into two categories: signaling kinases and transcription factors[3]. There are four isoforms in the Hsp90 family: GRP94, Hsp90α, Hap90β, and TRAP1[4]. Each isoform is a flexible homodimer with an N-terminal domain (NTD), middle domain (MD), and C-terminal domain (CTD). The CTD participates in homodimerization and both the NTD and MD are involved in ATP hydrolysis. When it comes to ATP hydrolysis, Hsp90 has three conformational changes: open, semi-open, and closed[1]. The NTD contains a deep ATP-binding pocket and a short segment called the "ATP lid." When Hsp90 binds ATP, this lid closes over the binding pocket, resulting in a chain of conformational changes throughout the protein that completes the active site of the "split ATPase"[3] (closed conformation)[5]. These conformational changes make ATP hydrolysis by Hsp90 very slow. ATP hydrolysis by Hsp90 is also regulated by interactions with client proteins, post-translational modifications, and the sequential binding and release of co-Chaperones[3]. Ultimately, Hsp90 is regulated by the heat-shock response (HSR) and heat-shock factor 1 (HSF1). The HSR is activated by stimuli such as bacterial/viral infections, elevated temperatures, and oxidative stress. During such stimuli, denatured protein levels increase, which converts the cytoplasmic non-DNA-binding HSF1 into a homotrimer with DNA-binding activity. HSF1, which before had a repressed association with Hsp90, is released, undergoes homotrimerization, and translocates to the nucleus where it binds to heat-shock elements (HSE). HSF1 then undergoes a series of phosphorylations that convert it into a transcription factor that proceeds to upregulate Hsp90 and other Chaperones and co-Chaperones[6].

Hsp90 is often overexpressed in cancer cells and is responsible for stabilizing many oncogenic proteins[5]. In fact, proteins associated with the 10 hallmarks of cancer are all dependent on Hsp90. This makes Hsp90 a promising target for cancer treatment. There have been several inhibitors identified that exhibit pan Hsp90 inhibition, meaning they inhibit all four isoforms. However, many of these have produced cardiotoxicity, gastrointestinal toxicity, and/or ocular toxicity effects. Pan Hsp90 inhibition also activates the HSR, which further induces Hsp90 expression[4]. Because of this, the dosage of inhibitor must be increased, but this isn't feasible because of the toxicity. Inhibitors that that target the NTD (NT-inhibitors) have been used in over 24 clinical trials for cancer treatment, and they bind to the ATP-binding pocket, inhibiting the Chaperone activity of Hsp90 and leading to client protein degradation. But, because of the activation of the HSR and the toxicity levels, most of these trials haven't been very successful. Therefore, Hsp90 isoform-selective inhibitors that allosterically inhibit the CTD and MD, as it is hoped that this will still inhibit ATPase and Chaperone activity without activating the HSR. There have been many potential allosteric binding sites in the CTD and MD identified via computer models, however most studies done use the closed form of Hsp90 as a template as that is the only computer model available. The published crystal structure of Hsp90 is not complete, as it lacks structural information of important functional regions.

One promising group of compounds are coumarins, which have been shown to bind to the CTD of Hsp90 without activating the HSR. But, the affinity hasn't been very high and it's difficult to develop high-affinity inhibitors without knowing all the structural information of Hsp90. In one study, a coumarin derivative, MDCC (7-diethylamino-3-[N-(2-maleimidoethyl)carbamoyl]coumarin), was used to label human Hsp90α so that the crystal structure of the MD and CTD (Hsp90αMC) with the label could be determined. MDCC, as a coumarin derivative, has a common coumarin core. There is an identified hydrophobic binding pocket which is a potential allosteric binding site for Hsp90 inhibitors with the common coumarin core. In this study, it was found from the MDCC-Hsp90αMC crystal structure that the binding of MDCC causes a conformational change that locks Hsp90 in an inactive hexamer by orienting a key residue, Arg400, away from the ATP-binding pocket. This makes it so that the stabilization of the NTD and MD required for optimal ATPase activity is impossible. It also inhibits productive client protein binding by blocking the CTD. The crystal structure also suggested that the Hsp90α structural regions of the catalytic loop and the CTD and Src loop are vital for ATP hydrolysis and client protein binding respectively[1]. This indicates that coumarin derivatives are promising candidates for Hsp90 inhibitors.

Another compound that has shown promise, and is one of the subjects of my Honors Thesis, is NSC145366. In a different study[5], they explored the biochemical mechanism by which NSC modulates Hsp90 function. Through different assays, it was shown that NSC interacts with human Hsp90α and Hsp90β via the CTD and inhibits both ATPase and Chaperone activity. It also didn't illicit a detectable HSR and showed broad activity across the NCI-60 cancer cell panel, however it was not as effective in NCI xenograft models. But, there is still a lot that could be learned from NSC, since it appears to have a unique mechanism of Hsp90 inhibition as a noncompetitive inhibitor with respect to ATP.

Another promising compound, and the second subject of my Honors Thesis, is bruceantin (BCT). BCT, an antimalarial agent, was used in a study[2] on castration-resistant prostate cancer (CRPC). Hsp90 is responsible for folding androgen receptor (AR), an androgen-activated transcription factor that functions in normal prostate development as well as prostate cancer (PCa) development and progression to metastatic CRPC. This makes AR, specifically full-length AR (AR-FL), and androgen synthesis common targets for PCa treatments. However, the common treatments become ineffective once the cancer progresses to CRPC because it is resistant. Because Hsp90 is responsible for activating and stabilizing AR, Hsp90 inhibitors could be effective targets for PCa and CRPC treatment. BCT is one of these Hsp90 inhibitors. In this study, it was shown to bind to Hsp90 and inhibit the chaperon activity. This was shown to lead to AR-FL and AR-variant 7 (AR-V7) degradation and appeared to inhibit CRPC tumor growth and metastasis in male nude mice. It is not known exactly where BTC binds, but it may bind to the ATP-binding pocket, which would make it a competitive inhibitor.

The objective of my Honors Thesis was to crystallize Hsp90 linked separately with BCT and NSC so that the structure can be further analyzed to determine if and how the inhibitors are binding to Hsp90. This will give insight into the mechanisms of the inhibitors and the possible effects on Hsp90 function.

Methods[1]

Protein expression and purification

Recombinant Hsp90 protein was expressed in *Escherichia coli* (cell line: Bl21gold DE3) and purified by Ni-NTA (nitrilotriacetic acid) affinity purification after homogenization of the bacterial cells. The Hsp90 was purified from the cell lysate with binding buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 10% glycerol, pH 7.8) and Ni-NTA affinity column and eluted with elution buffer (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, 10% glycerol, pH 7.8) and the His-tag was cleaved by TEV (tobacco etch virus) protease (mass ratio was 1:100) while dialyzing against loading buffer at 4℃ overnight. The Hsp90 was then collected from a second Ni-NTA column as the flowthrough.

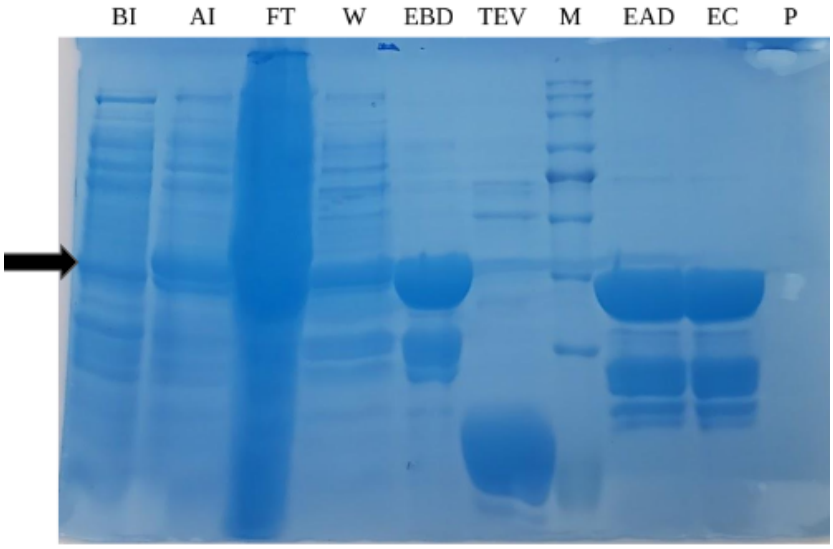


Figure 1

BI- Before Induction; AI- After Induction; FT- Ni I Flow Through; W- Ni I Wash; EBD- Ni I Elution Before Digestion; TEV- TEV; M- Molecular Weight Marker; EAD- Ni I Elution After Digestion; EC- Elution Concentrated; P- Permeate; Arrow points out Hsp90 for all lanes

The Hsp90 was then purified by Size Exclusion Chromatography (SEC) through the superset 200 SEC column and in SEC buffer (20 mM HEPES, 150 mM NaCl, pH 7.2).

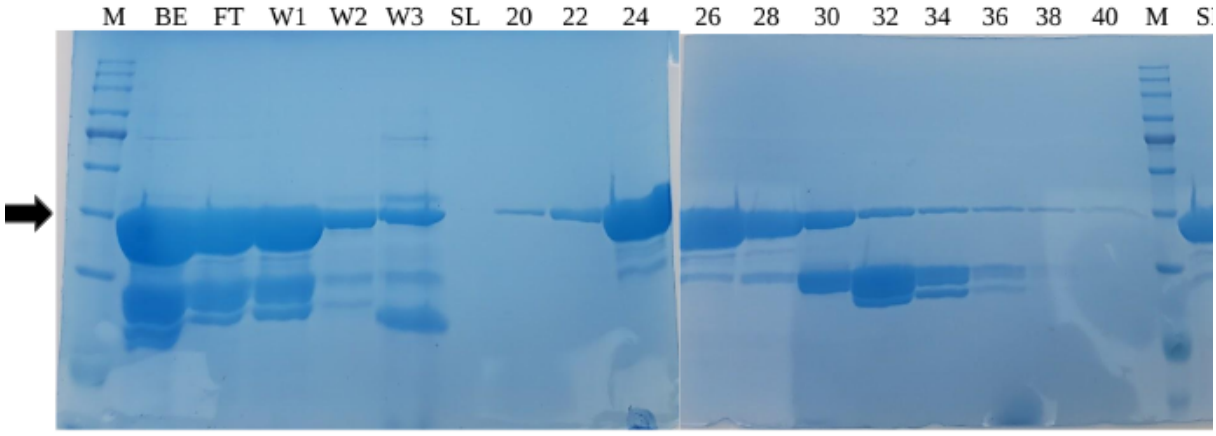


Figure 2

M- Molecular Weight Marker; BE- Buffer Exchange; FT- Ni II Flow Through; W1- Ni II Wash 1; W2- Ni II Wash 2; W3- Ni II Wash 3; SL- SEC Load; 20-40- Elution Fractions 20-40; SP- SEC Pool; Arrow points out Hsp90 for all

lanes

Crystallization of Hsp90 With BCT and NSC

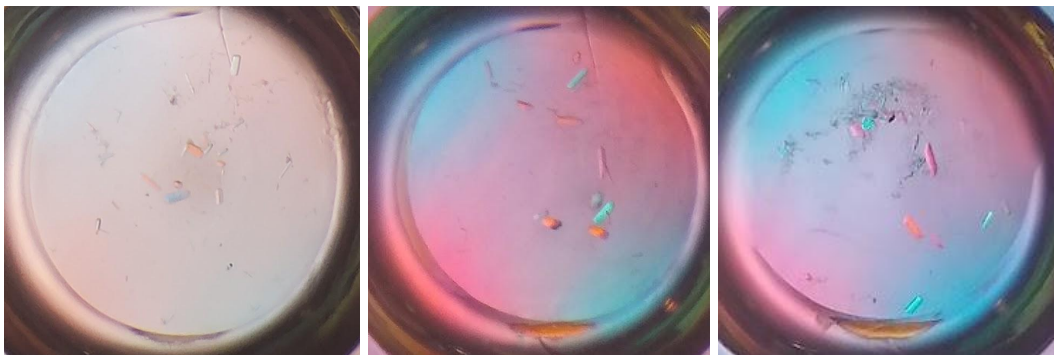
Hsp90 (range of concentrations) was mixed with 40 mM BCT and 40 mM NSC, and, later on, the Hsp90/NSC mixture was centrifuged for 10 minutes at 14,000 rpm to remove precipitate. The supernatant was used for crystallization. The complexes were crystallized using the sitting-drop vapor-diffusion method at room temperature (23ºC) in the conditions listed in Table 1.

	[Inhibitor] (mM)	Buffer	Buffer : Complex (uL)		[Inhibitor] (mM)	Buffer	Buffer : Complex (uL)
Hsp90/BCT	2	15% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5	Hsp90/NSC	2	12% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5
	2	18% PEG 3350, 0.1M ammonium citrate tribasic pH 6.5	0.5 : 0.5		2	15% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5
	2	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5		2	18% PEG 3350, 0.1M ammonium citrate tribasic pH 6.5	0.5 : 0.5
	5	18% PEG 3350, 0.1M ammonium citrate tribasic pH 6.5	0.5 : 0.5		5	18% PEG 3350, 0.1M ammonium citrate tribasic pH 6.5	0.5 : 0.5
	5	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5		2	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5
	5	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.7		4	18% PEG 3350, 20% glycerol, 0.1M ammonium citrate tribasic pH	0.5 : 0.5
	5	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.7 : 0.7		5	18% PEG 3350, 20% glycerol, 0.1M ammonium citrate tribasic pH	0.5 : 0.5
	7	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5		5	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5
	7	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.7		5	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.7
	7	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.7 : 0.5		5	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.7 : 0.7
	7	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.7 : 0.7		7	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5
	2	20% PEG 3350, 0.1M ammonium citrate tribasic pH 6.5	0.5 : 0.5		7	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.7
	2	20% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5		7	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.7 : 0.5
					7	18% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.7 : 0.7
					2	20% PEG 3350, 0.1M ammonium citrate tribasic pH 6.5	0.5 : 0.5
					2	20% PEG 3350, 0.1M ammonium citrate tribasic pH 7.0	0.5 : 0.5

Table 1
Crystallization conditions

Crystals were harvested and placed in cryo-protectant (20% glycerol, 5 mM BCT for Hsp90/BCT and 20% glycerol, 5 mM NSC for Hsp90/NSC) before freezing in liquid nitrogen.

Results



(a) (b) (c)

Figure 3

(a) Hsp90/BCT (2 mM) crystals, formed in 15% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.5:0.5
(b) Hsp90/BCT (2 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 6.5, 0.5:0.5
(c) Hsp90/BCT (2 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.5:0.5

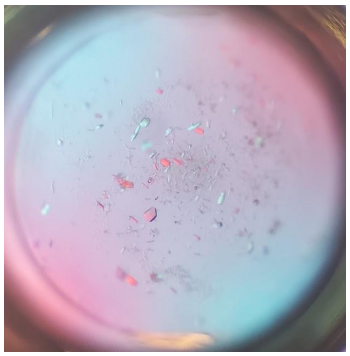
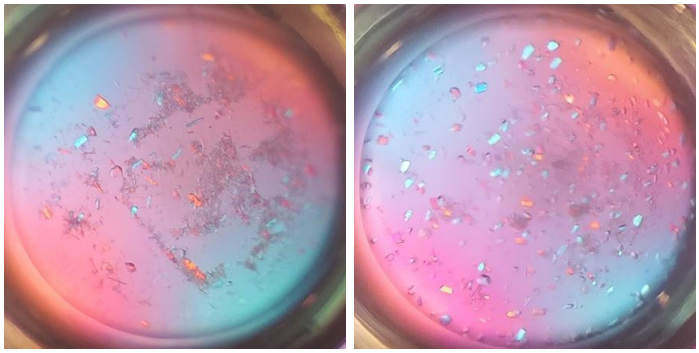


Figure 4

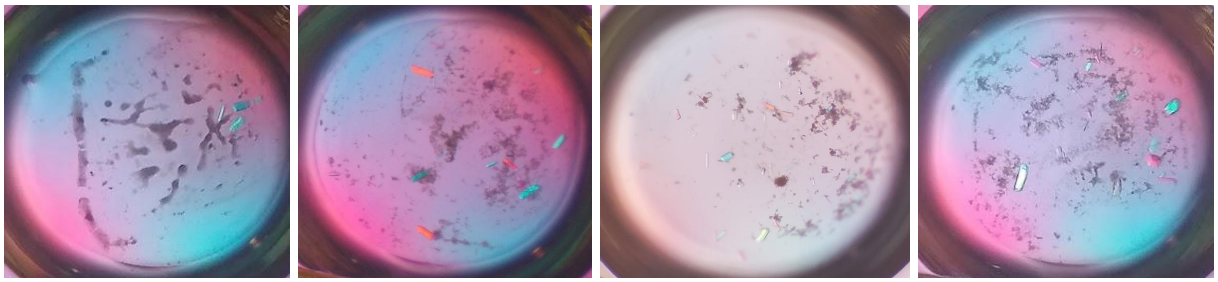
Hsp90/BCT (5 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.5:0.5



(a) (b)

Figure 5

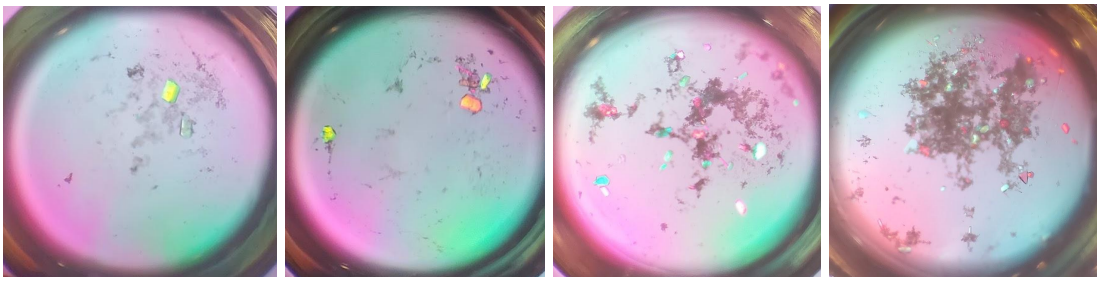
(a) Hsp90/BCT (7 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.7:0.5
(b) Hsp90/BCT (7 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.7:0.7



(a) (b) (c) (d)

Figure 6

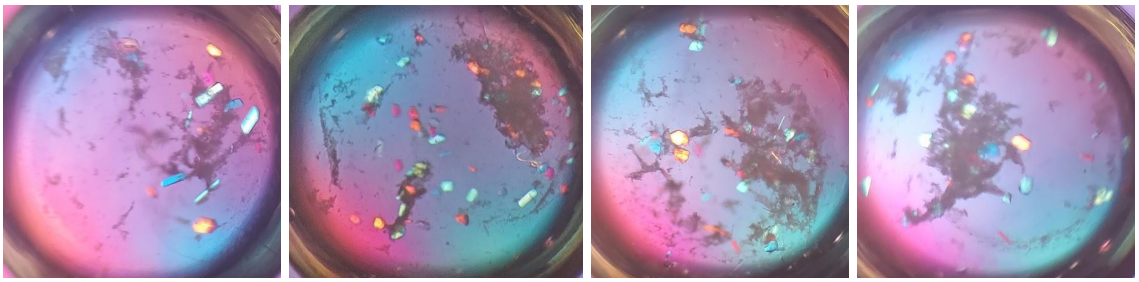
(a) Hsp90/NSC (2 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 6.5, 0.5:0.5
(b) Hsp90/NSC (2 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.5:0.5
(c) Hsp90/NSC (2 mM) crystals, formed in 20% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 6.5, 0.5:0.5
(d) Hsp90/NSC (2 mM) crystals, formed in 20% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.5:0.5



(a) (b) (c) (d)

Figure 6

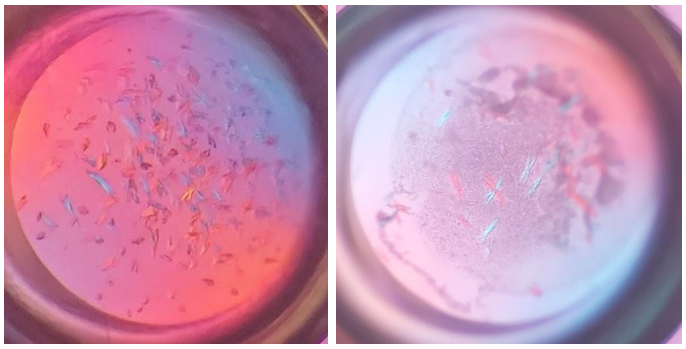
(a)-(c) Hsp90/NSC (5 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.5:0.5
(d) Hsp90/NSC (5 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.7:0.7



(a) (b) (c) (d)

Figure 7

(a) Hsp90/NSC (7 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.5:0.5
(b) Hsp90/NSC (7 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.5:0.7
(c) Hsp90/NSC (7 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.7:0.5
(d) Hsp90/NSC (7 mM) crystals, formed in 18% PEG3350, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.7:0.7



(a) (b)

Figure 8

(a)-(b) Hsp90/NSC (5 mM) crystals, formed in 18% PEG3350, 20% Glycerol, 0.1 M Ammonium Citrate Tribasic, pH 7.0, 0.7:0.5

Hsp90/BCT crystals that formed were, in general, smaller than Hsp90/NSC crystals. For both BCT and NSC, crystals developed after about 1 month in buffer without glycerol. In buffer with glycerol, Hsp90/NSC crystals developed after about 3 days and had a different morphology. Hsp90/BCT crystals appeared to be developing, but were very small and clustered together.

When both BCT and NSC crystals were harvested, many of them cracked when placed in the cryo-protectant and no data was obtained from X-ray diffraction because the crystals did not diffract well. This is because of the freezing conditions and because the stock solutions of BCT and NSC were mixed with DMSO, which made the protein complex unstable. For this reason, glycerol was added to the crystallization conditions.

Conclusion

Going forward, the stock solutions of BCT and NSC will be mixed with SEC buffer (the same buffer Hsp90 is kept in) instead of DMSO. Also, crystallization buffers will contain 20% glycerol, and further optimization is needed for Hsp90/BCT in order to obtain larger crystals. Once a sufficient number of crystals have been obtained under these conditions, they will be harvested and sent off for X-ray diffraction so the structure can be determined and analyzed. This will give insight into the mechanisms of inhibition of BCT and NSC and the possible effects on Hsp90 function. Based on how BCT and NSC inhibit Hsp90, specifically if they don't trigger the HSR, they may be good candidates for cancer treatment.

References

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