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Activity-Independent Discovery of Secondary Metabolites Using Chemical Elicitation and Cheminformatic Inference

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Abstract

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Author Contributions

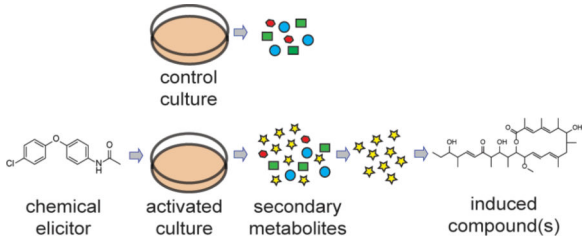
S.M.P. performed LC-MS and NMR, purification and structure elucidation of compounds, and chemical-genetic profiling in yeast. D.S. aided in NMR experiments and structure elucidation of oxohygrolidin. L.H. and M.Z. prepared crude extracts, assisted in LC-MS analyses, and tested antimicrobial activity. S.A.B. and J.L.R. performed and analyzed V-ATPase assay. S.L. and G.F. performed and analyzed the LysoTracker Red assay. J.T. and D.A. performed and analyzed the HSP90 ATPase assay. A.M. and R.L. aided in the structure elucidation of dinactin. B.S. performed the SEA target prediction. S.M.P and J.R.N. designed the project and wrote the manuscript.

The authors declare the following competing financial interest(s): B.K.S. is a founder of SeaChange Pharmaceuticals, which uses cheminformatic inference to predict targets.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00612. Supporting experimental procedures, germicidins and desferrioxamines commonly induced by Cl-ARC, dose-dependence of oxhygrolidin yields on Cl-ARC, antifungal versus antibacterial activities in crude extracts from WAC strains, SEA predictions for oxohygrolidin for the vacuolar ATPase using ChEMBL20, list of masses (base peak ions) of compounds induced by Cl-ARC, ^{13}C and ^{1}H NMR data of oxohygrolidin, 9-methylstreptimidone, and dinactin, and list of genes and their functions in *S. cerevisiae* (PDF)

Most existing antibiotics were discovered through screens of environmental microbes, particularly the streptomycetes, for the capacity to prevent the growth of pathogenic bacteria. This “activity-guided screening” method has been largely abandoned because it repeatedly rediscovers those compounds that are highly expressed during laboratory culture. Most of these metabolites have already been biochemically characterized. However, the sequencing of streptomycete genomes has revealed a large number of “cryptic” secondary metabolic genes that are either poorly expressed in the laboratory or that have biological activities that cannot be discovered through standard activity-guided screens. Methods that reveal these uncharacterized compounds, particularly methods that are not biased in favor of the highly expressed metabolites, would provide direct access to a large number of potentially useful biologically active small molecules. To address this need, we have devised a discovery method in which a chemical elicitor called Cl-ARC is used to elevate the expression of cryptic biosynthetic genes. We show that the resulting change in product yield permits the direct discovery of secondary metabolites without requiring knowledge of their biological activity. We used this approach to identify three rare secondary metabolites and find that two of them target eukaryotic cells and not bacterial cells. In parallel, we report the first paired use of cheminformatic inference and chemical genetic epistasis in yeast to identify the target. In this way, we demonstrate that oxohygrolidin, one of the eukaryote-active compounds we identified through activity-independent screening, targets the V1 ATPase in yeast and human cells and secondarily HSP90.



Many drugs have been discovered by screening culture supernatants of environmental microbes for compounds having specific biological activities.[1] Such “activity-guided” screens were the origin of antibacterials such as penicillin, vancomycin, streptomycin, tetracycline, daptomycin, and many others; antifungals such as amphotericin B, nystatin, and others; anticancer drugs such as doxorubicin and dactinomycin; and the immune modulator rapamycin. These compounds are referred to as “secondary” or “specialized” metabolites.

The complete set of secondary metabolites in nature has been referred to as the parvome.[2] Most of the known secondary metabolites were discovered through activity-guided screens against microbes, usually bacterial pathogens. As a result of the scale and success of previous screens, a central problem with this approach is that it results in the very frequent rediscovery of known compounds. Actinomycin D, streptomycin, streptothricin, and tetracycline can be expected to constitute 1–10% of the hits in typical activity-guided antibacterial screens. Chloramphenicol, cycloheximide, vancomycin, and erythromycin are also very common.[3,4] Genome sequencing has however revealed that most actinobacteria such as the streptomycetes, encode 20–50 secondary metabolic biosynthetic gene clusters.[5] Despite advances in *in silico* analysis,[6] the chemical structures and biological activities

generated by many of these pathways are unknown. These “cryptic” secondary metabolites are believed to represent an important opportunity for drug discovery.[7–9]

It is possible that the cryptic biosynthetic genes are simply not expressed at high levels during laboratory culture. In contrast, many of the known compounds are highly expressed, perhaps as part of the developmental life cycles of these microorganisms.[10,11] Indeed, it is possible that the biological activities of the highly expressed compounds could mask the presence of low abundance compounds. For example, if an extract contained an abundant known antibiotic and a low abundance novel antibiotic, it might be difficult to recognize the presence of the new compound. Under these circumstances, the presence of a mass that matches a known molecule could easily lead an investigator to miss the novel molecule. Because of these problems, simple activity-guided screening of this kind has been largely discarded for secondary metabolite discovery. Genetic tools have been developed to activate the cryptic biosynthetic genes;[7,12,13] however, these approaches are not easily scalable and do not overcome the masking of induced bioactivities by those that are already expressed. An unbiased discovery regimen permitting the detection of low abundance secondary metabolites without requiring prior knowledge of their biological activities could lead to the discovery of many unknown compounds of potential utility.

Identifying the molecular targets of newly purified metabolites is also a significant challenge. Chemical-genetic epistasis in model organisms has been established as a method of choice for identifying target pathways in bacteria[14] and yeast[15] but does not necessarily yield direct molecular targets. New methodology for efficiently identifying the targets of these compounds would therefore be an excellent companion technology for an unbiased screening regimen.

We report new approaches to both of these challenges. First, we have used a synthetic “elicitor” of secondary metabolism called Cl-ARC, a derivative of a previously discovered molecule called ARC2,[16] to enhance the yields of low abundance secondary metabolites. We demonstrate for the first time that Cl-ARC specifically enhances yields of secondary metabolites but has relatively little effect on the rest of the metabolomic profile. The resulting increase in yield makes it possible to distinguish secondary metabolites, without knowing their biological activity, against a background of other material that is present in vast excess. Importantly, this includes secondary metabolites with related activities. To simplify target identification, we report the first combined use of chemical-genetic epistasis and cheminformatic inference. Using this ensemble of approaches, we detected a large number of Cl-ARC-induced secondary metabolites and purified and identified structures for three of them. One of these molecules was active against both bacteria and eukaryotes, while the other two were specifically active against eukaryotes. We then demonstrate that one of the eukaryote-active compounds is an inhibitor of the vacuolar-type ATPase (V-ATPase) and has a secondary activity against HSP90.

This integrated use of unbiased screening and cheminformatic target identification is a broadly applicable solution to two of the central challenges of natural product discovery.

RESULTS AND DISCUSSION

Chemical Perturbation of Metabolism

To develop chemical elicitation as an activity-independent discovery method for secondary metabolites, we examined the effect of 10 μM Cl-ARC (Figure 1A) on 50 different strains. Forty wild isolates from the Wright Actinomycete Collection (WAC)[17] were grown on five growth media (MS, MYM, R5M, R2YE, and SAM), and 10 previously sequenced strains were grown on three growth media (MS, MYM, and R5M). All cultures were carried out in the presence and absence of Cl-ARC, in duplicate. Cultures were extracted with *n*-butanol, and the resulting 920 extracts (460 control and 460 Cl-ARC treated) were subjected to comparative LC-MS analysis. All the effects were reproduced in duplicate extracts.

Examination of the masses in the paired control and Cl-ARC treated extracts revealed at least one elicitor-induced compound in the majority of the strains. We induced 216 compounds corresponding to 46 distinct masses expressed as *m*/*z* values (Table S1). Two common effects were enhanced yields of *m*/*z* 197.1241 $[M + H]^+$ and *m*/*z* 183.1086 $[M + H]^+$, consistent with the germicidin A and B/C antibiotics,[18] and of *m*/*z* 561.3737 $[M + H]^+$ and *m*/*z* 601.3704 $[M + H]^+$, consistent with the unchelated masses of the iron siderophore desferrioxamines B and E.[19] These two effects were observed in seven and 19 strains, respectively. Effects on both compound families were observed in six strains (Figure S1).

Growth medium had a complex effect on Cl-ARC action. The elicitor induced the desferrioxamines during growth on R2YE and MS but not on SAM, R5M, or MYM where production of these siderophores was not observed. This is intriguing because it suggests that the production of these siderophores is under a level of control that is distinct from iron availability. In contrast, in three strains, Cl-ARC induced germicidins A and B/C regardless of the culture medium. Cl-ARC effects on the germicidins and desferrioxamines are consistent with our previous work in *Streptomyces coelicolor* and *Streptomyces pristinaespiralis.*[16,20]

Aside from the germicidins and desferrioxamines, Cl-ARC induced 109 other compounds (Figure 2, Table S1). Again, culture medium influenced the impact of the elicitor. We observed 36 induced compounds during growth on MYM, 25 on SAM, 20 on R5M, 18 on R2YE, and 10 on MS. Three compounds of mass *m*/*z* 754.4849 $[M + NH_4]^+$, *m*/*z* 768.5042 $[M + NH_4]^+$, and *m*/*z* 782.5157 $[M + NH_4]^+$ were induced by Cl-ARC in the strains WAC0171 and WAC0256 regardless of growth medium. These masses were also induced in WAC0172 and WAC0269, though only on MYM. These masses differ by increments of 14 Da (e.g., CH_2), suggesting that they are structurally related. Other examples of compounds that were induced in more than one strain include *m*/*z* 897.4247 $[M + H]^+$ (seven strains), *m*/*z* 243.2460 $[M + H]^+$ (seven strains), *m*/*z* 427.3080 $[M + NH_4]^+$ and *m*/*z* 614.3367 $[M + NH_4]^+$ (four strains), *m*/*z* 345.3050 $[M + H]^+$ (three strains), and *m*/*z* 923.6031 $[M + HCOO]^-$ and *m*/*z* 937.6196 $[M + HCOO]^-$ (three strains) (Table S1). In total, aside from the desferrioxamine- and germicidin-like masses, 40 distinct masses were induced in 29 of the 50 strains included in this study (Figure 2). We refer to the collection of induced compounds as the Cl-ARC “elicitome”. The fact that identical masses were induced in more than one

strain suggested that, for this collection of strains at least, we may be nearing saturation of the Cl-ARC-inducible subset of the parvome.

It is noteworthy that aside from the desferrioxamines and germicidins, none of the Cl-ARC-induced peaks had masses consistent with the known highest frequency compounds.[3] Moreover, while we observed common secondary metabolites like actinomycin D and cycloheximide in control extracts (confirmed by NMR, MS, and UV data), neither was induced by Cl-ARC. This, as well as the LC-MS data that showed that most Cl-ARC-induced peaks were relatively small, demonstrated that Cl-ARC enhances yields of many low abundance compounds.

Cl-ARC Specifically Induces Secondary Metabolites

To determine whether these Cl-ARC-induced masses were secondary metabolites, we selected compounds from three strains for purification, structure elucidation, and biochemical characterization. The compound *m*/*z* 575.3736 $[M + H]^+$ from *S. ghanaensis* ATCC 14672 was identified as the 16-membered macrolide oxohygrolidin (Figure 1B, Figure 3A, Table S2). Oxohygrolidin has not been previously reported from *S. ghanaensis*.[21] Another compound, *m*/*z* 308.1850 $[M + H]^+$, was elucidated as the glutarimide polyketide 9-methylstreptimidone[22] from *Streptomyces hygroscopicus* ATCC 53653 (Figure 1C, Figure 3B, Table S3). One of the related compounds from WAC0256 (described above) was identified using the ACD Structure Elucidator program as the cyclotetralactone dinactin (Figure 1D, Table S4). High-resolution MS and MS/MS data further revealed the other related compounds as the macrotetrolide antibiotics[23] nonactin, monactin, and trinactin, consistent with the inference that they are related molecules differing only in the number of $-CH_2$ groups at specific locations (Figure 1D, Figure 3C).

We examined the effect of varying Cl-ARC concentration on oxohygrolidin yields in *S. ghanaensis* cultures (Figure S2). The elicitor enhanced yields at concentrations as low as 1–5 μM, and there was a clear dose response effect at higher concentrations. Importantly, other compounds in the control extract (DMSO) were not influenced by the presence of the elicitor at any concentration measured. This supports the idea that the effect of the elicitor is restricted to a specific set of otherwise low abundance secondary metabolites. As far as we know, Cl-ARC is the first example of a synthetic compound having this capacity. These results suggest that the use of a chemical elicitor in an unbiased screening regimen can open up poorly sampled chemical diversity to purification and molecular characterization.

Unbiased Screening Reveals Low-Abundance Compounds Missed in Activity-Guided Screens

To compare activity guided screening with the unbiased detection of Cl-ARC-induced compounds, we tested crude extracts from 200 Cl-ARC treated and control cultures for biological activity against *Bacillus subtilis*, *Escherichia coli*, and *Saccharomyces cerevisiae*. We observed cell killing of one or more of the target organisms in 131 out of 400 extracts (Figure S3). Despite the fact that we observed at least one Cl-ARC-induced compound in most strains, the bioactivity of Cl-ARC and control strains were indistinguishable. Activity-

based screening therefore did not detect any Cl-ARC-induced antibacterial or antifungal compounds.

We then tested the three compounds that we had purified solely on the grounds that they were induced by Cl-ARC and found that oxohygrolidin and 9-methylstreptimidone inhibited the growth of yeast but not the prokaryotes, while the macrotetrolides (nactins) inhibited the growth of both. The minimum inhibitory concentrations of oxohygrolidin and 9-methylstreptimidone were 64 and 32 μg/mL against *S. cerevisiae*, respectively. This demonstrates that unbiased screening for elicitor-induced secondary metabolites overcomes the masking of low abundance compounds by common, high abundance compounds. While the antimicrobial activities of the Cl-ARC treated and control extracts were the same (due to constitutively produced, high abundance compounds), simply purifying the Cl-ARC-induced compounds allowed us to detect oxohygrolidin, 9-methylstreptimidone, and the nactins. Indeed, the control extracts from *S. hygroscopicus* (Figure 3B) and WAC0256 (Figure 3C) contained high concentrations of deoxynigericin and cycloheximide, respectively, neither of which was influenced by the elicitor and which were presumably the activities we detected in screening of these extracts for bioactivity.

Target Network Inference by Chemical-Genetic Profiling in Yeast

Most known antifungals target the fungal cell wall. The molecular targets of 9-methylstreptimidone and oxohygrolidin are unknown, while the nactins have been described as ionophores.[24] To identify a target pathway for oxohygrolidin, we exploited its specific activity against *S. cerevisiae*. It has been previously demonstrated that yeast strains bearing null mutations in genes encoding dispensable elements of a pathway exhibit altered susceptibility (hypersensitivity or resistance) to chemical inhibitors of the pathway's essential components, and this chemical-genetic epistasis has been exploited to identify target pathways of experimental drugs.[25] We therefore screened a library of 4309 *S. cerevisiae* strains each bearing a null mutation in one dispensable gene and identified 13 mutants that were hypersensitive to oxohygrolidin (Figure 4A).

To explore the relatedness of the mutations in these strains, we constructed a network map of their interactions. The resulting network revealed a high density of relatedness among these 13 genes (Figure 4B, Table S5) including many genes that are clearly involved in vesicle-mediated trafficking. The gene *VPS1* encodes a dynamin-like GTPase involved in vacuolar sorting, cytoskeleton organization, endocytosis, and other vesicle functions.[26,27] *VPS9* encodes a guanine nucleotide exchange factor (GEF) involved in Golgi-endosome trafficking and sorting.[28] The target guanine nucleotide binding proteins are members of the Rab family including Vps52; mutations in the *VPS52* gene were also sensitized to oxohygrolidin.[29] *VPS53* encodes a component of the Golgi-associated retrograde protein (GARP) complex that is required for the recycling of proteins from endosomes to the late Golgi.[29] This relatedness argued that the data are biologically relevant and that oxohygrolidin specifically targets intracellular trafficking.

To identify a molecular target for oxohygrolidin, we used the similarity ensemble approach (SEA).[30] This and related approaches[31–35] infer targets based on the similarity of a bait compound to known ligands of over 2500 targets in databases such as ChEMBL[36] or

WOMBAT.[37] The method, as described previously,[30,38] compares the topology of a bait ligand (oxohygrolidin in this case) to those of the known ligands for each of over 2500 targets. Tanimoto coefficients are measured to all ligands for any given target; these values are summed and compared with what would be expected for a ligand set of the same size but drawn at random from the entire library, here, for instance, ChEMBL. From the ratio of the summed Tc values to those expected at random, and their standard deviations, we can calculate Z-scores. By co-opting the BLAST statistical machinery,[30] the method then generates expectation values. The method has been tested by prospective prediction with over 200 compounds and confirmed against multiple unrelated targets:[30,39–43] overall, roughly half of the predictions have been confirmed experimentally. This rate is far above random, though admittedly almost all of the molecules tested have been synthetic, medicinal-chemistry like molecules, not the more functionally complex natural products like oxohygrolidin. Importantly, SEA provides evidence for target relationships that is orthogonal to that of chemical-genetic epistasis, and it occurred to us that combining these two approaches would be particularly powerful.

When we undertook a SEA screen of the ChEMBL12 database, we found that oxohygrolidin closely resembled two putative targets: the 100 nM (or better) ligand set of the vacuolar ATP synthase, with an *E*-value of 10^{-45}, and the 100 nM (or better) ligand set of HSP90, with an *E*-value 10^{-40}; no other targets were predicted at any significant *E*-value (similar results were found using ChEMBL20, Figure S4). The vacuolar ATP synthase caught our attention because of its role in organelle transport, especially vesicles, a function that is consistent with the chemical genetic epistasis network. Oxohygrolidin shares structural similarity with bafilomycin, a known V-ATPase inhibitor; however, such similarities can often be misleading. We therefore tested both predictions.

Oxohygrolidin Can Target V-ATPase and HSP90

To test the *in silico* target prediction of oxohygrolidin, we carried out *in vitro* assays with purified yeast vacuolar-type ATPase (V-ATPase) and HSP90. ATPase activity was monitored with an enzyme-coupled assay in which ATP hydrolysis is coupled to oxidation of NADH via an ATP regenerating system. Similar to the chemical probes bafilomycin A1 and concanamycin A known to target this enzyme, we found that oxohygrolidin essentially eliminated enzyme activity at 1 μM and had an estimated IC_{50} value of 0.25 μM (Figure 5). Conversely, 10 μM oxohygrolidin did not inhibit the yeast mitochondrial ATP synthase (data not shown), consistent with its specific inhibition of the V-ATPase.

To determine whether oxohygrolidin was active against the V-ATPases in higher eukaryotes, we examined its effect on lysosomal acidification in human cells, one of its known *in vivo* roles. We used fluorescent LysoTracker probes, which accumulate and fluoresce inside the lysosomal lumen when it is acidified, to compare the effect on HeLa cells with that of the known V-ATPase inhibitor concanamycin (Figure 6). Both compounds prevented the accumulation of the fluorophore in the lysosome in comparison to the control cells at 500 nM and to some extent at 100 nM (Figure 6). These results demonstrate that oxohygrolidin inhibits both yeast and mammalian V-ATPases as predicted.

To test the secondary target predicted for oxohygrolidin, HSP90, we investigated its ability to inhibit human HSP90α ATPase activity in an ATP/NADH coupled assay. Oxohygrolidin inhibited HSP90 with an IC_{50} of about 50 μM. Bafilomycin A1 and concanamycin A also inhibited the enzyme at similar levels (Figure 7). In contrast, these natural products did not have a strong effect on the yeast HSC82 ATPase at 25 μM, suggesting that the inhibition of HSP90, though weak, is specific. These experimental observations are consistent with the SEA predictions of V-ATPase and HSP90 as primary and secondary targets for oxohygrolidin, respectively. Indeed, this work is also the first report of HSP90 as a secondary target of bafilomycin A1 and concanamycin A in addition to their primary target in the V-ATPase.

In sum, we have shown that chemical elicitation can be used to reveal secondary metabolites in complex mixtures of cellular material. We focused on Cl-ARC, one of at least 15 known elicitors, and showed that it preferentially elevates yields of selected low abundance secondary metabolites. Crucially, aside from the germicidins and desferrioxamines, most of the compounds in the Cl-ARC elicitome are rare or unknown metabolites that were expressed at low levels. Common, high yield antifungals such as cycloheximide and actinomycin D did not respond to the elicitor. The specific induction of otherwise poorly expressed metabolites by Cl-ARC allowed us to develop a discovery method for natural products that is unbiased by the biological activity of the products themselves. Indeed, whereas most screening of streptomycetes has been aimed at finding antibacterials, we identified two eukaryote-active compounds and one ionophore in this way. Furthermore, the elevated yields of the elicitor-induced compounds made it easier to purify sufficient quantities so that we could structurally characterize three of them. Because the molecular target of Cl-ARC (FabI) is itself very highly conserved in the Actinobacteria, we anticipate that this elicitor will have similar effects on the secondary metabolites in most bacteria. It will therefore be important to explore the effect of Cl-ARC-assisted, unbiased screens to bacterial species isolated from novel environments. For example, applying Cl-ARC to species from marine sediments such as *Salinispora*[44] and diverse, taxonomically novel endosymbionts of marine invertebrates such as sponges[45] would be of considerable interest.

The use of cheminformatic target identification in tandem with yeast chemical genetic epistasis proved to be a powerful and complementary means of rapidly characterizing the mode of action of newly purified compounds. Yeast chemical-genetic epistasis indicated a possible target pathway for oxohygrolidin, vesicle-mediated trafficking, but did not identify a specific target. The chemical structure of oxohygrolidin resembles very closely bafilomycin, a plecomacrolide known to prevent translocation of protons across membranes by inhibiting the enzyme V-ATPase. However, compounds that share structural similarities may not always exhibit the same mode of action. Conversely, the similarity ensemble approach suggested two possible direct targets, the vacuolar ATPase and HSP90, but could not distinguish which of these was the most biologically relevant. Combining these two inferences clearly favored the first of the two, and the *in vitro* and *in vivo* assays confirmed that the V-ATPase is likely to be the direct target while HSP90 is a secondary target. Indeed, perusal of the chemical genetic epistasis data suggested that the *in vitro* HSP90/oxohygrolidin interaction is not likely to be biologically relevant at least under normal

growth conditions. For example, of the strains illustrating altered sensitivity to oxohygrolidin, none had mutations in genes directly involved in heat shock and the unfolded protein response. In contrast, many of the sensitizing mutations were in genes directly involved in cell biological processes that depend on the V-ATPase.

This work represents the first exhaustive investigation of the collection of secondary metabolites induced by a chemical elicitor, validating this approach to natural product discovery. As our collection of chemical elicitors expands, it should be possible to activate the production of most or all of the secondary metabolites in any given strain, ultimately making it possible to rapidly identify many cryptic secondary metabolites in many bacterial species. Pairing this with the use of the SEA approach and chemical genetic epistasis in model systems would, in turn, connect each new natural product to a specific molecular target and biochemical pathway.

METHODS

See Supporting Information for experimental details.

Bacterial Strains, Culture Conditions, and Compounds

Fifty strains were used for screening with Cl-ARC. The strains were grown on five different solid media (R5M, MYM, MS, R2YE, or SAM) for 7 days at 30 °C. Cl-ARC (*N*-[4-(4-chlorophenoxy)-phenyl]-acetamide, $C_{14}H_{13}NO_2Cl$) was synthesized previously as described by Craney et al.[16] The antibiotics concanamycin A, bafilomycin A1, and G-418 were purchased from Santa Cruz Biotechnology.

Comparative Metabolite Profiling by LC-MS Analysis

Strains were inoculated in the presence of either DMSO or 10 μM Cl-ARC in duplicate. Crude extracts were prepared and subjected to LC-MS analysis using an Agilent 1200 series LC system coupled to a Bruker micrOTOF II with an electrospray ionization source. The metabolite profiles were compared using the MZMine 2.10 software.[46]

Isolation, Purification, and Structure Elucidation of Compounds

Crude butanol extracts were prepared from 7-day old agar cultures of *S. ghanaensis* ATCC 14672, *S. hygroscopicus* ATCC 53653, and WAC0256. The extracts were subjected to prefractionation and purification using an Alliance 2695 HPLC series (Waters). High-resolution ESI-MS was performed on Waters Micromass Quattro Ultima and Xevo G2-S Q-Tof mass spectrometers. One- and two-dimensional NMR data were acquired on a Bruker Avance III 700 MHz NMR spectrometer equipped with a 5 mm QNP cryoprobe, operating at 700.17 MHz for ^{1}H NMR and 176.08 MHz for ^{13}C NMR. The ACD Structure Elucidator software[47] was used to elucidate the structure of dinactin.

Antibacterial and Antifungal Activity Testing of Extracts and Compounds

Crude extracts and purified compounds were tested by Kirby Bauer disk diffusion assay against *Bacillus subtilis* 168, *Escherichia coli* K-12, and *Saccharomyces cerevisiae* Y7092.

Minimum inhibitory concentration for oxohygrolidin against *S. cerevisiae* was determined using the standard microtiter broth dilution assay.

Chemical-Genetic Profiling in Yeast

A library of *S. cerevisiae* haploid deletion mutants (4309 strains) from BY4741 (*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*) were a gift from Brenda Andrews (University of Toronto). The mutants were pinned on 384-agar array plates containing either DMSO or oxohygrolidin. Strains that showed hypersensitivity to oxohygrolidin were further tested by serial spot dilution assay. A yeast interaction network was constructed using the GeneMania program.[48]

Target Prediction by the Similarity Ensemble Approach

The SMILES string for oxohygrolidin was used for a SEA (http://sea.bkslab.org) screen of the over 3000 targets in the ChEMBL12 database.[36] Targets with highly significant *E*-values against ligand sets, where binding was defined as 100 nM or better, were prioritized for testing.

V-ATPase Inhibition Assay

V-ATPase was purified as described previously[49] from the yeast strain SABY31. The activity of the V-ATPase in the presence of oxohygrolidin, concanamycin A, and bafilomycin A1 was determined with an enzyme-coupled ATPase assay.[50]

LysoTracker Red Staining Assay

HeLa cells were treated with oxohygrolidin and concanamycin A and stained with LysoTracker Red DND-99. LysoTracker Red was visualized using a diode-pump solid-state laser at 561 nm (Spectral Applied Sciences). Post acquisition cells were selected using the region of interest (ROI) tool in Volocity, and the total fluorescence/cell was determined.

HSP90 ATPase Assay

The ATPase activity of human Hsp90α in the presence of oxohygrolidin, concanamycin A, and bafilomycin A1 was measured using an ATP/NADH coupled assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Bérdy J. Bioactive microbial metabolites. J. Antibiot. 2005; 58:1–26. [PubMed: 15813176]

2. Davies J. How to discover new antibiotics: harvesting the parvome. Curr. Opin. Chem. Biol. 2011; 15:5–10. [PubMed: 21111668]
3. Baltz RH. Marcel Faber Roundtable: is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration? J. Ind. Microbiol. Biotechnol. 2006; 33:507–513. [PubMed: 16418869]
4. Ito T, Masubuchi M. Dereplication of microbial extracts and related analytical technologies. J. Antibiot. 2014; 67:353–360. [PubMed: 24569671]
5. Nett M, Ikeda H, Moore BS. Genomic basis for natural product biosynthetic diversity in the actinomycetes. Nat. Prod. Rep. 2009; 26:1362–1384. [PubMed: 19844637]
6. Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E, Weber T. antiSMASH 2.0–a versatile platform for genome mining of secondary metabolite producers. Nucleic Acids Res. 2013; 41:W204–212. [PubMed: 23737449]
7. Laureti L, Song L, Huang S, Corre C, Leblond P, Challis GL, Aigle B. Identification of a bioactive 51-membered macrolide complex by activation of a silent polyketide synthase in *Streptomyces ambofaciens*. Proc. Natl. Acad. Sci. U. S. A. 2011; 108:6258–6263. [PubMed: 21444795]
8. Hosaka T, Ohnishi-Kameyama M, Muramatsu H, Murakami K, Tsurumi Y, Kodani S, Yoshida M, Fujie A, Ochi K. Antibacterial discovery in actinomycetes strains with mutations in RNA polymerase or ribosomal protein S12. Nat. Biotechnol. 2009; 27:462–464. [PubMed: 19396160]
9. Murakami T, Burian J, Yanai K, Bibb MJ, Thompson CJ. A system for the targeted amplification of bacterial gene clusters multiplies antibiotic yield in *Streptomyces coelicolor*. Proc. Natl. Acad. Sci. U. S. A. 2011; 108:16020–16025. [PubMed: 21903924]
10. Yoon V, Nodwell JR. Activating secondary metabolism with stress and chemicals. J. Ind. Microbiol. Biotechnol. 2014; 41:415–424. [PubMed: 24326978]
11. Craney A, Ahmed S, Nodwell J. Towards a new science of secondary metabolism. J. Antibiot. 2013; 66:387–400. [PubMed: 23612726]
12. McKenzie NL, Thaker M, Koteva K, Hughes DW, Wright GD, Nodwell JR. Induction of antimicrobial activities in heterologous streptomycetes using alleles of the *Streptomyces coelicolor* gene absA1. J. Antibiot. 2010; 63:177–182. [PubMed: 20224601]
13. Gomez-Escribano JP, Bibb MJ. Heterologous expression of natural product biosynthetic gene clusters in *Streptomyces coelicolor*: from genome mining to manipulation of biosynthetic pathways. J. Ind. Microbiol. Biotechnol. 2014; 41:425–431. [PubMed: 24096958]
14. Typas A, Nichols RJ, Siegele DA, Shales M, Collins SR, Lim B, Braberg H, Yamamoto N, Takeuchi R, Wanner BL, Mori H, Weissman JS, Krogan NJ, Gross CA. High-throughput, quantitave analyses of genetic interactions in *E. coli*. Nat. Methods. 2008; 5:781–787. [PubMed: 19160513]
15. Ho CH, Magtanong L, Barker SL, Gresham D, Nishimura S, Natarajan P, Koh JL, Porter J, Gray CA, Andersen RJ, Giaever G, Nislow C, Andrews B, Botstein D, Graham TR, Yoshida M, Boone C. A molecular barcoded yeast ORF library enables mode-of-action analysis of bioactive compounds. Nat. Biotechnol. 2009; 27:369–377. [PubMed: 19349972]
16. Craney A, Ozimok C, Pimentel-Elardo SM, Capretta A, Nodwell JR. Chemical perturbation of secondary metabolism demonstrates important links to primary metabolism. Chem. Biol. 2012; 19:1020–1027. [PubMed: 22921069]
17. Thaker MN, Wang W, Spanogiannopoulos P, Waglechner N, King AM, Medina R, Wright GD. Identifying producers of antibacterial compounds by screening for antibiotic resistance. Nat. Biotechnol. 2013; 31:922–927. [PubMed: 24056948]
18. Aoki Y, Matsumoto D, Kawaide H, Natsume M. Physiological role of germicidins in spore germination and hyphal elongation in *Streptomyces coelicolor* A3(2). J. Antibiot. 2011; 64:607–611. [PubMed: 21792209]
19. Barona-Gómez F, Lautru S, Francou FX, Leblond P, Pernodet JL, Challis GL. Multiple biosynthetic and uptake systems mediate siderophore-dependent iron acquisition in *Streptomyces coelicolor* A3(2) and *Streptomyces ambofaciens* ATCC 23877. Microbiology. 2006; 152:3355–3366. [PubMed: 17074905]

20. Ahmed S, Craney A, Pimentel-Elardo SM, Nodwell JR. A synthetic, species-specific activator of secondary metabolism and sporulation in *Streptomyces coelicolor*. ChemBioChem. 2013; 14:83–91. [PubMed: 23239420]
21. Kretschmer A, Dorgerloh M, Deeg M, Hagenmaier H. The structures of novel insecticidal macrolides: bafilomycins D and E, and oxohygrolidin. Agric. Biol. Chem. 1985; 49:2509–2511.
22. Saito N, Kitame F, Kikuchi M, Ishida N. Studies on a new antiviral antibiotic, 9-methylstreptimidone. I. Physicochemical and biological properties. J. Antibiot. 1974; 27:206–214. [PubMed: 4366263]
23. Jizba J, Sedmera P, Zima J, Beran M, Blumauerová M, Kandybin NV, Samoukina GV. Macrotetrolide antibiotics produced by *Streptomyces globisporus*. Folia Microbiol. (Dordrecht, Neth.). 1991; 36:437–443. [PubMed: 1821868]
24. Asher IM, Phillies GD, Kim BJ, Stanley HE. Ion complexation in nonactin, monactin, and dinactin: a Raman spectroscopic study. Biopolymers. 1977; 16:157–185. [PubMed: 576586]
25. Andrusiak K, Piotrowski JS, Boone C. Chemical-genomic profiling: systematic analysis of the cellular targets of bioactive molecules. Bioorg. Med. Chem. 2012; 20:1952–1960. [PubMed: 22261022]
26. Smaczynska-de Rooij II, Allwood EG, Aghamohammadzadeh S, Hettema EH, Goldberg MW, Ayscough KR. A role for the dynamin-like protein Vps1 during endocytosis in yeast. J. Cell Sci. 2010; 123:3496–3506. [PubMed: 20841380]
27. Vater CA, Raymond CK, Ekena K, Howald-Stevenson I, Stevens TH. The VPS1 protein, a homolog of dynamin required for vacuolar protein sorting in *Saccharomyces cerevisiae*, is a GTPase with two functionally separable domains. J. Cell Biol. 1992; 119:773–786. [PubMed: 1429836]
28. Hama H, Tall GG, Horazdovsky BF. Vps9p is a guanine nucleotide exchange factor involved in vesicle-mediated vacuolar protein transport. J. Biol. Chem. 1999; 274:15284–15291. [PubMed: 10329739]
29. Conibear E, Stevens TH. Vps52p, Vps53p, and Vps54p form a novel multisubunit complex required for protein sorting at the yeast late Golgi. Mol. Biol. Cell. 2000; 11:305–323. [PubMed: 10637310]
30. Keiser MJ, Roth BL, Armbruster BN, Ernsberger P, Irwin JJ, Shoichet BK. Relating protein pharmacology by ligand chemistry. Nat. Biotechnol. 2007; 25:197–206. [PubMed: 17287757]
31. Paolini GV, Shapland RH, van Hoorn WP, Mason JS, Hopkins AL. Global mapping of pharmacological space. Nat. Biotechnol. 2006; 24:805–815. [PubMed: 16841068]
32. Bender A, Jenkins JL, Glick M, Deng Z, Nettles JH, Davies JW. “Bayes affinity fingerprints” improve retrieval rates in virtual screening and define orthogonal bioactivity space: when are multitarget drugs a feasible concept? J. Chem. Inf. Model. 2006; 46:2445–2456. [PubMed: 17125186]
33. Reker D, Perna AM, Rodrigues T, Schneider P, Reutlinger M, Mönch B, Koeberle A, Lamers C, Gabler M, Steinmetz H, Müller R, Schubert-Zsilavecz M, Werz O, Schneider G. Revealing the macromolecular targets of complex natural products. Nat. Chem. 2014; 6:1072–1078. [PubMed: 25411885]
34. Antolín AA, Mestres J. Distant Polypharmacology among MLP Chemical Probes. ACS Chem. Biol. 2015; 10:395–400. [PubMed: 25365788]
35. Yildirim MA, Goh KI, Cusick ME, Bárabasi AL, Vidal M. Drug-target network. Nat. Biotechnol. 2007; 25:1119–1126. [PubMed: 17921997]
36. Bento AP, Gaulton A, Hersey A, Bellis LJ, Chambers J, Davies M, Krüger FA, Light Y, Mak L, McGlinchey S, Nowotka M, Papadatos G, Santos R, Overington JP. The ChEMBL bioactivity database: an update. Nucleic Acids Res. 2014; 42:D1083–1090. [PubMed: 24214965]
37. Olah, M.; Mracec, M.; Ostopovici, L.; Rad, R.; Bora, A.; Hadaruga, N.; Olah, I.; Banda, M.; Simon, Z.; Mracec, M.; Oprea, TI. Wombat: world of molecular bioactivity. In: Oprea, TI., editor. Chemo informatics in Drug Discovery. Wiley-VCH Verlag GmbH & Co.; KGaA, Weinheim, FRG: 2005.
38. Hert J, Keiser MJ, Irwin JJ, Oprea TI, Shoichet BK. Quantifying the relationships among drug classes. J. Chem. Inf. Model. 2008; 48:755–765. [PubMed: 18335977]

39. Keiser MJ, Setola V, Irwin JJ, Laggner C, Abbas AI, Hufeisen SJ, Jensen NH, Kuijer MB, Matos RC, Tran TB, Whaley R, Glennon RA, Hert J, Thomas KL, Edwards DD, Shoichet BK, Roth BL. Predicting new molecular targets for known drugs. Nature. 2009; 462:175–181. [PubMed: 19881490]
40. DeGraw AJ, Keiser MJ, Ochocki JD, Shoichet BK, Distefano MD. Prediction and evaluation of protein farnesyltransferase inhibition by commercial drugs. J. Med. Chem. 2010; 53:2464–2471. [PubMed: 20180535]
41. Laggner C, Kokel D, Setola V, Tolia A, Lin H, Irwin JJ, Keiser MJ, Cheung CY, Minor DL, Roth BL, Peterson RT, Shoichet BK. Chemical informatics and target identification in a zebrafish phenotypic screen. Nat. Chem. Biol. 2012; 8:144–146. [PubMed: 22179068]
42. Lounkine E, Keiser MJ, Whitebread S, Mikhailov D, Hamon J, Jenkins JL, Lavan P, Weber E, Doak AK, Côté S, Shoichet BK, Urban L. Large-scale prediction and testing of drug activity on side-effect targets. Nature. 2012; 486:361–367. [PubMed: 22722194]
43. Lemieux GA, Keiser MJ, Sassano MF, Laggner C, Mayer F, Bainton RJ, Werb Z, Roth BL, Shoichet BK, Ashrafi K. In silico molecular comparisons of C. elegans and mammalian pharmacology identify distinct targets that regulate feeding. PLoS Biol. 2013; 11:e1001712. [PubMed: 24260022]
44. Ziemert N, Lechner A, Wietz M, Millán-Aguiñaga N, Chavarria KL, Jensen PR. Diversity and evolution of secondary metabolism in the marine actinomycete genus Salinispora. Proc. Natl. Acad. Sci. U. S. A. 2014; 111:E1130–1139. [PubMed: 24616526]
45. Abdelmohsen UR, Bayer K, Hentschel U. Diversity, abundance and natural products of marine sponge-associated actinomycetes. Nat. Prod. Rep. 2014; 31:381–399. [PubMed: 24496105]
46. Pluskal T, Castillo S, Villar-Briones A, Oresic M. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinf. 2010; 11:395.
47. Moser A, Elyashberg ME, Williams AJ, Blinov KA, Dimartino JC. Blind trials of computer-assisted structure elucidation software. J. Cheminf. 2012; 4:5.
48. Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, Chao P, Franz M, Grouios C, Kazi F, Lopes CT, Maitland A, Mostafavi S, Montojo J, Shao Q, Wright G, Bader GD, Morris Q. The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. Nucleic Acids Res. 2010; 38:W214–220. [PubMed: 20576703]
49. Benlekbir S, Bueler SA, Rubinstein JL. Structure of the vacuolar-type ATPase from *Saccharomyces cerevisiae* at 11-Å resolution. Nat. Struct. Mol. Biol. 2012; 19:1356–1362. [PubMed: 23142977]
50. Loetscher HR, deJong C, Capaldi RA. Modification of the F0 portion of the H+-translocating adenosinetriphosphatase complex of *Escherichia coli* by the water-soluble carbodiimide 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide and effect on the proton channeling function. Biochemistry. 1984; 23:4128–4134. [PubMed: 6237682]

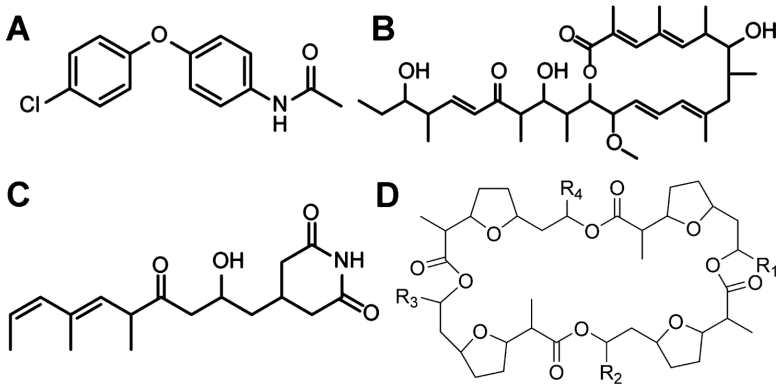

Figure 1.

Chemical structures of (A) Cl-ARC, (B) oxohygrolidin, (C) 9-methylstreptimidone, and (D) nonactin ($R_1 = R_2 = R_3 = R_4 = CH_3$), monactin ($R_1 = CH_2CH_3$, $R_2 = R_3 = R_4 = CH_3$), dinactin ($R_1 = R_3 = CH_2CH_3$, $R_2 = R_4 = CH_3$), and trinactin ($R_1 = R_2 = R_3 = CH_2CH_3$, $R_4 = CH_3$).

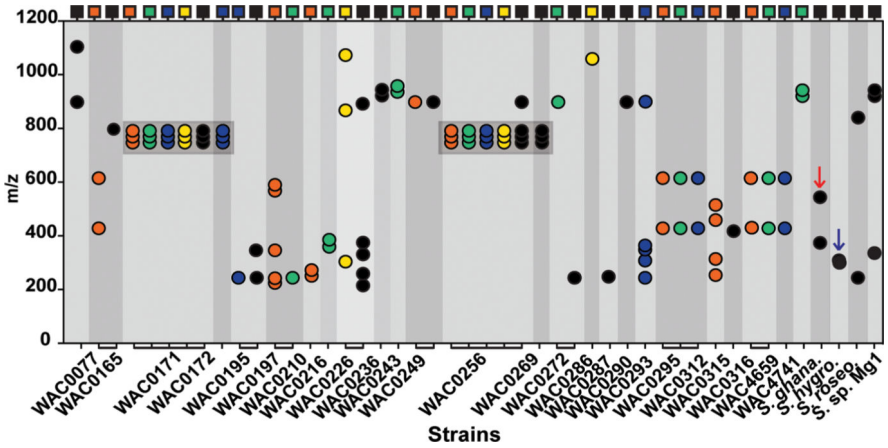

Figure 2.

Forty compounds indicated by their mass values (*m*/*z*) are induced in 29 strains in different media: MS (yellow), MYM (black), R2YE (green), R5M (blue), or SAM (red). Oxohygrolidin is indicated by a red arrow, 9-methylstreptimidone is indicated by a blue arrow, and the nactins are boxed. *S. ghana* = *S. ghanaensis* ATCC 14672; *S. hygro* = *S. hygroscopicus* ATCC 53653; *S. roseo* = *S. roseosporus* NRRL 15998.

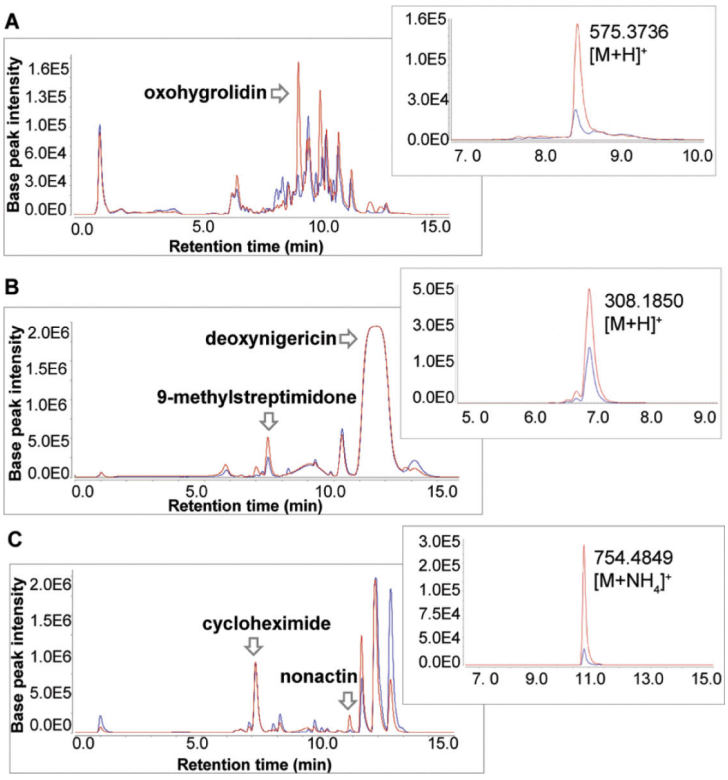

Figure 3.

Comparative metabolite profiling by LC-MS analysis. All strains were grown for 7 days in the presence of either DMSO (blue) or 10 μM Cl-ARC (red). The following induced compounds were observed: (A) oxohygrolidin from *S. ghanaensis* ATCC 14672, (B) 9-methylstreptimidone from *S. hygroscopicus* ATCC 53653, and (C) nonactin from WAC0256. Deoxynigericin and cycloheximide production were not affected by Cl-ARC.

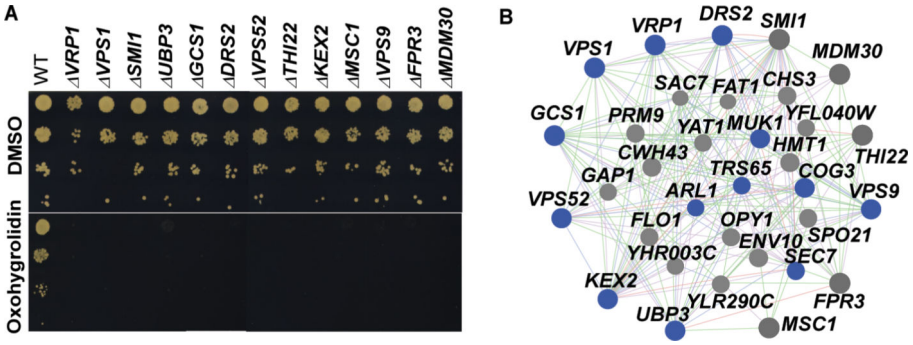

Figure 4.

Effect of oxohygrolidin on yeast deletion mutants. (A) Spot dilution plates showing hypersensitivity of mutants to oxohygrolidin (1/4 MIC) compared with DMSO. (B) Network depicting deleted genes from the hypersensitive strains (outermost circles) and their interactions. Genes that are involved in vacuolar transport are indicated in blue.

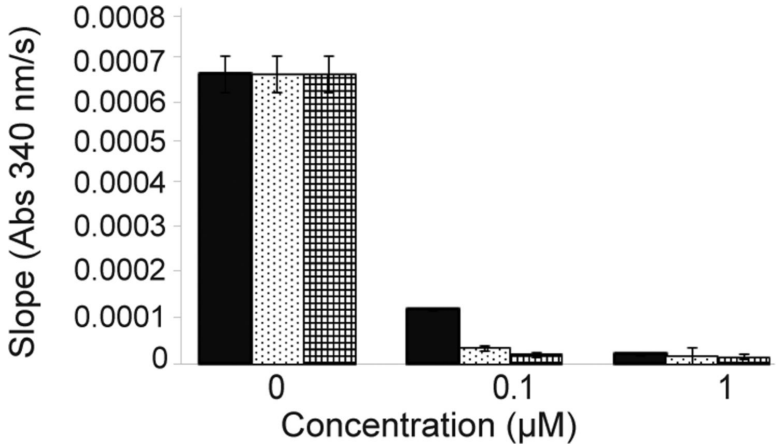

Figure 5.

Inhibition of yeast V-ATPase activity. Activity was measured by the rate of NADH loss in an enzyme-coupled assay. Oxohygrolidin (black bar) inhibited V-ATPase comparable to the plecomacrolides, bafilomycin A1(dotted bar), and concanamycin A (hatched bar).

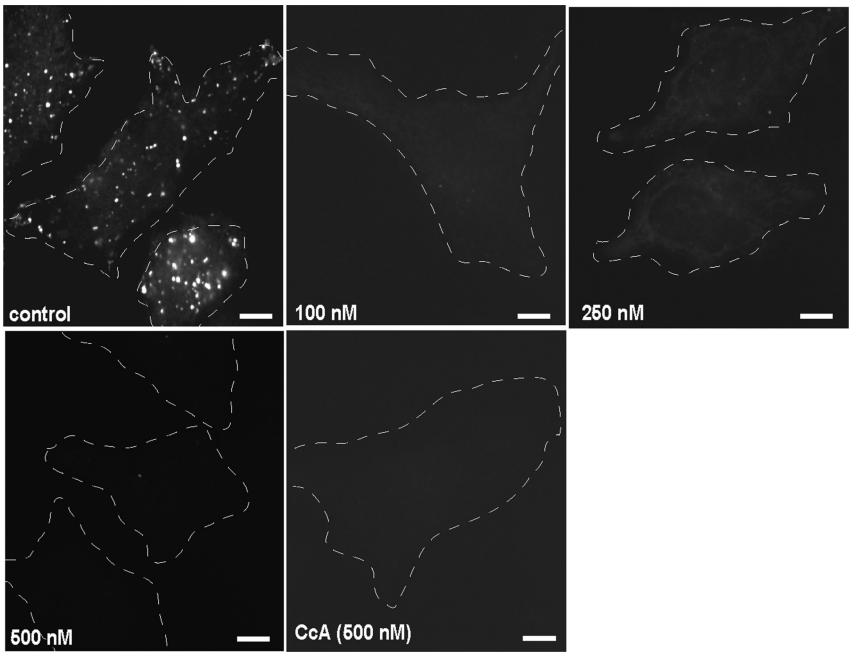

Figure 6.

Inhibition of lysosomal acidification. LysoTracker staining of HeLa cells treated with oxohygrolidin and concanamycin A (CcA) showed a decrease in lysosomal labeling compared with control (Ctrl) cells. Scale bar, 10 μm.

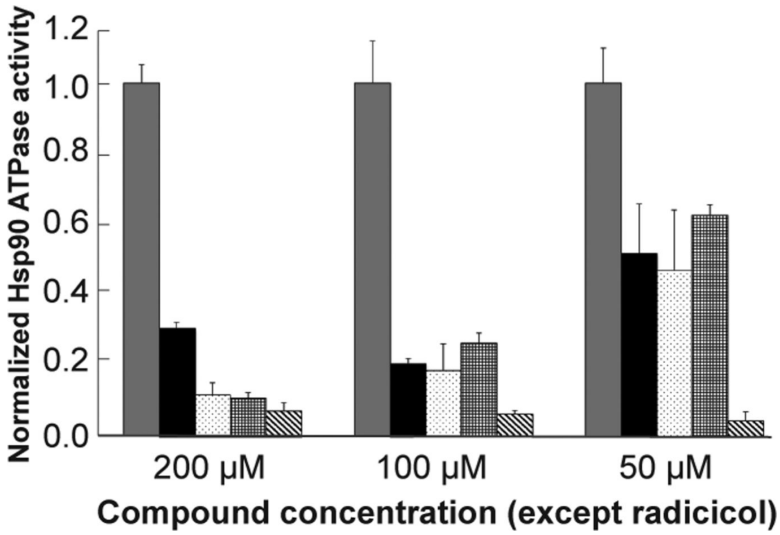

Figure 7.

Inhibition of human HSP90α ATPase activity in the presence of oxohygrolidin (black bar), bafilomycin A1 (dotted bar), and concanamycin A (hatched bar). Activity was measured using an ATP/NADH-coupled assay. DMSO (gray bar) and radicicol (20 μM, striped bar) were used as negative and positive controls, respectively.