

# Analogues of the Allosteric Heat Shock Protein 70 (Hsp70) Inhibitor, MKT-077, As Anti-Cancer Agents

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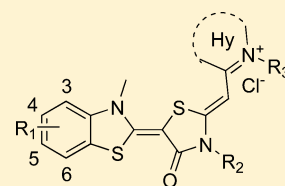
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## S Supporting Information

**ABSTRACT:** The rhodacyanine, MKT-077, has antiproliferative activity against cancer cell lines through its ability to inhibit members of the heat shock protein 70 (Hsp70) family of molecular chaperones. However, MKT-077 is rapidly metabolized, which limits its use as either a chemical probe or potential therapeutic. We report the synthesis and characterization of MKT-077 analogues designed for greater stability. The most potent molecules, such as **30** (JG-98), were at least 3-fold more active than MKT-077 against the breast cancer cell lines MDA-MB-231 and MCF-7 (EC<sub>50</sub> values of 0.4 ± 0.03 and 0.7 ± 0.2 μM, respectively). The analogues modestly destabilized the chaperone clients, Akt1 and Raf1, and induced apoptosis in these cells. Further, the microsomal half-life of JG-98 was improved at least 7-fold (*t*<sub>1/2</sub> = 37 min) compared to MKT-077 (*t*<sub>1/2</sub> < 5 min). Finally, NMR titration experiments suggested that these analogues bind an allosteric site that is known to accommodate MKT-077. These studies advance MKT-077 analogues as chemical probes for studying Hsp70s roles in cancer.

**KEYWORDS:** Breast cancer, mortalin, Hsp90, proteostasis, p53



Heat shock protein 70 (Hsp70) is an ATP-dependent molecular chaperone that plays essential roles in protein homeostasis.<sup>1,2</sup> There are 13 members of the Hsp70 family in mammals,<sup>3</sup> including the constitutively expressed, cytosolic heat shock cognate 70 (Hsc70, HSPA8), the stress-inducible, cytoplasmic heat shock protein 70 (Hsp72, HSP1A1), and the mitochondrial Hsp70 (mtHsp70, mot-2, HSPA9). These chaperones bind and stabilize a wide array of proteins, including many kinases and transcription factors involved in pro-survival signaling.<sup>2,4,5</sup> Accordingly, high levels of Hsp70s are associated with immortalized cells and poor prognosis in multiple types of cancer, including breast cancer, endometrial cancer, and cervical cancer.<sup>6</sup> Many cancer cells appear to become addicted to these high chaperone levels because dual silencing of Hsc70 and Hsp72 leads to cell death in tumor cells, but not normal fibroblasts.<sup>7</sup> For these reasons, Hsp70 has emerged as a potential target for anticancer agents.<sup>1</sup> Inhibition of Hsp70s might be especially powerful in combination with other chemotherapies because the levels of Hsp72 are dramatically increased after exposure to other therapies, such as Hsp90 inhibitors,<sup>8</sup> proteasome inhibitors,<sup>9</sup> and radiation.<sup>10</sup>

MKT-077 is a cationic rhodacyanine that was originally developed as a dye and later found to have promising antiproliferative activity against numerous cancer cell lines, including those derived from bladder carcinomas, colon carcinomas, breast carcinomas, melanomas, and pancreatic carcinomas.<sup>11–13</sup> Wadhwa and colleagues used a biotinylated version of MKT-077 to show that this compound derives its anticancer activity by binding to members of the Hsp70 family, including Hsc70 and mtHsp70.<sup>13–15</sup> Subsequent work showed that at least part of the antiproliferative activity of

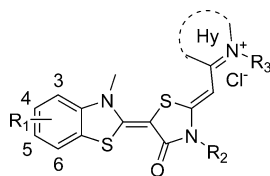
MKT-077 involved release of mtHsp70 from the tumor suppressor, p53.<sup>13</sup> Recently, we used NMR to discover that MKT-077 binds Hsc70 at an allosteric site within the nucleotide-binding domain.<sup>16</sup> Interestingly, this site is conserved in the major Hsp70 family members, including Hsp72 and mtHsp70, and it appears to be important in the chaperone's ATP hydrolysis cycle because an analogue of MKT-077, YM-01, disrupts nucleotide turnover and impacts chaperone functions.<sup>14,16,17</sup> However, MKT-077 and YM-01 are not toxic to fibroblasts or normal epithelial cells.<sup>18</sup> Thus, MKT-077 and its analogues only appear to be toxic to cells that are addicted to Hsp70s, reminiscent of the mechanisms invoked for Hsp90 and proteasome inhibitors.<sup>19,20</sup>

MKT-077 was found to be susceptible to rapid metabolism, with a lifetime of only 4.4 ± 1.0 min in mouse liver microsomes,<sup>21</sup> largely because of oxidation at the benzothiazole and pyridinium rings.<sup>22</sup> Guided by those studies, we used a previously reported synthetic route (Scheme 1)<sup>14</sup> to generate MKT-077 analogues designed to remove these metabolic liabilities. In these analogues, we retained the cationic character of MKT-077, but it should be noted that derivatives with neutral pyridines retain antiproliferative activity.<sup>22</sup> Briefly, the syntheses started with cyclization of substituted anilines with potassium ethyl xanthate, followed by methylation by iodomethane under mild basic conditions. The resulting benzothiazoles were activated by methyl *p*-toluenesulfonate and coupled with a series of N-substituted

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Table 1. Summary of the Antiproliferative Activities and Microsomal Stabilities of MKT-077 and Its Derivatives<sup>a</sup>

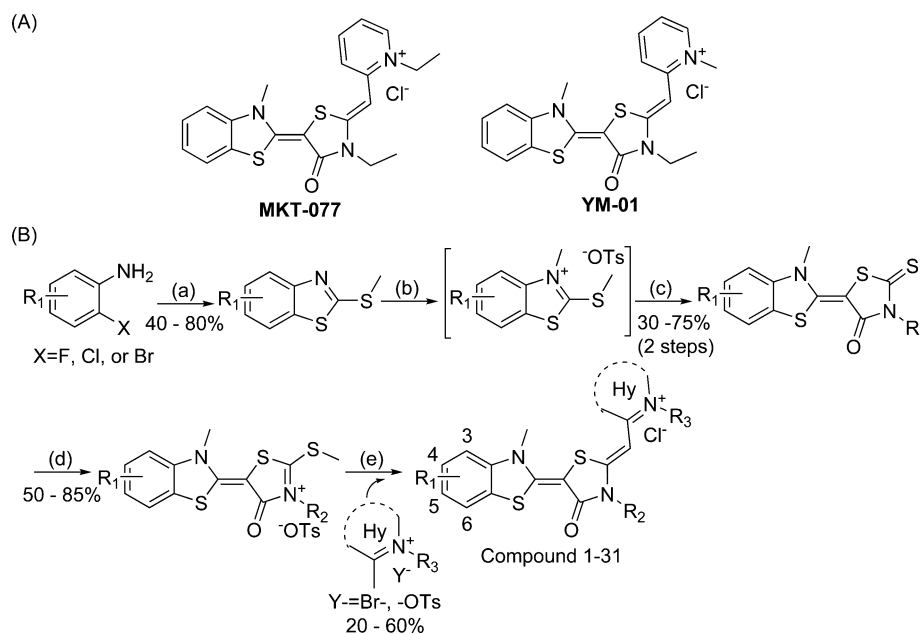
compd	R1	R2	R3	Hy	MDA-MB-231 EC <sub>50</sub> (μM)	MCF-7 EC <sub>50</sub> (μM)	MEF <sup>b</sup> EC <sub>50</sub> (μM)	microsome stability t <sub>1/2</sub> (min)
MKT-077	H	ethyl	ethyl	2-pyridyl	1.4 ± 0.2	2.2 ± 0.2	>50	<5
YM-01	H	ethyl	methyl	2-pyridyl	2.0 ± 0.2	5.2 ± 0.8	>50	<5
1	3-F	ethyl	methyl	2-pyridyl	4.0 ± 0.4	2.2 ± 0.4	>50	15
2	4-F	ethyl	methyl	2-pyridyl	7.7 ± 0.6	18 ± 4.4	>50	23
3	5-F	ethyl	methyl	2-pyridyl	5.6 ± 0.4	6.2 ± 0.8	>50	7.7
4	6-F	ethyl	methyl	2-pyridyl	6.2 ± 1.0	8.5 ± 3.0	>50	16
5	3-F	ethyl	ethyl	2-pyridyl	3.8 ± 0.2	1.0 ± 0.3	>30	NT <sup>c</sup>
6	4-F	ethyl	ethyl	2-pyridyl	13 ± 0.7	2.4 ± 0.6	>30	NT
7	5-F	ethyl	ethyl	2-pyridyl	8.3 ± 1.1	0.9 ± 0.3	>30	NT
8	6-F	ethyl	ethyl	2-pyridyl	2.8 ± 0.2	0.8 ± 0.1	27 ± 3.0	NT
9	3-Cl	ethyl	methyl	2-pyridyl	4.4 ± 0.6	7.8 ± 1.8	>50	NT
10	4-Cl	ethyl	methyl	2-pyridyl	4.6 ± 0.4	28 ± 4.3	>50	NT
11	5-Cl	ethyl	methyl	2-pyridyl	8.3 ± 0.7	9.5 ± 3.1	>50	8.2
12	6-Cl	ethyl	methyl	2-pyridyl	4.1 ± 0.5	5.2 ± 1.0	>50	NT
13	4-OMe	ethyl	methyl	2-pyridyl	2.8 ± 0.4	14 ± 2.6	>50	NT
14	5-OMe	ethyl	methyl	2-pyridyl	4.0 ± 0.4	11 ± 2.2	>50	10
15	4-CF <sub>3</sub>	ethyl	methyl	2-pyridyl	21 ± 4.5	>30	>50	NT
16	5-CF <sub>3</sub>	ethyl	methyl	2-pyridyl	19 ± 2.9	>30	>50	NT
17	H	allyl	methyl	2-pyridyl	3.6 ± 0.4	8.4 ± 2.4	>50	NT
18	H	benzyl	methyl	2-pyridyl	1.8 ± 0.3	2.9 ± 0.6	>50	NT
19	H	ethyl	propyl	2-pyridyl	1.6 ± 0.2	2.8 ± 0.8	>50	NT
20	H	ethyl	benzyl	2-pyridyl	1.0 ± 0.2	1.5 ± 0.2	>50	NT
21	H	ethyl	2-hydroxyethyl	2-pyridyl	24 ± 3.3	6.9 ± 2.1	>50	NT
22	H	ethyl	methyl	4-pyridyl	1.7 ± 0.1	19 ± 4.9	>50	NT
23	H	ethyl	methyl	2-pyrimidinyl	5.8 ± 0.8	9.1 ± 1.6	>50	NT
24	H	ethyl	methyl	2-thiazolyl	5.1 ± 0.3	0.7 ± 0.1	>50	NT
25	H	ethyl	ethyl	2-thiazolyl	2.2 ± 0.2	0.8 ± 0.1	>30	NT
26	H	ethyl	benzyl	2-thiazolyl	0.5 ± 0.1	0.6 ± 0.04	6.8 ± 0.4	NT
27 (JG-83)	3-F	ethyl	benzyl	2-thiazolyl	0.4 ± 0.03	1.0 ± 0.2	8.3 ± 0.7	NT
28 (JG-84)	3-Cl	ethyl	benzyl	2-thiazolyl	0.4 ± 0.02	0.8 ± 0.2	7.0 ± 0.6	8.8
29	4-Cl	ethyl	benzyl	2-thiazolyl	0.6 ± 0.1	0.8 ± 0.2	>30	NT
30 (JG-98)	5-Cl	ethyl	benzyl	2-thiazolyl	0.4 ± 0.03	0.7 ± 0.2	24 ± 1.3	37
31	6-Cl	ethyl	benzyl	2-thiazolyl	0.5 ± 0.1	1.0 ± 0.8	>30	NT

<sup>a</sup>Antiproliferative activity was measured using MTT assays. Results are the average of experiments performed in triplicate, and error is SEM. For stability studies, compounds (1 μM) were incubated with mouse liver microsomes and degradation monitored by LC-MS (see the Supporting Information). <sup>b</sup>From C57BL/6 mice. <sup>c</sup>Not tested or poorly soluble.

rhodanines. These products were methylated by methyl *p*-toluenesulfonate, followed by another coupling with an activated heterocycle. The products were dissolved in methanol and passed through a chloride exchange column to generate the final compounds 1–31 in overall yields between 20% and 40% (Table 1).

To compare the antiproliferative activities of these molecules, MKT-077, YM-01, and compounds 1–31 were evaluated in MDA-MB-231 and MCF7 breast cancer cells using MTT assays.<sup>23</sup> MKT-077 had EC<sub>50</sub> values of 1.4 ± 0.2 and 2.2 ± 0.2 μM against these cells. YM-01, which differs from MKT-077 only in the methyl substitution in the R<sub>3</sub> position, was only slightly weaker (EC<sub>50</sub> values of 2.0 ± 0.2 and 5.2 ± 0.8 μM, respectively) (Table 1). Consistent with the literature, neither molecule was toxic to mouse embryonic fibroblasts (MEFs) (EC<sub>50</sub> > 50 μM) and both had short half-lives in

microsomes (t<sub>1/2</sub> < 5 min). Introducing fluorine in the 3, 4, 5, or 6 positions of the benzothiazole (R<sub>1</sub>) of YM-01 (compounds 1–4) significantly improved stability (t<sub>1/2</sub> = 15, 23, 7.7, and 16 min, respectively). However, these substitutions also reduced potency (EC<sub>50</sub> values between 2.2 and 18 μM). When the same fluorinations were introduced in the context of the ethyl modification at the R<sub>3</sub> position (compounds 5–8), potency was only slightly improved (EC<sub>50</sub> between 0.8 and 13 μM). Replacing fluorines for chlorines in the YM-01 scaffold (compounds 9–12) did not improve potency and, in fact, further increased the EC<sub>50</sub> values to between 4.1 and 28 μM. Similarly, placing trifluoromethyl or methoxy groups at positions 4 and 5 (compounds 13–16) decreased activity to between 3 and more than 30 μM (Table 1). Together, these results suggested that small substitutions on the benzothiazole protected against metabolism but that larger groups reduced antiproliferative activity.

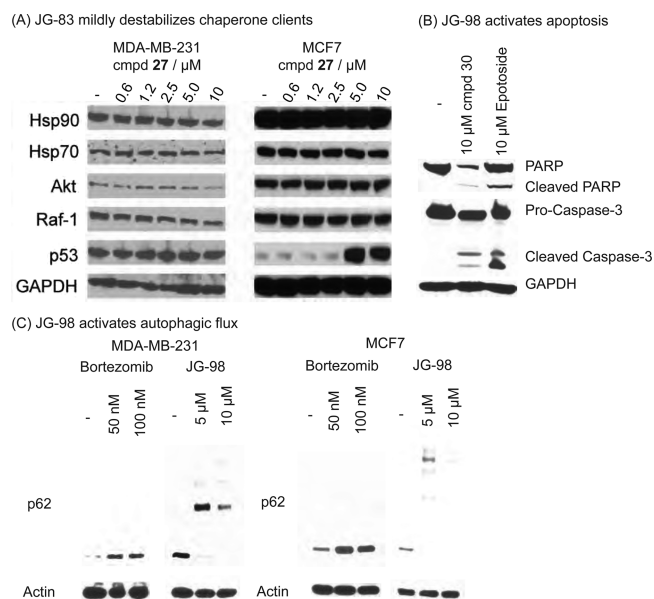
Scheme 1. Synthetic Route to MKT-077 and Analogues<sup>a</sup>

<sup>a</sup>(A) Chemical structures of MKT-077 and YM-01. (B) General synthetic route to analogues. (a) 1. Potassium ethyl xanthate, DMF, 4 h, 125 °C; 2. methyl iodide, triethylamine, ethanol, 1 h, 80 °C; (b) methyl *p*-toluenesulfonate, anisole, 125 °C; (c) N-substituted rhodanine, triethylamine, acetonitrile, 4 h, 25 °C; (d) methyl *p*-toluenesulfonate, DMF, 3 h, 135 °C; (e) 1. triethylamine, acetonitrile, 3 h, 80 °C, 2. Cl<sup>-</sup> ion exchange column.

Next, we made substitutions at the central rhodanine ring ( $R_2$ ) and the N-substitutions of the pyridinium ( $R_3$ ). Replacing the  $R_2$  ethyl group of MKT-077 with an allyl or benzyl group (compounds **17** and **18**) did not substantially affect activity against either cell line. Likewise, replacing the ethyl moiety of the pyridinium ( $R_3$ ) with a propyl group (compound **19**) did not significantly improve potency ( $EC_{50}$  values  $2.8 \pm 0.8$  and  $1.6 \pm 0.2$   $\mu\text{M}$  against MCF7 and MDA-MB-231 cells, respectively). However, increasing the size of this substituent to a benzyl group (compound **20**) improved activity by approximately 2-fold ( $EC_{50}$  values of  $1.5 \pm 0.2$  and  $1.0 \pm 0.2$   $\mu\text{M}$ ). The more hydrophilic 2-hydroxyethyl (compound **21**) decreased potency ( $EC_{50}$  values  $6.9 \pm 2.1$  and  $24 \pm 3.3$   $\mu\text{M}$ ), suggesting that hydrophobic groups were favored in this position.

Changing the position of the heteroatom in the pyridinium heterocycle (Hy) from the 2 to 4 position (compound **22**) did not significantly influence antiproliferative activity in MDA-MB-231 cells ( $EC_{50}$   $1.7 \pm 0.1$   $\mu\text{M}$ ), while the activity was reduced in MCF-7 cells ( $EC_{50}$   $19 \pm 4.9$   $\mu\text{M}$ ). Similarly, adding another nitrogen to the ring (compound **23**) reduced potency by approximately 2-fold, while replacing the pyridinium with a thiazolium (compound **24**) improved the potency by 3-fold in MCF-7 cells ( $EC_{50}$   $0.7 \pm 0.1$   $\mu\text{M}$ ) and slightly reduced activity in MDA-MB-231 cells ( $EC_{50}$   $5.1 \pm 0.3$   $\mu\text{M}$ ). To test whether the improvement in activity by the 2-thiazolyl group at  $R_3$  would be robust, we also generated analogues with this modification in the context of ethyl and benzyl substitutions at  $R_2$  (compounds **25** and **26**, respectively). These compounds followed the structure–activity trends of the earlier molecules, with the activity of compound **26** improved by 2-fold in MDA-MB-232 cells and 4-fold in MCF7 cells, with  $EC_{50}$  of  $0.5 \pm 0.1$  and  $0.6 \pm 0.04$   $\mu\text{M}$ , respectively.

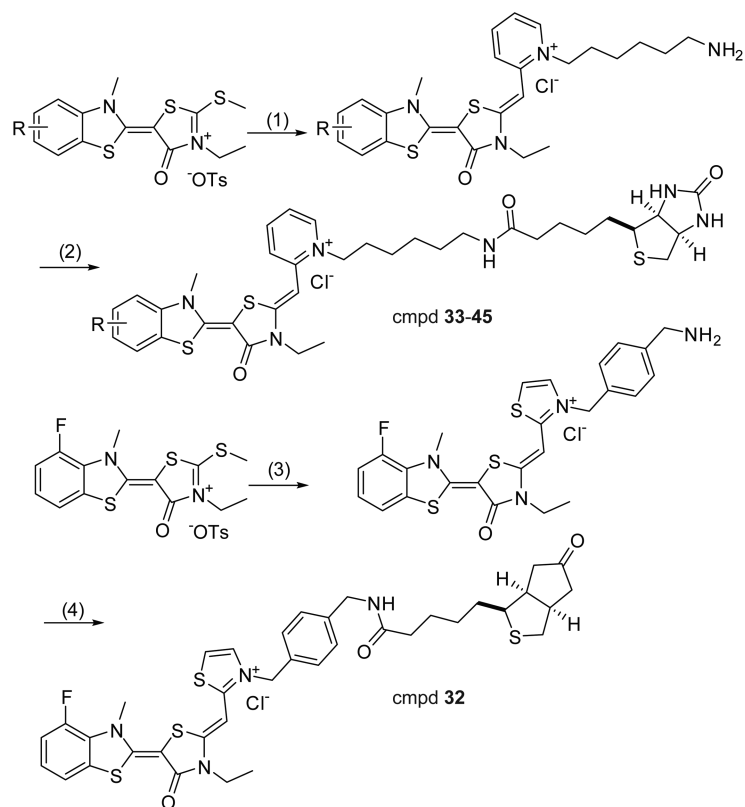
Together, these results suggested that a modest increase in antiproliferative potency might be gained by switching the pyridinium for a thiazolium. The results also suggested that appending a benzyl moiety to this ring might further promote



**Figure 1.** Treatment with MKT-077 analogue modestly affects the stability of chaperone clients and induces apoptosis. (a) Cells were treated for 24 h with the indicated concentration of compound 27 (JG-83). Results are representative of experiments performed in triplicate. (b) Compound 30 (JG-98) induces cleavage of caspase 3 and PARP in MDA-MB-231 cells after 48 h. Results are representative of experiments performed in duplicate. (c) Compound 30 (JG-98) induces oligomerization and reduction of p62 in MDA-MB-231 and MCF7 cells after 24 h. Results are representative of experiments performed in duplicate.

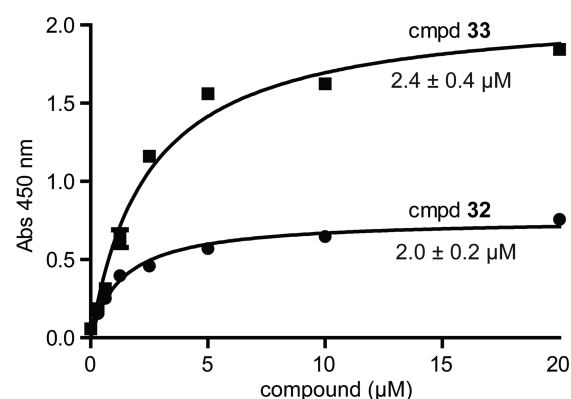
activity. To test these ideas, we combined the thiazolium and benzyl modifications with halogen replacements on the benzothiazole ring (compounds **27–31**). Each of these compounds had significantly improved potency compared to MKT-077, with  $EC_{50}$  values between 1.0 and 0.4  $\mu\text{M}$  (Table 1). For example, the compound that combines a 3-F group with a

## (A) Synthesis of biotinylated MKT-077 analogues



- (1) a. 1-(6-((*tert*-butoxycarbonyl)amino)hexyl)-2-methylpyridin-1-ium bromide, triethylamine; b. Cl ion exchange column; c. trifluoroacetic acid  
 (2) NHS-Biotin, triethylamine  
 (3) a. 3-(4-((*tert*-butoxycarbonyl)amino)methyl)benzyl)-2-methylthiazol-3-ium bromide, triethylamine; b. Cl ion exchange column; c. trifluoroacetic acid  
 (4) NHS-Biotin, triethylamine

## (B) YM01-biotin and JG83-biotin have similar affinity for Hsc70



## (C) Biotinylated analogues bind Hsc70

compound	R	$K_D$ / $\mu\text{M}$
<b>33</b> (YM01-biotin)	H	$2.0 \pm 0.2$
<b>34</b>	3-F	$10.9 \pm 4.4$
<b>35</b>	4-F	$2.3 \pm 0.5$
<b>36</b>	5-F	$1.5 \pm 0.2$
<b>37</b>	6-F	$1.9 \pm 0.4$
<b>38</b>	3-Cl	$7.7 \pm 1.0$
<b>39</b>	4-Cl	$4.0 \pm 0.5$
<b>40</b>	5-Cl	$1.7 \pm 0.3$
<b>41</b>	6-Cl	$2.6 \pm 0.6$
<b>42</b>	4-OMe	$0.9 \pm 0.1$
<b>43</b>	4-CF <sub>3</sub>	$0.4 \pm 0.1$
<b>44</b>	5-OMe	$5.4 \pm 0.7$
<b>45</b>	5-CF <sub>3</sub>	$1.0 \pm 0.2$

**Figure 2.** Biotinylated MKT-077 analogues bind Hsc70. (A) Synthetic route to biotinylated MKT-077 analogues. (B) Biotinylated versions of YM-01 (33) and JG-83 (32) bind human Hsc70 with a similar affinity, as judged by ELISA. Results are the average of at least three experiments performed in duplicate. The error is SEM. (C) Binding affinities ( $K_D$ ) of compounds 33–45 to Hsc70 by ELISA. See the Supporting Information for details.

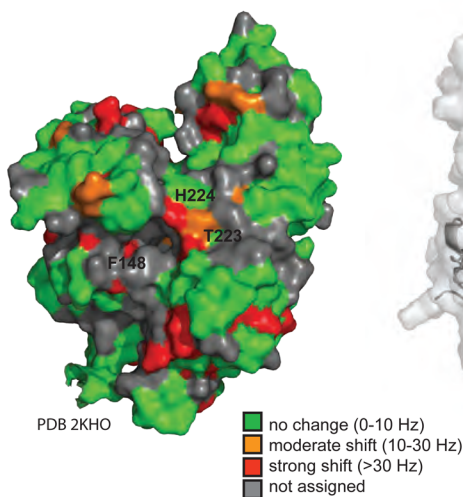
benzyl substituted 2-thiazolyl (compound 27; JG-83) was 2-fold more potent against MCF7 cells ( $EC_{50} = 1.0 \pm 0.2 \mu\text{M}$ ) and more than 3-fold improved against MDA-MB-231 cells ( $EC_{50} = 0.4 \pm 0.03 \mu\text{M}$ ). Likewise, the equivalent compound with a 5-Cl substitution (compound 30; JG-98) had a potency of  $0.4 \pm 0.03 \mu\text{M}$  against MDA-MB-231 cells and an  $EC_{50}$  value of  $0.7 \pm 0.2 \mu\text{M}$  for MCF7 cells. Some of these compounds, including JG-83 and JG-98, had increased activity against MEFs compared to MKT-077, but the selectivity for cancer cells was still estimated to be between 8- and 20-fold. Importantly, JG-98 had a microsomal lifetime of 37 min, which is at least 7-fold better than MKT-077 or YM-01.

To explore whether these compounds affected the stability of known Hsp70 clients, we treated MDA-MB-231 and MCF-7 cells with JG-83 for 24 h and then performed Western blots for p53, Akt1, and Raf1.<sup>24</sup> We found that Akt1 and Raf1 levels were reduced ~25% in MDA-MB-231 and MCF7 cells (Figure 1a). The magnitude of the loss in pro-survival clients is not as dramatic as is commonly observed for inhibitors of Hsp90.<sup>25</sup> It is possible that other clients are more dramatically affected. For example, in the MCF7 cells, the levels of mutated p53<sup>26</sup> were also significantly elevated at 5 and 10  $\mu\text{M}$  of JG-83, suggesting that reactivation of the tumor suppressor might be a relevant mechanism in this cell line. Importantly, we found that the levels

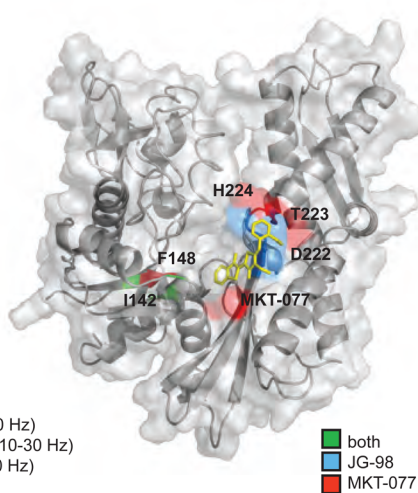
of Hsp70 and Hsp90 were not elevated by JG-83 in either cell line (Figure 1a), confirming that MKT-077 analogues do not elevate a stress response.<sup>14</sup> To probe whether these compounds could induce apoptosis, we treated MDA-MB-231 cells with JG-98 and measured cleavage of caspase-3 and PARP after 48 h. Both apoptotic mediators were activated (Figure 1b), suggesting that JG-98 triggers apoptosis. Finally, we examined whether JG-98 had an effect on autophagy. Treatment of both MDA-MB-231 and MCF7 cells with JG-98 strongly affected autophagic flux, as indicated by an increase in p62 oligomerization and a reduction in p62 monomer (Figure 1c). Together, these results support the idea that Hsp70 is a hub for multiple cell survival pathways.<sup>1</sup>

Next, we wanted to explore whether the MKT-077 derivatives retained binding to Hsc70 in vitro. To test this idea, we synthesized a biotinylated version of JG-83 (compound 32), using a modification of the previous synthetic route (Figure 2A). Using an ELISA,<sup>14</sup> the affinity ( $K_D$ ) of 32 for purified, human Hsc70 was calculated to be  $2.4 \pm 0.4 \mu\text{M}$  (Figure 2B), which was indistinguishable from the affinity of Hsc70 for a biotinylated version of YM-01 ( $K_D = 2.0 \pm 0.2 \mu\text{M}$ ). Thus, the 3-fold stronger antiproliferative activity of JG-83 did not seem to arise from tighter Hsc70 binding in vitro. To explore this relationship further, we generated biotinylated versions of an additional subset of MKT-077 analogues (compounds 33–45) (Figure 2A)

(A) JG-98 binds to DnaK by NMR



(B) JG-98 and MKT-077 share the same binding site



(C) The JG-98 binding site is conserved across Hsp70 family members

	140	141	142	143	144	145	146	147	148	149	150
<b>DNAK</b>	A	V	I	T	V	P	A	Y	F	N	D
<b>HSPA8</b>	A	V	V	T	V	P	A	Y	F	N	D
HSPA2	A	V	I	T	V	P	A	Y	F	N	D
HSPA9	A	V	I	T	V	P	A	Y	F	N	D
<b>HSPA1A</b>	A	V	I	T	V	P	A	Y	F	N	D
HSPA1L	A	V	I	T	V	P	A	Y	F	N	D
HSPA5	A	V	V	T	V	P	A	Y	F	N	D
HSPA6	A	V	I	T	V	P	A	Y	F	N	D
HSPA12A	W	V	I	T	V	P	A	I	W	K	Q
HSPA12B	W	V	L	T	V	P	A	I	W	K	Q
HSPA13	A	V	I	S	V	P	A	E	F	D	L
HSPA14	V	V	I	T	V	P	F	D	F	G	E

	219	220	221	222	223	224	225	226	227
<b>DNAK</b>	T	N	G	D	T	H	L	G	G
<b>HSPA8</b>	T	A	G	D	T	H	L	G	G
HSPA2	T	A	G	D	T	H	L	G	G
HSPA9	T	N	G	D	T	F	L	G	G
<b>HSPA1A</b>	T	A	G	D	T	H	L	G	G
HSPA1L	T	A	G	D	T	H	L	G	G
HSPA5	T	N	G	D	T	H	L	G	G
HSPA6	T	A	G	D	T	H	L	G	G
HSPA12A	T	G	G	P	Y	G	S	L	G
HSPA12B	S	G	G	P	Y	G	A	V	G
HSPA13	M	S	G	N	N	K	L	G	G
HSPA14	T	N	T	D	D	N	I	G	G

bold = residues sensitive to JG-98 by NMR

**Figure 3.** JG-98 binds to a conserved, allosteric site on an Hsp70 family member. (A) Titration of JG-98 into  $^{15}\text{N}$  DnaK $_{1-388}$  revealed chemical shifts, with a strong cluster in a deep pocket near F148. (B) JG-98 is predicted to bind the same allosteric pocket that accommodates MKT-077. The docked configuration of MKT-077 is shown (see text). (C) Alignment of DnaK and human Hsp70 family members, highlighting the cluster of residues sensitive to treatment with JG-98 (bold). Invariant residues are shown in red, conserved residues in orange, and nonconserved residues in white. Note that there is also high conservation outside the sensitive residues.

and measured their affinities for Hsc70 in the ELISA format.<sup>14</sup> The results suggested that modifications at the 4', 5', and 6' positions (compounds 35–37 and 39–41) were generally well tolerated ( $K_D$  values between 1.5 and 4.0  $\mu\text{M}$ ) and that fluorine or chlorine at the 3' position (compounds 34 and 38) only modestly weakened affinity ( $K_D = 10.9 \pm 4.4$  and  $7.7 \pm 1.0$   $\mu\text{M}$ , respectively) (Figure 2C). Thus, Hsc70 binding affinity alone was not a good predictor of potency in MTT assays, possibly due to differences in allosteric efficiency<sup>27</sup> and/or differences in cellular permeability.

To further explore binding to Hsp70, JG-98 was titrated into an aqueous solution of  $^{15}\text{N}$ -labeled DnaK nucleotide binding domain (DnaK $_{1-388}$ ) and its interactions with the protein monitored by NMR. We used DnaK, a prokaryotic orthologue of Hsp70, in these studies because of its good behavior in solution and the availability of a large number of NMR resonance assignments.<sup>28</sup> Titrating JG-98 (200  $\mu\text{M}$ ) into the nucleotide-binding domain of  $^1\text{H}$ - $^{15}\text{N}$  DnaK $_{1-388}$  (160  $\mu\text{M}$ ) showed strong chemical shifts ( $\Delta\text{Hz} > 30$ ) in the TROSY-HSQC experiment. Strong shifts appeared in a deep, hydrophobic pocket formed by Ile142, Phe148, and other residues (Figure 3A), and additional shifts were seen in nearby surface residues: Gly222, Asp223, and Thr224. A similar region was previously implicated in the binding of MKT-077 to Hsc70<sup>16</sup> and an overlay of the two sets of compound-sensitive residues suggested that MKT-077 and JG-98 interact with the same site (Figure 3B). In addition, treatment with JG-98 led to scattered chemical shifts in the IIA, IB, and IIB subdomains of DnaK (see Figure 3A). The major sites perturbed by JG-98 (residues I142, F148, D222, T223, and H224) are highly conserved among prokaryotic and eukaryotic Hsp70s, and they are nearly invariant in the major family members, such as Hsc70 (HSPA8), Hsp72 (HSPA1A), and mtHsp70 (HSPA9) (Figure 3C). Thus, compounds of this class will likely have affinity for nearly all of the family members, as previously suggested.<sup>16</sup>

In conclusion, a library of MKT-077 derivatives was synthesized and evaluated against two breast cancer cell lines. Compounds such as 27 (JG-83) and 30 (JG-98) had improved potencies against breast cancer cells and extended lifetimes in liver microsome studies. Further, JG-98 bound to a conserved, allosteric site on Hsp70. These studies advance the rhodacyanines as potential anticancer agents and improve their utility as chemical probes for studying Hsp70s roles in cancer.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

Compound characterization and methods for syntheses and biological studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### 👤 Author Contributions

X.L., S.R.S., J.C., A.A., Z.T.Y., and E.R.P.Z. performed experiments. All authors contributed to interpreting the results and preparing the manuscript.

### 🗒 Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

Hsp70, heat shock protein 70; MEF, mouse embryonic fibroblast; ELISA, enzyme-linked immunosorbent assay; PARP, poly ADP ribose polymerase

## ■ REFERENCES

- (1) Evans, C. G.; Chang, L.; Gestwicki, J. E. Heat Shock Protein 70 (Hsp70) as an emerging drug target. *J. Med. Chem.* **2010**, *53*, 4585–4602.
- (2) Goloudina, A. R.; Demidov, O. N.; Garrido, C. Inhibition of HSP70: A challenging anti-cancer strategy. *Cancer Lett.* **2012**, *325*, 117–124.
- (3) Kampinga, H. H.; Craig, E. A. The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 579–592.
- (4) Sherman, M.; Gabai, V.; O'Callaghan, C.; Yaglom, J. Molecular chaperones regulate p53 and suppress senescence programs. *FEBS Lett.* **2007**, *581*, 3711–3715.
- (5) Gabai, V. L.; Budagova, K. R.; Sherman, M. Y. Increased expression of the major heat shock protein Hsp72 in human prostate carcinoma cells is dispensable for their viability but confers resistance to a variety of anticancer agents. *Oncogene* **2005**, *24*, 3328–3338.
- (6) Ciocca, D. R.; Calderwood, S. K. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress Chaperones* **2005**, *10*, 86–103.
- (7) Powers, M. V.; Clarke, P. A.; Workman, P. Dual targeting of HSC70 and HSP72 inhibits HSP90 function and induces tumor-specific apoptosis. *Cancer Cell* **2008**, *14*, 250–262.
- (8) Guo, F.; Rocha, K.; Bali, P.; Pranpat, M.; Fiskus, W.; Boyapalle, S.; Kumaraswamy, S.; Balasis, M.; Greedy, B.; Armitage, E. S. M.; Lawrence, N.; Bhalla, K. Abrogation of heat shock protein 70 induction as a strategy, to increase antileukemia activity of heat shock protein 90 inhibitor 17-allylamino-demethoxy geldanamycin. *Cancer Res.* **2005**, *65*, 10536–10544.
- (9) Grossin, L.; Etienne, S.; Gaborit, N.; Pinzano, A.; Courmil-Henrionnet, C.; Gerard, C.; Payan, E.; Netter, P.; Terlain, B.; Gillet, P. Induction of heat shock protein 70 (Hsp70) by proteasome inhibitor MG 132 protects articular chondrocytes from cellular death in vitro and in vivo. *Biorheology* **2004**, *41*, 521–534.
- (10) Sadekova, S.; Lehnert, S.; Chow, T. Y. K. Induction of PBP74/mortalin/Grp75, a member of the hsp70 family, by low doses of ionizing radiation: a possible role in induced radioresistance. *Int. J. Radiat. Biol.* **1997**, *72*, 653–660.
- (11) Chiba, Y.; Kubota, T.; Watanabe, M.; Matsuzaki, S. W.; Otani, Y.; Teramoto, T.; Matsumoto, Y.; Koya, K.; Kitajima, M. MKT-077, localized lipophilic cation: Antitumor activity against human tumor xenografts serially transplanted into nude mice. *Anticancer Res.* **1998**, *18*, 1047–1052.
- (12) Koya, K.; Li, Y.; Wang, H.; Ukai, T.; Tatsuta, N.; Kawakami, M.; Shishido, T.; Chen, L. B. MKT-077, a novel rhodacyanine dye in clinical trials, exhibits anticarcinoma activity in preclinical studies based on selective mitochondrial accumulation. *Cancer Res.* **1996**, *56*, 538–543.
- (13) Wadhwa, R.; Sugihara, T.; Yoshida, A.; Nomura, H.; Reddel, R. R.; Simpson, R.; Maruta, H.; Kaul, S. C. Selective toxicity of MKT-077 to cancer cells is mediated by its binding to the hsp70 family protein mot-2 and reactivation of p53 function. *Cancer Res.* **2000**, *60*, 6818–6821.
- (14) Wang, A. M.; Miyata, Y.; Klinedinst, S.; Peng, H.-M.; Chua, J. P.; Komiyama, T.; Li, X.; Morishima, Y.; Merry, D. E.; Pratt, W. B.; Osawa, Y.; Collins, C. A.; Gestwicki, J. E.; Lieberman, A. P. Activation of Hsp70 reduces neurotoxicity by promoting polyglutamine protein degradation. *Nat. Chem. Biol.* **2013**, *9*, 112–118.
- (15) Tikoo, A.; Shakri, R.; Connolly, L.; Hirokawa, Y.; Shishido, T.; Bowers, B.; Ye, L. H.; Kohama, K.; Simpson, R. J.; Maruta, H. Treatment of ras-induced cancers by the F-actin-bundling drug MKT-077. *Cancer J.* **2000**, *6*, 162–168.
- (16) Rousaki, A.; Miyata, Y.; Jinwal, U. K.; Dickey, C. A.; Gestwicki, J. E.; Zuiderweg, E. R. P. Allosteric drugs: The interaction of antitumor compound MKT-077 with human Hsp70 chaperones. *J. Mol. Biol.* **2011**, *411*, 614–632.
- (17) Koren, J., III; Miyata, Y.; Kiray, J.; O'Leary, J. C., III; Nguyen, L.; Guo, J.; Blair, L. J.; Li, X.; Jinwal, U. K.; Cheng, J. Q.; Gestwicki, J. E.; Dickey, C. A. Rhodacyanine derivative selectively targets cancer cells and overcomes tamoxifen resistance. *PLoS One* **2012**, *7*, e35566.
- (18) Wadhwa, R.; Colgin, L.; Yaguchi, T.; Taira, K.; Reddel, R. R.; Kaul, S. C. Rhodacyanine dye MKT-077 inhibits in vitro telomerase assay but has no detectable effects on telomerase activity in vivo. *Cancer Res.* **2002**, *62*, 4434–4438.
- (19) Didelot, C.; Lanneau, D.; Brunet, M.; Joly, A.-L.; De Thonel, A.; Chiosis, G.; Garrido, C. Anti-cancer therapeutic approaches based on intracellular and extracellular heat shock proteins. *Curr. Med. Chem.* **2007**, *14*, 2839–2847.
- (20) Duerfeldt, A. S.; Blagg, B. S. J. Hsp90 inhibition: elimination of shock and stress. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4983–4987.
- (21) Tatsuta, N.; Suzuki, H.; Mochizuki, T.; Koya, K.; Kawakami, M.; Shishido, T.; Motoji, N.; Kuroiwa, H.; Shigematsu, A.; Chen, L. B. Pharmacokinetic analysis and antitumor efficacy of MKT-077, a novel antitumor agent. *Cancer Chemother. Pharmacol.* **1999**, *43*, 295–301.
- (22) Miyata, Y.; Li, X.; Lee, H.-F.; Jinwal, U. K.; Srinivasan, S. R.; Seguin, S. P.; Young, Z. T.; Brodsky, J. L.; Dickey, C. A.; Sun, D.; Gestwicki, J. E. Synthesis and initial evaluation of YM-08, a blood–brain barrier permeable derivative of the heat shock protein 70 (Hsp70) inhibitor MKT-077, which reduces Tau levels. *ACS Chem. Neurosci.* **2013**, *4*, 930–939.
- (23) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63.
- (24) Balaburski, G. M.; Leu, J. I. J.; Beeharry, N.; Hayik, S.; Andrade, M. D.; Zhang, G.; Herlyn, M.; Villanueva, J.; Dunbrack, R. L., Jr.; Yen, T.; George, D. L.; Murphy, M. E. A modified HSP70 inhibitor shows broad activity as an anticancer agent. *Mol. Cancer Res.* **2013**, *11*, 219–229.
- (25) Caldas-Lopes, E.; Cerchiatti, L.; Ahn, J. H.; Clement, C. C.; Robles, A. L.; Rodina, A.; Moullick, K.; Taldone, T.; Gozman, A.; Guo, Y.; Wu, N.; de Stanchina, E.; White, J.; Gross, S. S.; Ma, Y.; Varticovski, L.; Melnick, A.; Chiosis, G. Hsp90 inhibitor PU-H71, a multimodal inhibitor of malignancy, induces complete responses in triple-negative breast cancer models. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 8368–8373.
- (26) Wasielewski, M.; Elstrodt, F.; Klijn, J. G. M.; Berns, E. M. J. J.; Schutte, M. Thirteen new p53 gene mutants identified among 41 human breast cancer cell lines. *Breast Cancer Res. Treat.* **2006**, *99*, 97–101.
- (27) Assimon, V. A.; Gillies, A. T.; Rauch, J. N.; Gestwicki, J. E. Hsp70 protein complexes as drug targets. *Curr. Pharm. Des.* **2013**, *19*, 404–417.
- (28) Bertelsen, E. B.; Chang, L.; Gestwicki, J. E.; Zuiderweg, E. R. P. Solution conformation of wild-type E. coli Hsp70 (DnaK) chaperone complexed with ADP and substrate. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 8471–8476.