A duplexed phenotypic screen for the simultaneous detection of inhibitors of the molecular chaperone heat shock protein 90 and modulators of cellular acetylation

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Abstract
Histone deacetylases (HDACs), histone acetyltransferases (HATs), and the molecular chaperone heat shock protein 90 (HSP90) are attractive anticancer drug targets. High-throughput screening plays a pivotal role in modern molecular mechanism-based drug discovery. Cell-based screens are particularly useful in that they identify compounds that are permeable and active against the selected target or pathway in a cellular context. We have previously developed time-resolved fluorescence cell immunosorbent assays (TRF-Cellisas) for compound screening and pharmacodynamic studies. These assays use a primary antibody to the single protein of interest and a matched secondary immunoglobulin labeled with an europium chelate (Eu). The availability of species-specific secondary antibodies labeled with different lanthanide chelates provides the potential for multiplexing this type of assay. The approach has been applied to the development of a 384-well duplexed cell-based screen to simultaneously detect compounds that induce the co-chaperone HSP70 as a molecular marker of potential inhibitors of HSP90 together with those that modulate cellular acetylation (i.e., potential inhibitors of histone deacetylase or histone acetyltransferase activity). The duplexed assay proved reliable in high-throughput format and ~64,000 compounds were screened. Following evaluation in secondary assays, 3 of 13 hits from the HSP70 arm were confirmed. Two of these directly inhibited the intrinsic ATPase activity of HSP90 whereas the third seems to have a different mechanism of action. In the acetylation arm, two compounds increased cellular acetylation, one of which inhibited histone deacetylase activity. A third compound decreased cellular histone acetylation, potentially through a novel mechanism of action. [Mol Cancer Ther 2007;6(3):1112–22]

Introduction
High-throughput screening is an integral part of contemporary mechanism-based drug discovery and provides an effective strategy for identifying compounds with activity against specific molecular targets, the pharmacologic properties of which can be subsequently optimized by medicinal chemistry (1). The process has been applied successfully to the development of novel anticancer agents (2). Biochemical screens use isolated targets such as enzymes, cofactors, or membrane components, which are usually biochemically pure and well characterized. Cell-based or phenotypic assays (3) use cultured whole cells, which contain all the components of the myriad pathways that are associated with cell functions such as growth, division, differentiation, and apoptosis. In cell-based assays, modulation of the target by small molecules could result from inhibition or activation of one or more of the many molecular components in a complex pathway or network. Mechanistic deconvolution of the compound is required for hits from cell-based screens and can be challenging (4).

The molecular chaperone heat shock protein 90 (HSP90) is an attractive anticancer drug target because it maintains the conformation, stability, and function of several oncogenic client proteins such as CRAF, ERBB2, AKT, and cyclin-dependent kinase-4 (CDK4; refs. 5, 6). HSP90 inhibition leads to proteasomal degradation of client proteins, disruption of signaling pathways, and antitumor activity (5–9). Several HSP90 inhibitors have been described (10, 11) including the natural products radicicol and geldanamycin. The semisynthetic analogue 17-allylamino-17-demethoxygeldanamycin, the first HSP90 inhibitor to enter clinical trials, has shown evidence of target inhibition and clinical activity (12). The properties of purine-based HSP90 inhibitors continue to be improved by structure-aided analogue synthesis and X-ray crystallography (13–15). All these compounds inhibit the ATPase activity of HSP90 by
binding to the NH2-terminal ATP-binding pocket (15–17). In addition, we have identified a novel 3,4-diarylpyrazole HSP90 inhibitor, CCT018159, in a biochemical screen designed to detect HSP90 ATPase inhibitors (18). This has been optimized by structure-based design (19, 20).

There is increasing interest in other regions of the HSP90 molecule (21). The crystal structure of the COOH terminus of HSP90 was determined as part of the overall structure of the yeast HSP90-p23-Sha1 “closed” chaperone complex (22). Compound interaction in this area may be inhibitory as suggested for novobiocin (23) and molybdate (24). The HSP90N isoform can activate CRAF despite lacking the NH2-terminal ATPase site (25). Furthermore, inhibition of the active site in the COOH-terminal region may regulate the activity of the NH2-terminal region (26). These COOH-terminal active sites may be ATPases (26) but their exact nature is unknown. Alternatively, identification of compounds preventing HSP90 interaction with its co-chaperones, such as AHA1 (27), p23 (28), and CDC37 (29), or with its client proteins (30) maybe a useful strategy for HSP90 inhibition and could potentially provide compounds with distinct molecular and cellular properties (6).

The molecular signature of HSP90 inhibition includes depletion of client proteins and also induction of HSP70 through an effect on heat shock factor 1 (9, 12). We have used increased HSP70 expression as an end point in one arm of a duplexed cell-based phenotypic screen as a molecular marker of HSP90 inhibition. We reasoned that inhibitors identified in this cell-based screen might inhibit HSP90 ATPase activity directly or via the other mechanisms described above. It was also possible that HSP70 levels could be altered by additional mechanisms controlling its expression.

Histone deacetylases (HDAC) are zinc-dependent enzymes that deacetylase lysine residues on the NH2-terminal “tails” of histones and other cellular proteins (31). These alterations induce changes in chromatin structure and modify protein-DNA interactions. Inhibition of HDACs results in histone hyperacetylation, leading to transcriptional activation or repression of genes, many of which are involved with malignancy (32–35). Several HDAC inhibitors have been described, including the hydroxamic acids trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), LAQ-824, PDX-101, the benzamide MS-275, and the cyclic depsipeptide FR901228. These compounds induce cell cycle arrest and apoptosis, have antitumor effects in vivo (36), and several are in clinical trials (36).

In addition to inhibition of histone deacetylation, treatment with HDAC inhibitors causes hyperacetylation of α-tubulin and other cellular proteins. Cell motility is dependent on α-tubulin acetylation (37) and this may be an important factor in metastasis and angiogenesis (38). HDAC6 is an α-tubulin deacetylase (37, 38), and tubacin, a specific inhibitor of this enzyme, was found using a high-throughput cytoblot screen (39). Interestingly, HSP90 is hyperacetylated following exposure of cells to HDAC inhibitors, disrupting the function of the molecular chaperone (40), which leads to loss of client proteins. Acetylation of HSP90 is mediated via inhibition of HDAC6 (41, 42).

Histone acetyltransferases (HATs) catalyze the acetylation of specific histone lysine residues (43). With HDACs, they are important regulators of gene expression via modulation of histone acetylation (35). Many cellular proteins, in addition to histones, are also HAT substrates, including p53 (44). Aberrant expression and mutation of several HATs are associated with malignancy (45, 46) and represent additional targets for anticancer drug discovery. Few inhibitors have been described, however (44). Using a biochemical screen (47), we have identified a series of thiol-reactive isothiazolones that inhibit p300/CREB binding protein–associated factor (PCAF) and p300, resulting in increased cellular acetylation and reduced growth inhibition (48).

For all three molecular targets, HSP90, HDACs, and HATs, there is continued scope for discovery of new inhibitors, in particular, non-ATPase inhibitors of HSP90 novel classes of HDAC inhibitors (e.g., non-hydroxamic acid), that may have improved pharmaceutical properties and chemically tractable inhibitors of HATs.

Here, we report the development of a duplexed phenotypic screen to identify HSP90 inhibitors and modulators of cellular acetylation and the results obtained from a compound library screen. The cytoblot assay (49) detects apoptotic changes using specific antibodies on fixed and permeabilized cells and has been applied to several high-throughput screens (39, 50). This format has been adapted to develop a time-resolved fluorescent (TRF) variant of the cytoblot using europium (Eu) chelate-labeled secondary antibodies (TRF-Cellisas; refs. 51, 52). The format was used as the basis for high-throughput screening in 96-well plates (53). The TRF end point provides the potential for multiplexing screens (54), enabling more efficient reagent use. We hypothesized that HSP90 inhibition would be indicated by increased cellular HSP70 whereas inhibition of HAT and HDAC activity would be revealed by either reduced or increased cellular acetylation respectively.

In common with other cell-based screens, secondary assays were required to provide preliminary deconvolution of the mechanism of action of identified hits (Fig. 1). From a 64,000-compound collection, the duplexed screen and subsequent secondary assays identified validated hits in both arms of the screen. Three of these increased HSP70 expression (HSP70 inducer) and two inhibited the ATPase activity of HSP90. From the other arm of the screen, three modulators of cellular acetylation (acetylation modulator) were identified. One compound reduced cellular acetylation but did not inhibit HAT enzymatic activity. Two compounds increased acetylation and one showed weak inhibition of HDAC activity.

**Materials and Methods**

**Materials**

HCT116 human colon tumor cells were obtained from American Type Culture Collection via LGC Promochem,
Duplexed Cell-Based Screens for Modulators of HSP90 and Acetylation

Figure 1. Scheme for cascade of secondary assays to investigate the mode of action of the hits from the duplexed screen.

Tedddington, United Kingdom and were free of Mycoplasma as determined by nested PCR. Geldanamycin and TSA (Sigma-Aldrich Co. Ltd., Poole, United Kingdom) were stored in aliquots of 25 mmol/L stock in DMSO at −80°C. Inducible HSP70 (HSP72) monoclonal antibody (SPA810) was obtained from Biocom (Bressgen; York, United Kingdom); polyclonal rabbit antibody to acetylated proteins (Ab193) from Abcam Ltd. (Cambridge, United Kingdom); DELFIA assay buffer, Eu-labeled antimouse immunoglobulin G (IgG; A2014), samarium (Sm)–labeled antirabbit IgG, and enhancement solution from Perkin-Elmer Life Sciences (Beaconsfield, United Kingdom); polyclonal rabbit antibody to acetylated histone H3 from Upstate (Milton Keynes, United Kingdom); DELFIA assay buffer, Eu-labeled antimouse immunoglobulin G (IgG; AD0124), samarium (Sm)–labeled antirabbit IgG, and enhancement solution from Perkin-Elmer Life Sciences (Beaconsfield, United Kingdom). A mixture was shaken for 2 min and incubated for 30 min at 37°C. The medium was aspirated and the cells were washed with PBS, trypsinized, neutralized, and counted. Cells were subsequently diluted to ~36,000/mL and dispensed into 384-well microtitre plates (2,000 per well) using a Multidrop dispenser (Thermo Electron, Basingstoke, Hants, United Kingdom) and incubated for 29 h before compound addition.

Addition of Compounds and Controls

For screening, 320 compounds (3 μL of a 200 μmol/L solution in 2% DMSO) were added to the central wells of each 384-well plate (10.3 μmol/L compound and 0.1% DMSO final concentration in the well) using a Multidrop. Plates were routinely screened in batches of 25 to 30. Compounds were replaced by 3 μL of 2% DMSO in three additional plates included at the start, middle, and end of each batch of plates.

TRF-Cellisa

The plates in TRF-Cellisa incubation steps were placed in plastic boxes lined with damp paper. Reagents were added with a Multidrop (Thermo Electron). Plates were washed in an automatic plate washer [PlateWash 96/384 (Perkin-Elmer Life Sciences), Tecan 384 (Tecan, Reading, United Kingdom), or WellWash4 (Thermo Electron)]. Following incubation, medium was “flicked” out by hand. Cells were fixed and permeabilized with 0.25% Triton X-100 (50 μL) for 30 min at 37°C. The wells were washed once with 80-μL PBS and blocked for 30 min (50 μL of 5% dried milk in PBS) followed by a single wash as before. Primary and secondary antibodies were added together (50 μL) in 5% dried milk/assay buffer and incubated overnight at 4°C. Plates were washed thrice with water/Tween 20 (80 μL) and enhancement solution (50 μL) was added. After shaking (15 min), TRF was measured in a Victor2 1420 reader (Perkin-Elmer Life Sciences) using the Sm/Eu dual label mode. The plates were washed once with PBS (80 μL), BCA reagent was added (50 μL), and the mixture was shaken for 2 min and incubated for 30 min at 37°C. Absorption was read at 570 nm.

Analysis of Screen Data

Eu and Sm counts per minute were normalized for protein concentration by dividing by the BCA absorption at 570 nm (reagent blank subtracted). Results from plates acetylation modulator (AM)-1 and AM2 were obtained from ChemDiv (San Diego, CA). All other hit compounds were from the Cancer Research UK Compound Collection.

Cell Culture

HCT116 human colorectal cells were grown in T175 flasks (Corning, VWR, Poole, Dorset, United Kingdom) to 60% to 70% confluence in DMEM supplemented with 4,500 mg/mL glucose, 10% fetal bovine serum, 200 mmol/L L-glutamine, and 5 mL nonessential amino acids in a humidified atmosphere of 5% CO2 at 37°C. The medium was aspirated and the cells were washed with PBS, trypsinized, neutralized, and counted. Cells were subsequently diluted to ~36,000/mL and dispensed into 384-well microtitre plates (2,000 per well) using a Multidrop dispenser (Thermo Electron, Basingstoke, Hants, United Kingdom) and incubated for 29 h before compound addition.
where there was poor discrimination (<120%) between mean values of negative and positive controls were repeated. Primary hits were identified using OMMM and RS³ (Accelrys, Cambridge, United Kingdom) to analyze these data. Hits were defined as compounds that gave responses of more than 150% (HSP90), more than 200% (acetylation) or less than 50% (acetylation), as compared to the DMSO control. Hits were categorized as HSP70 inducers or acetylation modulators. HSP70 inducers could be inhibitors of HSP90 acting on ATPase or non-ATPase sites. Acetylation modulators could be inhibitors of HATs or HDAC inhibitors. On the other hand, hits identified against either of the assay readouts could have alternative cellular mechanisms.

**Hit Confirmation and Secondary Assays**

Hits were cherry picked using a Multiprobe (Perkin-Elmer Life Sciences) into 96-well plates and confirmed using individual 96-well TRF-Cellisas (51, 52) for HSP70 and acetylation. Cells were plated out [8,000 per well (200 µL)]. Compounds (20 µL to give a final concentration of 20 µmol/L) were added to the central 80 wells. Four wells of control 0.1% DMSO and four wells of control inhibitor (1 µmol/L geldanamycin for HSP70 and 0.33 µmol/L TSA for acetylation) were included on either side of each plate. Cells were fixed and permeabilized with fixing solution (200 µL). After washing (200 µL) and blocking (50 µL) as before, primary antibodies (SPA 810 and Ab193, both 1 µg/mL) in PBS were added and incubated for 1.5 h at 37°C. Eu-labeled antimouse IgG (0.2 µg/mL) or antirabbit IgG (0.2 µg/mL) was added to the HSP70 or acetylation assays, respectively (1 h at 37°C). Plates were washed, enhanced, and read using Eu time-resolved mode and counts normalized as before.

**Growth Inhibition Assay**

The growth inhibitory effects (GI₅₀) of confirmed hits were determined using the sulforhodamine B growth inhibition assay (55) in HCT116 colon cancer cells over 72 h.

**HSP90 ATPase Assay**

Confirmed HSP70 inducer hits were assayed for ATPase inhibition in a malachite green assay for the measurement of inorganic phosphate using recombinant yeast HSP90 enzyme (18).

**HDAC Activity Assay**

Compounds that raised cellular acetylation were assayed for HDAC inhibition using HeLa nuclear extract (Cilbiotech S.A., Mons, Belgium) and substrate and developer (KI-104 and KI-105, respectively; from Biomol International, LP, Exeter, United Kingdom) according to manufacturer's instructions.

**HAT Activity Assay**

The potential HAT inhibitor was tested for activity against PCAF enzyme using the filter-based assay as previously described (47).

**Western Blotting**

HCT116 cells were treated with confirmed hit compounds, 0.33 µmol/L TSA, 0.33 µmol/L geldanamycin, or DMSO control. Whole-cell lysates were prepared on ice [50 mmol/L Tris-HCl buffer (pH 8.5) containing 1% NP40, 2 mmol/L phenylmethylsulfonyl fluoride, 10 µg/mL each aprotinin and leupeptin, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and 1 mmol/L β-glycerophosphate] and protein concentration was determined by BCA assay. Equivalent protein concentrations were loaded onto precast gels followed by electrophoresis and transferred to nitrocellulose membranes. Membranes were probed with appropriate primary antibodies followed by secondary IgGs labeled with horseradish peroxidase. After addition of chemiluminescent reagent, the protein bands were visualized on high performance film (Hyperfilm, GE Healthcare) and quantified using ImageQuant 5.0 (GE Healthcare).

**Results**

**Assay Development**

We have previously described individual TRF-Cellisas for HSP70 and cellular acetylation (48, 51, 52). In preparation for the duplexed screen, initial studies were carried out in 96-well plates using these established assay conditions. Antibody concentrations were optimized by standard checkerboarding experiments (data not shown). Additionally, the time at which the positive controls geldanamycin and TSA (chemical structures shown in Fig. 2) were added after cells were plated and the subsequent duration of compound incubation were optimized to show the maximum difference in both Eu and Sm counts between treated and untreated cells. Table 1

![Figure 2. Chemical structures of the HSP90 ATPase activity inhibitors geldanamycin and CCT018159 and the HDAC inhibitor TSA.](https://example.com/figure2.png)
shows the change in Eu and Sm after treatment with geldanamycin and TSA at different times. TSA clearly modulated cellular acetylation at all time points tested. However, because the HSP70 response to geldanamycin was higher (~2-fold) at later time points, it was decided to treat cells 29 h following plating out and to fix the cells 48 h later.

Initially, both primary and both secondary antibodies were added together in two separate steps in the duplexed assay. Twenty-five microliters of each primary antibody [anti-HSP70 antibody (1 μg/mL) and anti–acetylated protein polyclonal antibody (1 μg/mL)] diluted in PBS were added to each well and the plates incubated for 1.5 h. Plates were washed thrice with deionized water containing 0.1% Tween 20 and incubated with Eu-labeled antimouse IgG (0.2 μg/mL) and Sm-labeled antirabbit IgG (0.17 μg/mL) diluted in assay buffer and incubated for 1 h. Using these conditions, a concentration-response relationship was obtained when HCT116 cells were treated with either geldanamycin (Fig. 3A) or TSA (Fig. 3B). In the screen, these compounds were used as positive controls at concentrations of 1.0 and 0.3 μmol/L, respectively.

For high-throughput screening, the duplexed assay was adapted to 384-well format. Growth curves for HCT116 cells cultured in 384-well plates showed that cells, plated as described in Materials and Methods (2,000 per well), were in log phase by 36 h and became confluent after 96 h. This allowed ample time to treat the cells for the required time during logarithmic growth. To increase throughput, the two primary and the two secondary antibodies were combined before addition to the plate, which was subsequently incubated overnight at 4°C, eliminating the need for one reagent addition and wash step. In the final duplexed screen, HCT116 cells were grown for 29 h, treated with compounds (10 μmol/L) or controls, and fixed 48 h later.

### Duplexed Screen Performance

Using the final duplexed assay protocol described in Materials and Methods, 224 plates containing ~64,000 compounds were screened. Figure 4 shows the mean normalized Sm and Eu results obtained from the control wells on each plate of one screen batch after treatment with TSA (0.3 μmol/L) or geldanamycin (1 μmol/L), respectively. Both are compared with the corresponding values for the wells on each plate that contained cells treated with DMSO vehicle (0.1%).

The overall between-plate mean HSP70 response to 1 μmol/L geldanamycin was 148.8 ± 12.8% [coefficient of variation (CV), 8.5%] and the change in acetylation after

<table>
<thead>
<tr>
<th>Time from plating to compound addition (h)</th>
<th>Incubation with compound (h)</th>
<th>Eu response to geldanamycin 1 μmol/L, % control (mean ± SD)</th>
<th>Sm response to TSA 0.3 μmol/L, % control (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>5</td>
<td>108.9 ± 16.2</td>
<td>27.5 ± 9.1</td>
</tr>
<tr>
<td>24</td>
<td>24</td>
<td>93.4 ± 8.5</td>
<td>73.7 ± 13.1</td>
</tr>
<tr>
<td>24</td>
<td>48</td>
<td>84.05 ± 12.6</td>
<td>274.7 ± 20.7</td>
</tr>
<tr>
<td>24</td>
<td>72</td>
<td>217.4 ± 19.7</td>
<td>204.5 ± 16.7</td>
</tr>
<tr>
<td>29</td>
<td>24</td>
<td>116.4 ± 14.6</td>
<td>142.3 ± 10.3</td>
</tr>
<tr>
<td>29</td>
<td>48</td>
<td>196.2 ± 27.7</td>
<td>301.6 ± 13.8</td>
</tr>
<tr>
<td>29</td>
<td>72</td>
<td>222.6 ± 50.7</td>
<td>244.3 ± 13.9</td>
</tr>
</tbody>
</table>

Note: Eu and Sm signals were normalized for protein in the well and percent increase from DMSO controls was determined.

### Figure 3

Changes in HSP70 expression (A) and cellular acetylation (B) in HCT116 cells treated with increasing concentrations of geldanamycin (GA; 0.25–8 μmol/L) and TSA (0.031–1 μmol/L), respectively, measured in the duplexed assay. Columns, mean; bars, SD. Open columns, DMSO controls.
treatment with 0.3 μmol/L TSA was 210 ± 25.8% (CV, 12.2%) when compared with the average results from DMSO-treated control cells (100%). These responses are consistent with those obtained using nonduplexed assays (2-fold increase for HSP70 and 2.5-fold increase for cellular acetylation). The mean within-plate variation was acceptable at 16.9% and 13.4% for geldanamycin and TSA, respectively. BCA assay absorbance values were used as a measure of cell number in each well. BCA readings from the DMSO-only control plates (CV, 16.4%) indicated reproducible cell plating and growth during the course of the assay.

**Screen Results**

Primary hits were selected as those that resulted in a cellular response equal to or greater than that of the positive controls, geldanamycin and TSA, on the same plate. The hits were cherry picked into 96-well plates. Compounds with activity in at least three further nonduplexed assays were assigned as confirmed hits and progressed through the secondary assays (Fig. 1).

**HSP70 Inducers**

There were 13 confirmed hits from the HSP70 arm of the screen. One of these was the 3,4-diarylpzazole compound CCT018159 (refs. 18, 19; chemical structure shown in Fig. 2), which had previously been identified from an overlapping compound collection using a biochemical screen for inhibitors of the NH2-terminal ATPase site of HSP90 (18). Identification of this compound has already led to a successful drug development program (19, 20). Analogues of CCT018159 that were previously shown to have weak ATPase inhibition at 40 μmol/L (18) were not identified as hits at the concentration (10 μmol/L) used in the duplexed screen. The identification of CCT018159 confirmed that the phenotypic duplexed screen was capable of identifying compounds that are known to act directly as HSP90 ATPase inhibitors.

The remaining 12 confirmed hits were tested as shown in Fig. 1. Six of these compounds were poorly soluble, showed little growth inhibition in HCT116 cells over 72 h, and were not pursued further. For the remaining compounds, H11 to H16, Table 2 shows the effect on HSP70 determined in a nonduplexed assay and GI50 values for growth inhibition in HCT116 cells together with their chemical structures. Of these compounds, only H11 [the trimethoxylchalcone CC002151; 1-(3,4,5-trimethoxyphenyl)-3-phenylprop-2-en-1-one] showed a clear concentration-dependent induction of HSP70 in HCT116 cells by both TRF-Cellisa and immunoblotting (Fig. 5).

**Table 2.** Chemical structures and data for HSP70 induction and growth inhibition (sulforhodamine B assay for 72 h) in HCT116 human colorectal tumor cells for compounds H11 to H16

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>% Increase in HSP70*</th>
<th>GI50 (μmol/L ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H11 CC002151</td>
<td><img src="http://example.com/structure1" alt="Structure" /></td>
<td>195</td>
<td>2.5 ± 0.8</td>
</tr>
<tr>
<td>H12 CC003862</td>
<td>Unpurified natural product</td>
<td>197</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>H13 CC000119</td>
<td><img src="http://example.com/structure2" alt="Structure" /></td>
<td>242</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>H14 CC003539</td>
<td><img src="http://example.com/structure3" alt="Structure" /></td>
<td>284</td>
<td>0.07 ± 0.06</td>
</tr>
<tr>
<td>H15 CC002986</td>
<td><img src="http://example.com/structure4" alt="Structure" /></td>
<td>116</td>
<td>1.9 ± 1.4</td>
</tr>
<tr>
<td>H16 CCT026194</td>
<td><img src="http://example.com/structure5" alt="Structure" /></td>
<td>183</td>
<td>0.02 ± 0.01</td>
</tr>
</tbody>
</table>

* n = 3–6.

1 The structure shown is of a close analogue of the hit CC000119.
Compounds HI1–HI6 were also tested for inhibition of intrinsic ATPase activity of HSP90 in the malachite green assay of inorganic phosphate release from ATP (18). Compound HI6 (CCT026194; the bisamide of 2,6-diaminobenzothiazole) showed weak inhibitory activity (36.3 ± 12% at 100 μmol/L). The remaining compounds, including CC002151, were inactive in this biochemical assay at concentrations up to 100 μmol/L.

With Western blotting, decreased expression of HSP90 client proteins (CRAF, CDK4, and ERBB2) was observed in cells treated with CCT026194 (Fig. 6A) but increased expression of HSP70 could not be shown reproducibly. Only the trimethoxychalcone (HI6: CC002151) showed both of these key molecular features characteristic of HSP90 inhibition at 24- and 48-h compound exposure (Fig. 6B). Thus, the results obtained with CC002151 show that, as well as identifying inhibitors of the ATPase activity of HSP90, the duplexed screen can identify compounds that induce protein expression changes consistent with HSP90 inhibition but by a mechanism other than direct ATPase inhibition.

Acetylation Modulators

Following confirmation assays, three acetylation modulators were identified (Table 3). Two compounds, AM1 and AM2 [CCT035764, 2,7-dimethylacridine-3,6-diamine (diaminodimethylacridine), and CCT030493, 4-(bis(2-methoxyethyl)amino)-N-(6-methoxybenzo[d]thiazol-2-yl)benzamide (2-amino-6-methoxybenzothiazole derivative)] caused an increase (140% and 160%, respectively), whereas the third, AM3 [CC000475; 4-(benzo[d]thiazol-2-yl)-4-ethoxycyclohexa-2,5-diene (1,4-cyclohexadiene derivative)], resulted in a complete loss of cellular acetylation. Figure 7A shows the effects of DMSO, TSA, and compounds AM1 to AM3 on histone acetylation in HCT116 cells as measured in a TRF-Cellisa. Compounds CCT035764 (AM1) and CCT030493 (AM2) increased histone acetylation by 140% and 120% (P = 0.0001 and 0.004, respectively) compared with a 200% change following TSA treatment. AM3 (CCT00475) markedly reduced histone acetylation to 17% of DMSO control. This is unlikely to be due to cytotoxicity because all compounds were tested at the same multiple of their respective GI50 values and the results are normalized to total cellular protein. In addition, many compounds that were shown to be toxic by the BCA measurement in the screen did not decrease cellular acetylation. CCT00475 did not inhibit the HAT activity of PCAF in a biochemical assay (47) at concentrations as high as 100 μmol/L.

![Figure 5](image-url) Effect of CCT002151 on HSP70 expression in HCT116 cells. A, cells were treated with increasing concentrations (0.6–10 μmol/L) of CCT002151 (○) for 48 h. Geldanamycin (1 μmol/L; ●) was used as a positive control. Points, mean; bars, SD; measured in the nonduplexed TRF-Cellisa. B, Western blotting. HCT116 cells were treated with geldanamycin (0.3 μmol/L; lanes 3 and 4) or CC002151 (5 and 10 μmol/L; lanes 5–8) for 24 or 48 h. Lanes 7 and 2, untreated controls. GAPDH was used as a loading control.

![Figure 6](image-url) Compounds that induce the molecular signature of HSP90 inhibition. Western blots showing changes in expression of HSP70 and the HSP90 client proteins in HCT116 cells after treatment with CCT026194 (lane 3; 0.05 μmol/L), geldanamycin (lane 2; 0.3 μmol/L), and DMSO control (lane 1; 0.15%) for 24 h (A) and with CC002151 (lane 3; 10 or 15 μmol/L), geldanamycin (lane 2; 0.3 μmol/L), and DMSO control (lane 1; 0.15%) for either 24 or 48 h (B). GAPDH was used as a loading control.
Compounds AM1 and AM2 were tested for inhibition of HDAC activity using HeLa cell nuclear extract as the source of enzyme. Compound AM1 (CCT035764) displayed weak activity at 200 μmol/L (30.8 ± 19.2% inhibition) whereas AM2 (CCT030493) was inactive. However, when HCT116 cells were treated with CCT030493 for 48 h and analyzed by immunoblotting, increased acetylation of both histone and α-tubulin (190% and 130%, respectively, by densitometry; Fig. 7B) was observed. Thus, the duplex screen can identify compounds that increase or decrease protein acetylation by mechanisms other than direct inhibition of HDAC or HAT enzymatic activity.

Discussion

We have used a duplexed phenotypic screen to identify compounds that cause protein expression changes that are characteristic of HSP90 inhibition as well as modulators of cellular acetylation in human colon carcinoma cells. The compounds identified were of various structural types and inhibited cell proliferation. Other compounds in the screen that markedly reduced total cellular protein had no effect on HSP70 expression or cellular acetylation.

The reproducibility of the duplexed cell-based assay was highly acceptable, considering the overall assay complexity. The protein measurement from DMSO control plates showed that the plating and growth of the cells was reproducible. The variation in the cellular response to geldanamycin and TSA was also highly acceptable. One of the challenges of simultaneously testing for compound effects on several distinct targets in a cell-based screen is the selection of conditions for detection of different phenotypic changes at one time point. Individual target responses to potential inhibitors could be dependent on a number of factors, such as effects on the cell cycle. In this case, the increase in cellular acetylation resulting from treatment with the HDAC inhibitor TSA occurred earlier than the geldanamycin-induced increase in HSP70 used as the marker of HSP90 inhibition (Table 1). This is consistent with rapid and highly dynamic histone and protein acetylation (56), in contrast to HSP90 inhibitor–induced HSP70 expression, which occurs more slowly (8, 9). Even with a longer treatment time chosen for maximum HSP70 response, each batch of compounds was assayed within a week. The duplexed screen was more economical than running two separate phenotypic screens. The choice of assay readout is critical in screen design. TRF is sensitive and less prone to background interference than standard fluorescence, luminescence, or color end points (52, 54). Furthermore, antibodies labeled with lanthanide chelates, exhibiting different emission spectra, offer good opportunities for duplexing assays. The number of plate repeats due to occasional poor discrimination between positive and negative control wells was in keeping with less complex screens (53).

One of the major advantages of cell-based screens is their ability to identify cell-permeable active compounds. Unlike biochemical screens, however, secondary assays are required to deconvolute the mode of action of hits (1, 4). In this case, a scheme was required that would initially rule in or rule out direct inhibition of the three targets (HSP90, HDACs, or HATs). The assays used to probe the action of the compounds (Fig. 1) provided useful information to help understand their overall phenotypic effect on cells (i.e., growth inhibition). Normalization of protein expression changes to total cellular protein minimizes the identification of purely cytotoxic compounds. We have previously shown that these changes are not produced by known cytotoxic agents (51, 57).

Hits were successfully detected in both arms of the screen but, interestingly, none were hits in both. Perhaps surprisingly, geldanamycin increased acetylation by ~75% throughout the screen whereas TSA did not increase HSP70. The latter was unexpected because HDAC inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>% Change in acetylation*</th>
<th>GI50 (μmol/L ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1</td>
<td>CCT035764</td>
<td>240% increase</td>
<td>0.4 ± 0.03</td>
</tr>
<tr>
<td>AM2</td>
<td>CCT030493</td>
<td>260% increase</td>
<td>&gt;100</td>
</tr>
<tr>
<td>AM3</td>
<td>CC000475</td>
<td>100% decrease</td>
<td>0.98 ± 0.4</td>
</tr>
</tbody>
</table>

*n = 3–6.

Table 3. Chemical structures and data for cellular acetylation and growth inhibition (sulforhodamine B assay for 72 h) in HCT116 cells for compounds AM1 to AM3
inhibit HSP90 activity, resulting in both induced HSP70 expression and depletion of client proteins (40–42). There is compelling evidence of a complex association between chromatin and HSP90 (58, 59). The effect of HSP90 inhibitors on cellular acetylation remains to be determined in more detail. We have shown using gene expression microarrays and proteomic analysis that HSP90 inhibition results in the altered expression of a number of chromatin-regulating genes and proteins (60).

The identification of CCT018159 as a hit in the HSP70 arm of the screen was encouraging. This compound was previously discovered in our biochemical screen as a HSP90 ATPase inhibitor (18). X-ray crystallography confirmed that it binds to the HSP90 NH2-terminal ATPase pocket (19, 20). HI6 (CCT026194), identified as a weak inhibitor of ATPase in the secondary assay cascade, showed only 30% inhibition (at 40 μmol/L) of isolated HSP90 in the biochemical screen (18) and was not picked as a hit on that occasion. The discrepancy between the ability of CCT026194 to cause an induction of HSP70 in the present screen but not reproducibly by subsequent Western blotting was most likely due to its low potency against HSP90. Effects other than on HSP90 may contribute to the growth inhibitory properties of CCT026194. This compound is a 2,6-bisamide derivative of benzothiazole and was reported as a hit in a cytotoxicity screen for anticancer agents although its antitumor properties in human tumor xenografts were disappointing (61). The two amide groups were subsequently varied, leading to a compound with improved activity in both the in vitro cell proliferation assay and xenograft studies. CCT026194 is closely related to the benzothiazole derivative BMS-243117, which is a nanomolar inhibitor of LCK, a protein tyrosine kinase required for T-cell antigen receptor signaling (62).

HI1 (CC002151) did not inhibit HSP90 ATPase activity, was growth inhibitory, and, at 24 and 48 h, induced the pattern of protein expression changes characteristic of HSP90 inhibition (Fig. 7). This finding suggests an indirect effect on HSP90. Further studies are required to determine the exact mechanism of action; in particular, whether the reduced expression of HSP90 clients is due to posttranslational destabilization through enhanced proteasomal-mediated degradation or to effects at the mRNA level. Despite the lack of precise mode of action, the identification of CC002151 was pleasing because we originally hypothesized that this duplexed screen had the potential to find putative non-ATPase inhibitors of HSP90 chaperone function. CC002151, a trimethoxychalcone, is one of a group of known thiol-reactive antimitotic compounds (63) that inhibit tubulin, disrupting microtubule assembly, which leads to G2-M arrest and cytoskeleton disruption (64). Compounds of this type have features present in known antimitotic agents such as combretastatin A-4, colchicine, and podophyllotoxins, which bind to tubulin, thereby disrupting microtubule assembly (63). Chalcones inhibit the expression of vascular cell adhesion molecule-1 in response to cytokines such as tumor necrosis factor-α (65). Interestingly, HSP90 is believed to play a role in glucocorticoid receptor nucleocytoplasmic shuttling, which requires an intact cytoskeleton (66). Mechanistic deconvolution could initially focus on whether CCT002151 interacts with the HSP90 multichaperone complex; if so, at what site; and how this activity relates to other observed cellular effects of this group of compounds.

In addition to compounds that affected HSP70 expression, three hits were identified from the acetylation arm of the screen (acetylation modulators). AM1 and AM2 induced cellular acetylation and were considered as putative HDAC inhibitors, whereas AM3 lowered acetylation, a molecular event known to occur following HAT inhibition (48). All three compounds were growth inhibitory. AM1 (CCT035764, diaminodimethylacridine or acridine yellow) inhibited HDAC activity, albeit with relatively

![Figure 7](https://example.com/figure7.png)

**Figure 7.** Compounds that induce changes in histone and cellular acetylation. A, effect of 0.3 μmol/L TSA and 15 μmol/L of compounds AM1 (CCT035764), AM2 (CCT030493), and AM3 (CC000475) for 48 h in HCT116 cells on acetylated histone measured in a nonduplexed, 96-well TRF-Cellisa compared with DMSO (0.1%). Columns, mean (n = 4–7); bars, SD. *, P < 0.001; **, P < 0.004 (unpaired t test). B, Western blot showing expression of acetylated histone and acetylated α-tubulin in HCT116 cells after treatment with TSA (lane 2; 0.3 μmol/L), CCT030493 (AM2; lane 3; 15 μmol/L), and DMSO (lane 1; 0.15%) for 48 h. GAPDH was used as a loading control.
low potency. This compound has a planar structure typically found in compounds that intercalate DNA and is known to inhibit telomerase by binding to the DNA/ DNA heteroduplex formed during the enzyme catalytic cycle (67). Aminoacridines, including compound AM1, are also inhibitors of protein kinase C (68). Interestingly, histone and nonhistone protein acetylation is increased on at least some lysine residues in response to DNA damage (69–71). The 2-amino-6-methoxybenzothiazole derivative AM2 (CCT030493) showed no direct inhibition of HDAC activity. However, cells treated with CCT030493 showed increased levels of histone acetylation and, to a lesser extent, α-tubulin acetylation by immunoblotting. This compound may therefore act as a HDAC inhibitor intra-cellularly or, alternatively, may modulate other proteins, corepressors, or coactivators present in multiprotein complexes with HDACs (32–34).

AM3 (CC000475; a 1,4-cyclohexadienone derivative) decreased cellular acetylation in the screen, indicating that it was a potential HAT inhibitor. It showed no direct inhibitory activity against the representative HAT, PCAF, although it may inhibit other HATs. Compounds of this structural type are cytotoxic, with potent antitumor activity in HCT116 cells (72). They induce apoptosis by interaction with thioredoxin (73) and glutathione (74) possibly through a double Michael addition interaction with sulfur nucleophiles (75). It is interesting that a series of isothiazolones, identified from an overlapping collection of compounds in a biochemical screen for inhibitors of HAT activity, are thought to react irreversibly with thiol groups (48). Recently described inhibitors of GCN5 HAT activity are also likely to be thiol reactive (76). Further studies are required to investigate the mechanism by which both total cellular acetylation and histone H3 acetylation are reduced, including assessment of the effects on other histone marks and total histone isoform expression.

In summary, a duplexed, cell-based phenotypic screen has been used to simultaneously identify putative inhibitors of HSP90 as measured by increased expression of the co-chaperone HSP70, as well as modulators of cellular acetylation. The screen successfully identified compounds that altered expression of HSP70 and HSP90 client proteins, apparently acting either directly on HSP90 ATPase or in a non-ATPase manner, as well as modulators of histone acetylation. Our experience shows the feasibility of this approach for future drug discovery projects.

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References
Duplexed Cell-Based Screens for Modulators of HSP90 and Acetylation


A duplexed phenotypic screen for the simultaneous detection of inhibitors of the molecular chaperone heat shock protein 90 and modulators of cellular acetylation

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