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Brief Article

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Synthesis and Biological Activities of a New Class of Heat Shock Protein 90 Inhibitors, Designed by Energy-Based Pharmacophore Virtual Screening

Antonino Lauria*, Ilenia Abbate, Carla Gentile, Francesca Angileri, Annamaria Martorana, Anna Maria Almerico

Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche "STEBICEF", Sezione di Chimica Farmaceutica e Biologica - Università di Palermo, Via Archirafi 32, I-90123 Palermo, Italy.

KEYWORDS. Energy-Based Pharmacophore Virtual screening, aminocyanopyridines, Hsp90 inhibitors, competitive binding assay.

ABSTRACT: The design through Energy-Based Pharmacophore Virtual screening has led to aminocyanopyridine derivatives as efficacious new inhibitors of Hsp90. The synthesized compounds showed a good affinity for Hsp90 ATP binding site in the competitive binding assay. Moreover, they showed an excellent antiproliferative activity against a large number of human tumor cell lines. Further biological studies on the derivative with the higher EC50 confirmed its specific influence on the cellular pathways involving Hsp90.

In the last decade the inhibition of a protein that regulates multiple signal transduction pathways in tumor cells has become an attractive target for cancer therapy. Heat Shock Protein 90 (Hsp90) is one of the most promising molecular targets for such an approach.[1] In fact, it is an ubiquitous molecular chaperone protein involved in the folding, reorganization and assembling of many key mediators for signal transduction. Hsp90 plays a crucial role in several biological processes such as cell proliferation, differentiation, apoptosis or stress response.[2]

Inhibition of the ATPase activity of Hsp90 disrupts an ongoing "folding" cycle, including multiple cochaperone proteins, and in turn leads to the destabilization, ubiquitination, and ultimately proteasomal degradation of client proteins.[3] Many Hsp90 client proteins are oncogenic proteins, overexpressed in cancer, often in mutated forms, and are responsible for unrestricted cancer cell proliferation and survival. The harsh environmental conditions of the tumor microenvironment, such as hypoxia and nutrient deficiency, contribute to the destabilization of proteins and further escalate their dependence on Hsp90, as supported by the higher Hsp90 levels and the higher ATPase activity found in tumor cells.[4,5] On the other hand, the molecular signature of Hsp90 inhibition, well established in cancer cell lines, denotes an increased expression of Hsp72 coupled with the depletion of oncogenic Hsp90-client proteins.[6,7]

Many natural-occurring compounds, such as Geldanamycin or Radicicol, act as Hsp90 inhibitors;[8] however, to date, only few compounds, as the 17-allylaminogeldanamycin (17-AAG) and the synthetic derivative SNX-5422, exerted a potent antitumor activity in a preclinical model and are currently in clinical trials.[9] Thus, it is easy to understand how much important is the research of new Hsp90 inhibitors with high selectivity and low toxicity.

In the last years, the *in silico* methodologies helped to design compounds with biological activities having different biological targets (proteins, DNA, etc.).[10-16] Also in the studies on Hsp90, different approaches were used to investigate the binding mode in the N-terminal domain.[17,18]

Herein we present the synthesis and the biological activities of a new class of Hsp90 inhibitors, designed through Energy-Based Pharmacophore virtual screening.

DESIGN

The virtual screening protocol was started by selecting four sets of known Hsp90 inhibitors, belonging to the oxazole, naphthol, and pyrazole classes (Figure 1).[19-21]

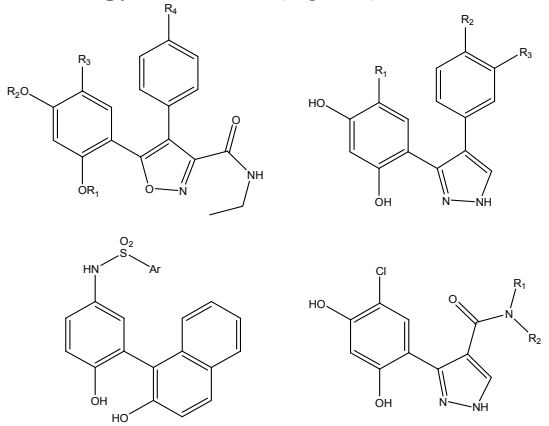


Figure 1. Pyrazole, naphthol, and oxazole Hsp90 inhibitors classes.

Each class was submitted to docking studies into the ATPase binding site of Hsp90 (PDB id: 1UYF). The top scored docked structure was selected for each class. Thus four energy-based pharmacophore hypotheses were built through E-pharmacophore facility, as available in the MAESTRO package (Version 9.3, Schrödinger, LLC, New York, NY, 2012). The four hypotheses were built in single mode, with seven maximum numbers of selected features (Figure 2).

Likewise, a large *in house* database of heterocyclic compounds (~100.000 structures) was docked with the same parameters used for the known Hsp90 inhibitor classes. The best hits (10%) were matched with the four energy based pharmacophore hypotheses.

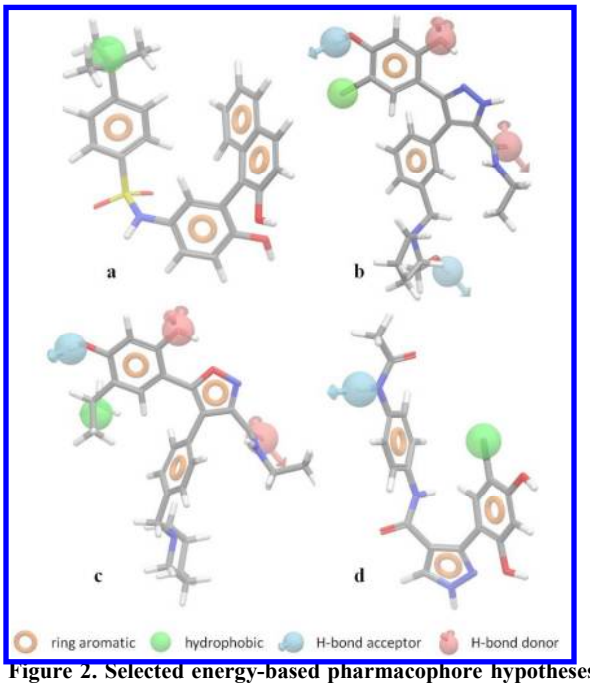


Figure 2. Selected energy-based pharmacophore hypotheses for naphthol (a, 5 points), pyrazole (b, 7 points; d, 5 points), and oxazole (c, 7 points) Hsp90 inhibitors classes, docked into Hsp90 ATPase binding site (PDB id: 1UYF).

The structures were ranked on the basis of the fitness scores and by fixing four as the minimum number of pharmacophore sites to be matched

The best scores were achieved for the class of 2-amino-3-cyanopyridine **1** (Figure 3a), obtained through the matching with the energy-based hypothesis built on the top scored docked structure (Figure 2, **b**) belonging to pyrazole class.

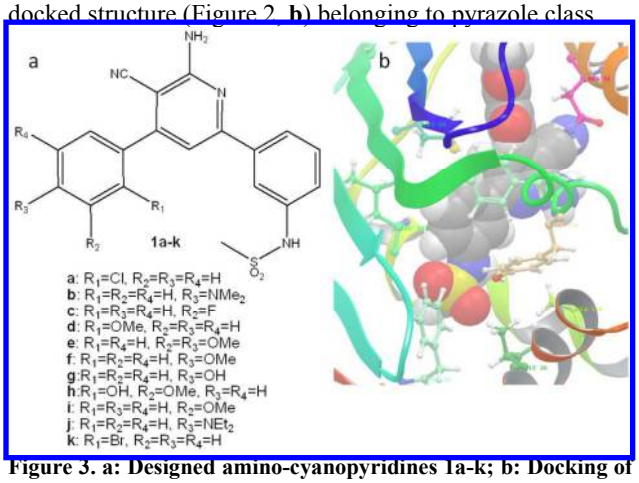


Figure 3. a: Designed amino-cyanopyridines 1a-k; b: Docking of the best hit compound 1h into the Hsp90 ATPase site (PDB id: 1UYF).

The analysis of the amino acids, involved in the binding to Hsp90 ATPase site of **1h**, the natural ligand of UYF, the geldanamycin (complexed in PDB id: 1YET), and the pyrazole derivative **b** used in the selected pharmacophore hypothesis (Figure 2) is reported in table 1.

It emerges that Asn51, Phe138, and Tyr139 are essential in the interactions with the Hsp90 ATPase site.

Moreover, the designed ligand **1h**, with respect to the other inhibitors, binds the Hsp90 ATPase site through two amino acids different respect to the other ligands (Phe22 and Ile26). This suggests a different binding mode if compared to that of the known ligands.

Table 1. Analysis of critical amino acids involved in the binding into Hsp90 ATPase site.

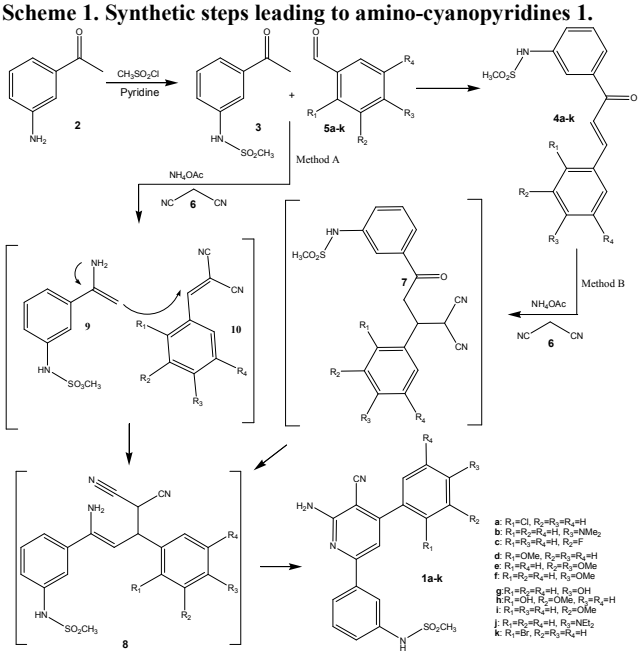
Amino acid	Phe22	Ile26	Asn51	Ala55	Asp93	Met98	Leu103	Leu107	Ala111	Phe138	Tyr139	Val150	Thr184
1h	✔	✔	✔			✔	✔		✔	✔	✔		
b			✔	✔			✔	✔	✔	✔	✔		✔
1UYF			✔		✔			✔	✔	✔	✔	✔	✔
1YET			✔	✔	✔	✔		✔		✔	✔		✔

In fact, the analysis of the binding mode of **1h** (Figure 3b) evidences the occurrence of several interactions with the active site, underlining the central role of some amino acid residues such as Phe22, Ile26, Asn51, Met98, Leu103, Ala111, Phe138, and Tyr 139, which provide direct non-covalent interactions.

SYNTHESIS

To optimize the yields of each derivative, the synthetic approach of the designed compounds was carried out through two different pathways (Scheme 1).

Scheme 1. Synthetic steps leading to amino-cyanopyridines 1.



The first synthetic route (method A), leading to the amino-cyanopyridines **1**, involved the introduction of a sulfamic moiety on aminoacetophenone **2** to give **3**, by reaction with methanesulfonyl chloride.[22] The α,β-unsaturated ketones **4** were synthesized through a base catalyzed Claisen-Schmidt condensation of **3** and aldehydes of type **5**. The chalcones **4** were reacted with malononitrile **6**, in the presence of ammonium acetate and anhydrous ethanol as solvent, to afford 2-amino-3-cyanopyridines **1a-k** in good yields. The reaction proceeds through Michael addition of the malononitrile anion to the α,β-unsaturated ketone to give the adduct **7**. The for-

mation of the Schiff base, which rearranges to the enamine **8**, can be followed by cyclization and subsequent dehydration, to give the 2-amino-3-cyanopyridines of type **1**.

The second synthetic approach (method B), bypasses the chalcone intermediates **4a-k** exploiting a one-pot coupling synthesis of four components. The mixture of aromatic aldehydes **5**, acetophenone **3**, malononitrile **6**, and ammonium acetate is dissolved in dry dioxane and refluxed overnight. The reaction probably proceeds via intermediate **9**, formed from acetophenone **3** and ammonium acetate, which reacts with alkylidenemalonitrile **10** (from condensation of aromatic aldehyde with malononitrile) to give **8**. The above reported cycloaddition, isomerization, and aromatization afforded the 2-amino-3-cyanopyridine **1**. This approach allowed the isolation of derivatives **1d-f,h,i** in higher yields and in short reaction time with respect to method A.

BIOLOGY

Hsp90 Competitive Binding Assay: The designed and synthetized compounds were tested to verify the ability to bind purified human Hsp90. The apparent binding affinities (EC50) of the selected amino-cyanopyridine derivatives **1a-k** for Hsp90 were determined using a competition-binding assay (Table 2).

Table 2. Apparent binding affinities (EC50) of selected amino-cyanopyridine derivatives 1a-k for Hsp90.

Cmd	R_1	R_2	R_3	R_4	EC50 (nM)
1a	Cl	H	H	H	950 ± 35
1b	H	H	NMe_2	H	371 ± 28
1c	H	F	H	H	$>10^4$
1d	OMe	H	H	H	634 ± 22
1e	H	OMe	OMe	H	867 ± 27
1f	H	H	OMe	H	541 ± 29
1g	H	H	OH	H	$>10^4$
1h	OH	OMe	H	H	252 ± 23
1i	H	OMe	H	H	$>10^4$
1j	H	H	NEt_2	H	783 ± 19
1k	Br	H	H	H	$>10^4$
17-AAG					99 ± 17

The apparent binding affinity (EC50) was evaluated using a competition-binding assay as described in supporting information. Data are the mean ± SD of n separate experiments carried out in triplicate. Statistically significant vs control with $p<0.005$.

Seven out eleven of the tested compounds showed binding affinity in the sub-micromolar concentration, with the highest activity for **1h**.

Antiproliferative screening: The hit compounds **1a-k** were submitted to the Developmental Therapeutics Program of the National Cancer Institute (DTP NCI) screening on the full NCI DTP human tumor cell lines panels.

Four out eleven compounds passed the selection criteria of the DTP NCI protocol and were tested twice to five-dose concentration screening. All results are reported in supporting information and summarized in table 3 and table 4.

All the screened compounds showed anti-proliferative activity against the tested cell lines in the mid-nanomolar range and derivative **1b** was the most active (MG_MID = 55 nM). The evaluation of the data for this class of compounds with respect to MG_MID values at GI50, TGI and LC50 levels confirmed that all tested 2-amino-3-cyanopyridines present both high antiproliferative activity and very low toxicity, with LC50>10μM for almost all the human tumor cell lines (supporting information).

Table 3. Overview of the GI50, TGI, and LC50 values (μM) for the selected compounds 1a,b,f,h.

Cmd		GI50	TGI	LC50
1a	MG_MID	0.676	14.454	56.234
	range	5.623-0.240	51.286-2.239	>100-23.442
1b	MG_MID	0.055	8.710	89.125
	range	0.955-0.013	>100-0.056	>100-8.128
1f	MG_MID	0.117	11.749	91.201
	range	1.230-0.016	>100-0.437	>100-19.498
1h	MG_MID	0.186	35.481	93.325
	range	0.776-0.024	>100-0.076	>100-14.125

[a]GI50 is the concentration that inhibits 50% net cell growth. [b]TGI is the concentration giving total growth inhibition. [c]LC50 is the concentration leading to 50% net cell death. MG_MID (mean graph midpoint) is the arithmetic mean value for all tested human cancer cell lines for each separate experiment.

Table 4. DTP NCI human tumor cell lines panels overview (GI50).

Panel cell line (μM)	**1a**	**1b**	**1f**	**1h**
Breast Cancer	0.46	0.10	0.14	0.26
CNS Cancer	0.51	0.04	0.08	0.16
Colon Cancer	0.55	0.04	0.10	0.15
Leukemia	0.58	0.04	0.10	0.14
Melanoma	0.66	0.06	0.08	0.12
Non-Small Cell Lung	1.10	0.06	0.13	0.25
Ovarian Cancer	0.98	0.08	0.22	0.21
Prostate Cancer	0.78	0.03	0.13	0.13
Renal Cancer	0.66	0.05	0.13	0.29

Analysing further the biological results, it is possible to evidence the excellent activity of **1b** against all cell lines of the melanoma (MG_MID = 0.06 μM), CNS (MG-MID = 0.04 μM), prostate (MG_MID = 0.03 μM) and colon (MG_MID = 0.04 μM) cancer subpanels. Significant results were obtained in the case of derivative **1f**, especially against the cell lines belonging to CNS, melanoma and colon subpanels, with GI50 always below 10^{-1}μM. Further, it should be underlined as the derivative **1h** showed the lowest cytotoxicity, with the largest number of human tumor cell lines (55 out 58) with LC50>100 μM. Moreover, **1h** evidenced an excellent activity against the full melanoma panel (GI50=0.12 μM) and the NCI-H522 and SF-295 cell lines with GI50 values of 24 nM and 43 nM, respectively.

Degradation of Hsp90 client proteins, up-regulation of Hsp72, and cell cycle distribution: Among the amino-cyanopyridines, the derivative**1h**, showing high antiproliferative activity, low cytotoxicity, and the best EC50 value (252 ± 23 nM), was selected for further biological screenings to elucidate its specific influence on the cellular pathways involving Hsp90. In particular, to link the high binding affinity of **1h** to cellular activity, three *in vitro* assays were set up using the human cancer cell line MCF7, an estrogen receptor positive breast tumor cell line able to express high level of Hsp90,[23] and included in NCI DTP human tumor cell lines panels.

The antiproliferative activity shown by derivative **1h** could be due to the breakdown of multiple cell survival pathways, as a consequence of destabilization of Hsp90 client proteins. To test this hypothesis, we evaluated the effects of **1h** on the degradation of some typical Hsp90 client proteins (Cdk4, c-Raf, and ErbB2) and on up-regulation of Hsp72 in MCF7 cells, by Western blotting analysis on total extracts following a 24 h cells exposure to various concentrations of the tested compound ranges from 2 to ½ of its GI50 value (274 nM, supporting information). The derivative **1h** causes dramatic depletion of the examined client proteins, inducing a very strong increase in the expression levels of the chaperone Hsp72, as shown in figure 4.

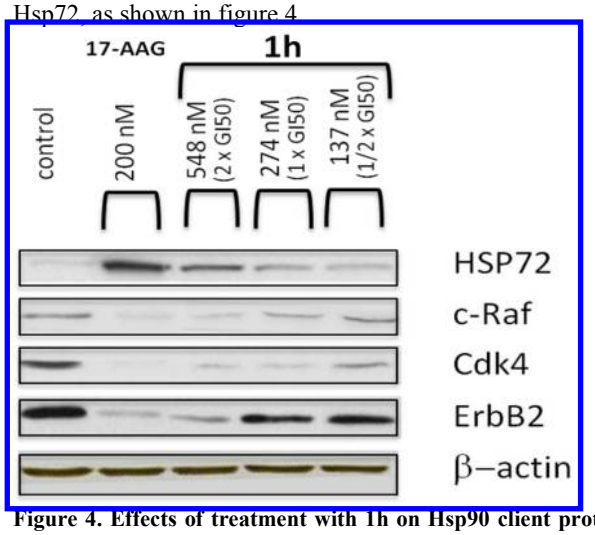


Figure 4. Effects of treatment with 1h on Hsp90 client proteins (Cdk4, ErbB2, c-Raf) levels and Hsp72 expression in MCF-7 cells. MCF-7 cells were exposed to 200 nM 17-AAG or 1h at 2, 1, or ½ of its GI50 value. Total cellular extracts were obtained 24 h after treatment. β-actin is shown as a control for protein loading.

In particular, **1h** at 2-fold of its GI50 concentration showed almost complete knockdown of Hsp90 client proteins. However, Cdk4 was consistently the most sensitive client protein, as measured by both rapid disappearances, whereas ErbB-2 levels were less affected. The depletion was dose-dependent and was achieved with potency similar to that of the reference compound (17-AAG) when tested in same experimental conditions.

Antitumor effects can be achieved by cell death and/or cell cycle arrest. Treatment with derivative **1h** for 24 h did not yield necrosis in MCF7 cells, as demonstrated by negative response to LDH activity assay (data not shown). Hsp90 inhibition induces cell cycle arrest, but the stage of cell cycle block by Hsp90 inhibitors is cell line- and tumor type-dependent.[6] G2/M arrest is seen in some epithelial cancer cell lines, including MCF7 cells.[24-27] To probe the mechanism of cell growth inhibition by derivative **1h** we examined its effects on cell-cycle distribution, by DNA staining with propidium iodide (PI). Cell cycle analysis was performed after 24 h of incubation with **1h** derivative in order to detect the shifts in cell cycle distribution before a significant amount of cells underwent apoptosis (Figure 5). Untreated MCF7 cells showed a normal diploid distribution presenting fast proliferation characteristics, with S + G2/M phase cells accounting for about 35% of the total cells. Treatment with **1h** for 24 h arrests cell cycle at G2/M phase in a dose-dependent manner.

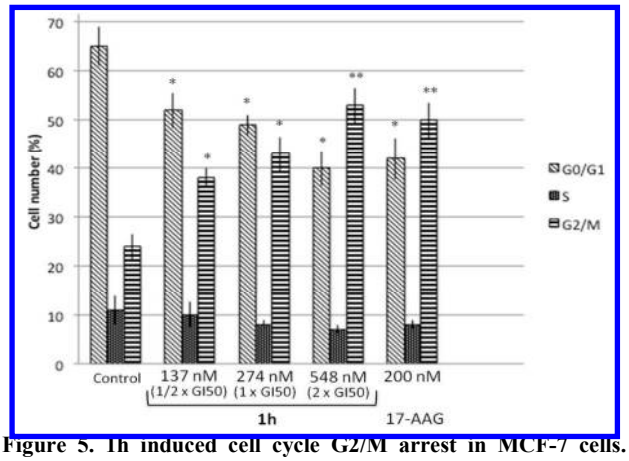


Figure 5. 1h induced cell cycle G2/M arrest in MCF-7 cells. MCF-7 cells were exposed to 200 nM of 17-AAG or for 1h at 2, 1, and ½ of its GI50 value. Then the cells were collected and DNA was stained with PI. DNA content was analysed by flow cytometry for cell cycle distribution. Results shown are mean ± SD for three independent experiments. * p<0.05; ** p<0.001 as compared to controls.

An increase in G2/M phase cells from 24% to 53% ($p < 0.0001$) was observed as a result of 24 h **1h** cells exposure to 2 x GI50 (0.548 μM). The marked accumulation in the G2/M cell cycle phase was coupled to a decrease in the G0/G1 phase cells (65% vs. 40%, $p < 0.05$) more pronounced than the decrease in the S phase (11% vs. 7%). When the same treatment was performed for 48 h, a new population sub-G0/G1 appeared with a parallel decrease of all the populations (data not shown).

In light of these findings, the cellular activities of the amino-cyanopyridine derivative **1h** are deeply interesting. The EC50 values for their apparent binding affinities are comparable to the GI50 value in MCF7 cells and at the similar nanomolar concentration **1h** is very effective, causing Hsp90-client protein depletion, Hsp72 up-regulation and G2/M cell cycle arrest. Collectively our results show good correlation between Hsp90 binding affinity and cellular activities, suggesting that the effects of **1h** on MCF7 cells were consequence of their Hsp90 chaperone inhibitory properties.

SUMMARY

The Energy-based pharmacophore virtual screening protocol was used to identify the amino-cyanopyridine derivatives **1a-k** as a new class of Hsp90 inhibitors. The competitive binding assay confirmed the Hsp90 binding capability of the synthetized compound at the sub-micromolar concentration.

The antiproliferative activity against a panel of nearly 60 cell lines was evaluated by NCI in the DTP protocol. The tested amino-cyanopyridines showed high potency and low toxicity. The hit amino-cyanopyridine**1h** revealed responses consistent with Hsp90 inhibition, including arrest of cell cycle at G2/M phase and down-regulation of Hsp90 client protein levels. In conclusion, the promising biological properties of these compounds justify further investigation to elucidate the detailed mechanism of action.

ASSOCIATED CONTENT

Supporting Information. Additional experimental procedures and biological screening method, the yields, melting point, purity, ^{1}H and ^{13}C NMR data for all the compounds, and the detailed pro-

tocol of molecular modeling and the results of all biological assays. This material is available free of charge via the Internet at http://pubs.acs.org."

AUTHOR INFORMATION

Corresponding Author

* Phone number: +390916167210; Fax number: +390916230923
Email_Address: antonino.lauria@unipa.it

ABBREVIATIONS

Hsp90, Heat Shock Protein 90; **17-AAG**, 17-AllylAminoGeldanamycin; **PI**, Propidium Iodide; **DTP**, Developmental Therapeutics Program; **NCI**, National Cancer Institute; **GI50**, Growth Inhibition of 50%; **TGI**, Total Growth Inhibition; **LC50**, Letal Concentration 50%; **MG_MID**, Mean Graph MIDpoint.

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