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Antitumor/Antifungal Celecoxib Derivative AR-12 is a Non-Nucleoside Inhibitor of the ANL-Family Adenylating Enzyme Acetyl CoA Synthetase

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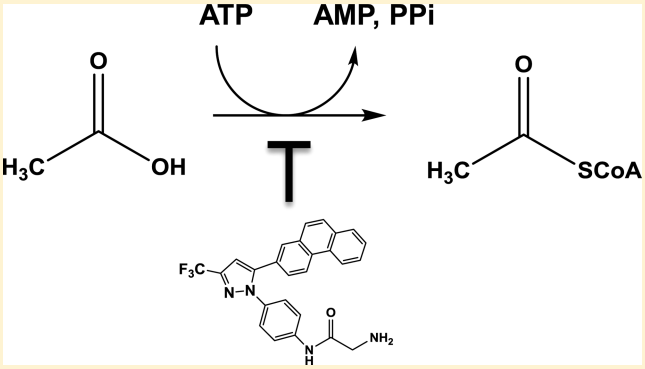
Supporting Information

ABSTRACT: AR-12/OSU-03012 is an antitumor celecoxib-derivative that has progressed to Phase I clinical trial as an anticancer agent and has activity against a number of infectious agents including fungi, bacteria and viruses. However, the mechanism of these activities has remained unclear. Based on a chemical-genetic profiling approach in yeast, we have found that AR-12 is an ATP-competitive, time-dependent inhibitor of yeast acetyl coenzyme A synthetase. AR-12-treated fungal cells show phenotypes consistent with the genetic reduction of acetyl CoA synthetase activity, including induction of autophagy, decreased histone acetylation, and loss of cellular integrity. In addition, AR-12 is a weak inhibitor of human acetyl CoA synthetase ACCS2. Acetyl CoA synthetase activity is essential in many fungi and parasites. In contrast, acetyl CoA is primarily synthesized by an alternate enzyme, ATP-citrate lyase, in mammalian cells. Taken together, our results indicate that AR-12 is a non-nucleoside acetyl CoA synthetase inhibitor and that acetyl CoA synthetase may be a feasible antifungal drug target.



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Cancer, invasive fungal infections, and parasitic disease all result from the growth of pathogenic eukaryotic cells within a eukaryotic host. In addition, viruses and intracellular bacteria such as *Salmonella* use host cell processes to replicate and/or evade the immune system. The development of drug therapies that target eukaryotic pathogens or selectively interfere with host processes required for pathogenesis, while avoiding significant host toxicity, is a significant challenge. In recent years, enormous advances have been made in the identification of new, targeted small molecules for the treatment of cancer, a development that was initiated by the discovery of the protein kinase inhibitor Gleevec.[1] Unfortunately, the same cannot be said for the development of new classes of antifungal or parasitic agents.[2,3] Indeed, the total number of antifungal drug classes in current clinical use is less than the number of new anticancer or HIV classes developed in the last 15 years. Similarly, there are currently no host-directed therapies for infectious diseases in use, although interest in this area is increasing.

Recently, the notion of repurposing drugs either approved or developed for one medical condition to the treatment of a new disease has gained traction as a drug discovery strategy.[4] The rationale for this approach is that drugs typically have more than one cellular effect or target, allowing one to exploit that activity in the context of a new disease. As a repurposing approach to antifungal drug discovery, we screened a set of anticancer protein kinase inhibitors for molecules with fungicidal activity toward *S. cerevisiae*, *C. albicans*, and *C. neoformans* (minimum inhibitory activity [MIC] 4 μg/mL or 10 μM). From this screen, we found that OSU-03012, now called AR-12 (Figure 1A), was active against both species.[5] Because phase I clinical trials of AR-12 as an anticancer agent indicate that serum levels near the fungal MIC are safely achievable in patients (S. Proniak, personal communication), AR-12 appeared to be a promising candidate for repurposing as an antifungal agent.

In addition to its antifungal activity, AR-12 has been shown to have activity against intracellular bacteria such as *Salmonella* and *Francisella*; at least a portion of this activity is due to its ability to modify host cells through the inhibition of Akt activity.[6,7] Similarly, Mohr et al. have shown that AR-12 reduces the replication of hemorrhagic fever viruses such as Lassa, Marburg, and Ebola; because AR-12 does not affect viral

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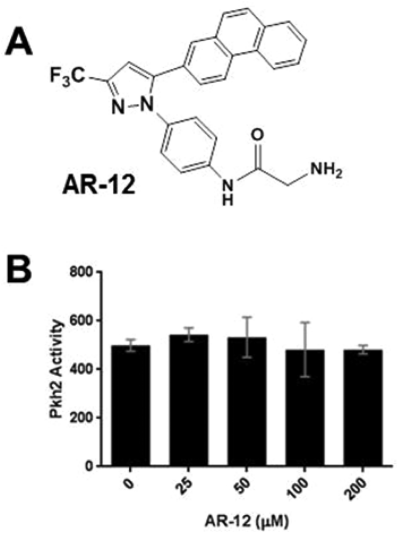

Figure 1. AR-12 does not inhibit the *C. neoformans* PDK1 ortholog in vitro. (A) Structure of AR-12. (B) Fungal PDKI *C. neoformans* Pkh2 purified from *E. coli* was treated with the indicated concentration of AR-12. Raw activity in arbitrary light units generated with the ADP Glo Assay (Promega) is depicted.

particle assembly or entry, it appears to target host cell processes required for viral replication.[8] Consistent with these observations, other groups have shown that AR-12 is active against a wide range of different viral pathogens when combined with phosphodiesterase inhibitors such as sildenafil.[9] Thus, AR-12 appears to have a wide range of biological effects in eukaryotic cells.

AR-12 is structurally derived from clinically used cyclooxygenase 2 (Cox2) inhibitor celecoxib but has no Cox2 activity.[10] Initial mechanism of action studies indicated that AR-12 inhibited protein kinase PDK1, a key enzyme in the survival and proliferation of cancer cells. Subsequently, it was also proposed to inhibit p21-activated kinases.[11] Nagashima et al. reported that they had been unable to observe the inhibition of PDK1 in vitro.[12] As described below, we were also unable to observe the inhibition of human PDK1 or a fungal ortholog by AR-12. Booth et al. have reported that AR-12 reduces GRP78/BIP/HSPA5/Dna K expression as part of its mechanism of action in both eukaryotic and prokaryotic cells, although the molecular target mediating those effects has not been identified.[9] As such, the mechanistic basis for the biological activity of AR-12 has remained unclear. Here, we describe a yeast-based chemical genetic approach that led to the identification acetyl CoA synthetase (Acs) as a target of AR-12. Furthermore, we show that AR-12 is a time-dependent inhibitor of Acs. As such, it represents a novel non-nucleoside inhibitor of this member of the acyl-CoA synthetase/nonribosomal peptide synthetase/luciferase (ANL) family of acyl-AMP-forming adenylation enzymes.[13]

RESULTS

Homozygous Deletion Profile of AR-12 with a Library of *C. albicans* Transcription Factor Mutants Indicating That the Molecule Affects Carbon Metabolism. To determine the mechanism of the antifungal activity of AR-12 (Figure 1A), we expressed and purified the kinase domain of *C. neoformans* Phk2-02, a previously characterized PDK1 ortholog.[14,15] As shown in Figure 1B, kinase phosphorylated the PDK1-tide substrate, but its activity was not significantly affected by AR-12 at very high concentrations of AR-12. Similar results were obtained with commercially available human PDK1 (data not shown). We therefore undertook a chemical-genetic approach to characterizing the mechanism of AR-12's antifungal activity.

First, we screened the publically available collection of 165 *C. albicans* homozygous transcription factor mutants (Materials and Methods). Chemical-genetic data from collections of homozygous deletion mutant libraries (also known as homozygous profiling, or HOP)[16] provides information related to the general pathways that are either targeted by the molecule or are required for the cell to buffer the effects of the molecule. To identify mutants with altered susceptibility to AR-12, we used competitive growth assays to compare each deletion mutant to a wild-type reference strain in the presence and absence of AR-12. (Figure S1 depicts a schematic representation of the assay.) A fitness score (FS) was developed (Materials and Methods) to normalize the effect of the mutation on the growth rate in the absence of AR-12 and thereby evaluate the comparative fitness of the mutant with respect to the reference strain in the presence of AR-12. The assay and fitness score were validated using well-characterized fluconazole-hypersensitive mutant *upc2*Δ/Δ,[17] which, as expected, showed a strong fitness defect with fluconazole in the competitive growth assay (FS = 2.1).

A relatively low stringency cutoff for mutants with altered susceptibility to AR-12 was used given the small size of the library and the ease of retesting: FS > 0.6 (hypersusceptible) or <−0.6 (resistant). A total of 35 mutants were identified in the initial screen; a scatter plot of the primary screen is shown in Figure 2A, along with the positions of representative mutants that were subsequently confirmed to have altered AR-12 susceptibility. We independently retested the 35 mutants in duplicate, and 12 mutants (5 hypersusceptible/7 resistant) showed reproducibly altered susceptibility to AR-12 (Table 1) using the competitive growth assay. The mean fitness defects are indicated in Table 1; each mutant shown in Table 1 had a fitness defect that met the criteria for a hit on each replicate. The relatively high false positive rate was expected due to the low stringency cutoff, and not all strains shown to be outside the cutoff in Figure 2A were confirmed.

Gene ontology (GO) analysis of the set of mutants revealed that all were involved in the regulation of primary and cellular metabolic processes ($P = 6 \times 10^{-11}$), macromolecule metabolic processes ($P = 3 \times 10^{-11}$), and a variety of other metabolic functions; a full list of the GO terms is provided in Table S2. Of the 12 mutants shown in Table, 9 have been characterized experimentally while the remaining are annotated on the basis of the function of orthologs in the model yeast *S. cerevisiae*. Five mutants (*nrg1*Δ/Δ, *mnl1*Δ/Δ, *mig1*Δ/Δ, *bcr1*Δ/Δ, and *upc2*Δ/Δ) involve transcription factors[18,19] with experimentally characterized roles in the regulation of genes related to carbon metabolism (*nrg1*Δ/Δ, *mnl1*Δ/Δ, *mig1*Δ/Δ, and *bcr1*Δ/Δ) or mitochondrial function (*upc2*Δ/Δ). As discussed above, the results of a HOP screen provide information regarding pathways and processes that are affected by a small molecule but generally do not provide a specific target. Nearly half of the mutants (5/12) identified in the screen are related to carbon metabolism and mitochondrial function. On the basis of this correlation, it seemed possible that AR-12 may directly or indirectly target a process related to central carbon metabolism.

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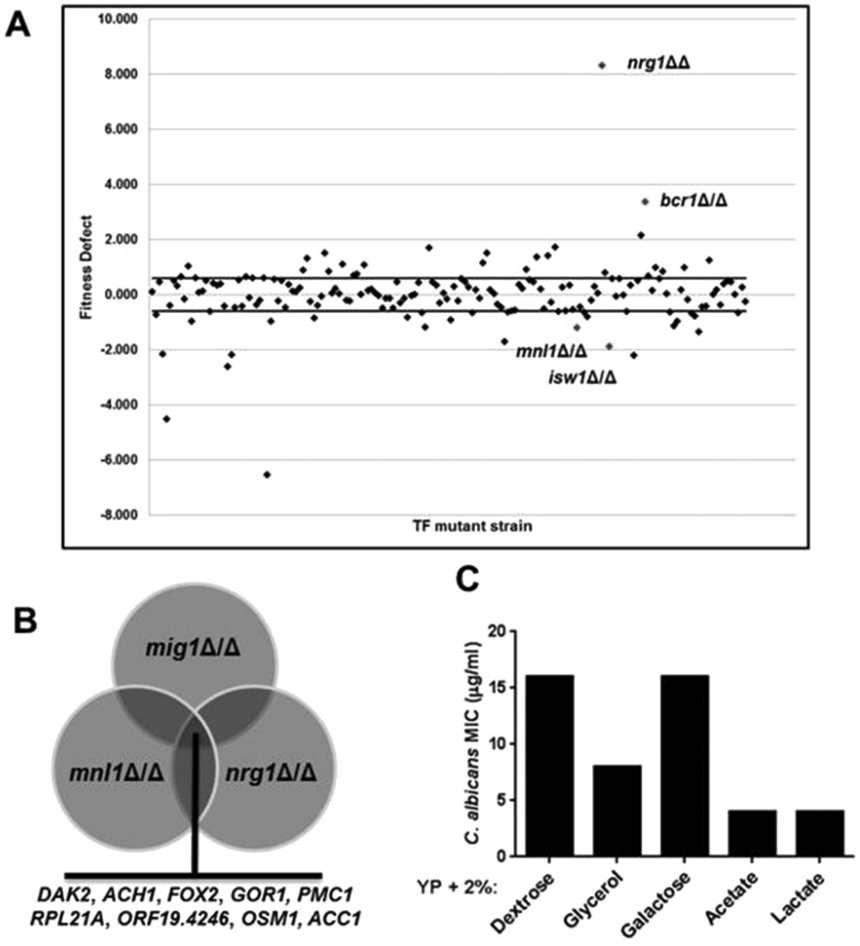

Figure 2. Screen of a homozygous transcription factor deletion library indicates that AR-12 may target carbon metabolism. (A) Scatter plot of the primary screen of the homozygous transcription factor deletion library. The dark line indicates the cutoff for resistant and hypersusceptible mutants (fitness score ±0.6; see Materials and Methods for a definition of the fitness defect). Representative hits that were confirmed are noted in red with gene names. (B) Indicated genes are regulated by *C. albicans* Mig1, MnM, and Nrg1. (C) The minimum inhibitory concentration (MIC) of SC5314 was determined in yeast peptone medium supplemented with the indicated carbon source. The data represent at least two biological replicates. The MICs were identical in all replicates.

Table 1. *C. albicans* Transcription Factor Mutants with Altered Susceptibility to AR-12

transcription factor deletion mutant	CGD[1] gene name	fitness defect score[2]	CGD description of function
orf19.391	*UPC2*	1.7 (0.5)	regulator of ergosterol biosynthetic genes and sterol uptake; binds *ERG2* promoter; induced by ergosterol depletion
orf19.4318	*MIG1*	1.3 (0.4)	repressor; regulates genes for carbon source utilization
orf19.7150	*NRG1*	25 (18)	regulates chlamydospore formation/hyphal gene induction/virulence
orf19.723	*BCR1*	3.5 (1.5)	regulates a/alpha biofilm formation, matrix, cell-surface-associated genes
orf19.3252	*DAL81*	1.2 (0.1)	ortholog of *S. cerevisiae* Dal81, regulation of nitrogen-degradation genes
orf19.3190	*HAL9*	−1.1 (0.3)	gene in zinc cluster region of Chr. 5; induced by Mnl1 in weak acid
orf19.7518	*ZCF38*	−1.7 (0.9)	putative Zn(II)2Cys6 transcription factor
orf19.2745	*UME7*	−3.0 (2.3)	similar to *S. cerevisiae* Ume6p involved in the regulation of meiotic genes
orf19.6121	*MNL1*	−1.3 (0.4)	required for adaptation to weak acid stress; activates a subset of the genes that are repressed by Nrg1
orf19.7401	*ISW2*	−2.5 (1.5)	ortholog of *S. cerevisiae* Isw2, an ATPase involved in chromatin remodeling
orf19.3736	*KAR4*	−1.1 (0.3)	ortholog of *S. cerevisiae* Kar4; role in karyogamy; opaque-specific, a-specific
orf19.4767	*ZCF28*	−1.0 (0.1)	required for yeast cell adherence to silicone substrate; spider biofilm-induced

[a]Candida Genome Database gene name and annotation. [b]Fitness defect score: >0.6 = hypersensitive, <−0.6 = resistant. Parentheses indicate the SEM of biological replicates. Each replicate assay for a listed mutant was above or below definition of hit.

One possible mechanism by which the deletion of a transcription factor mutant might modulate the activity of a small molecule is that the transcription factor may contribute to the expression of the target of the drug. To further explore this possibility, we focused on the three transcription factors in our set of mutants (Mig1, Nrg1, and Mnl1) that have been

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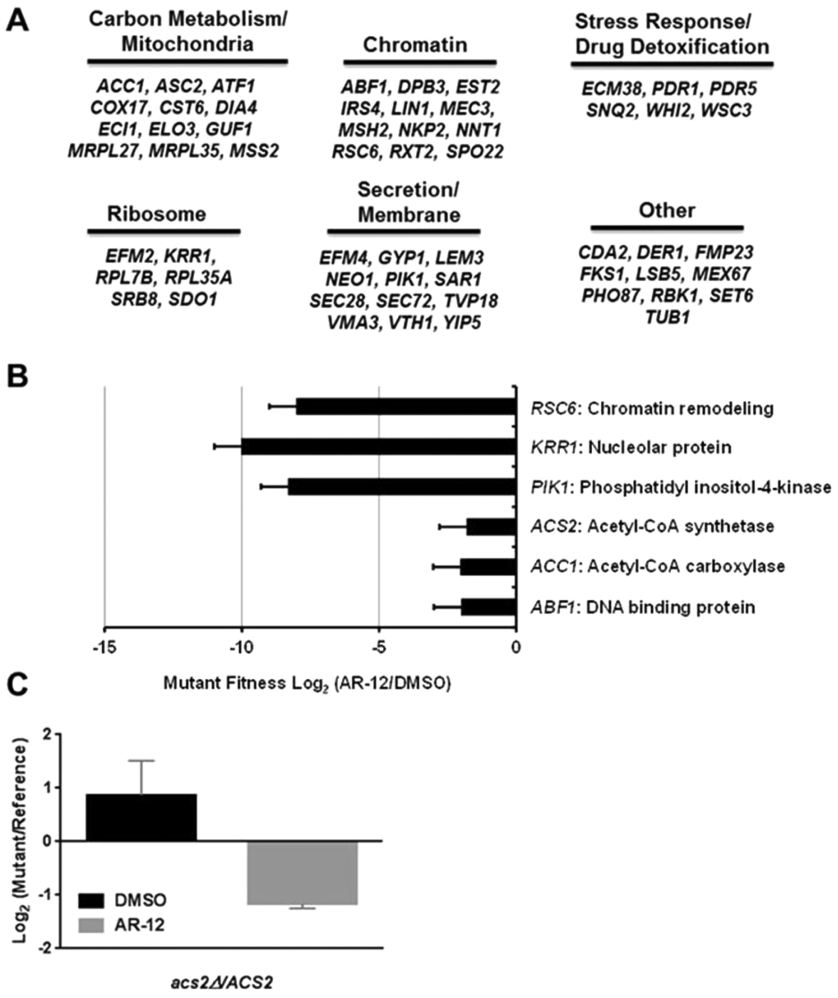

Figure 3. Chemical-induced haploinsufficiency identifies acetyl CoA synthetase as a candidate target for AR-12. (A) *S. cerevisiae* heterozygous deletion mutants of the listed genes show altered AR-12 susceptibility in a chemical-induced haploinsufficiency screen. (B) Essential genes with fitness defects in the screen. Bars indicate mean fitness defects with error bars indicating the standard deviation of four independent experiments. (C) Relative growth of *C. albicans acs2*Δ/*ACS2* normalized to reference strain in the presence and absence of AR-12. Data are from two to four replicates with a standard deviation indicated by error bars.

characterized by transcriptional profiling.[18,19] We identified genes regulated by all three transcription factors because such genes might provide more specific clues to the pathways or targets affected by AR-12. As shown in Figure 2B, nine genes are regulated by all three transcription factors and the set is enriched for genes related to monocarboxylic acid metabolism (*ACC1*, *ACH1*, *FOX2*, *GOR1*; $P = 0.00079$), glyoxylate metabolism (*FOX2*, *GOR1*: $P = 0.00068$), and fatty acid metabolic processes (*ACC1*, *ACH1*, *FOX2*: $P = 0.0028$). Three genes (*ACC1*, *ACH1*, and *FOX2*) are directly involved in acetyl coenzyme A (CoA) metabolism. These data and analyses support the hypothesis that pathways involved in carbon metabolism may modulate the ability of *C. albicans* to tolerate the effects of AR-12.

As an initial experimental test of the hypothesis that carbon metabolism may be involved in the mechanism for the antifungal activity of AR-12, we examined the effect of the culture-carbon source on the activity of AR-12 toward *C. albicans*. AR-12 was equally active against strains in the presence of glucose and galactose (Figure 2C). In the presence of glycerol, the activity of AR-12 was increased modestly (2-fold) while it was 4-fold more active against *C. albicans* cultivated in the presence of either acetate or lactate. Interestingly, one of the transcription factors identified in the screen, Mnl1, plays a role in the weak acid response, and another, Hal9, is directly regulated by Mnl1.[18,19] Finally, we tested the activity of AR-12 under anaerobic conditions using *C. albicans*. The MIC of AR-12 (4 μg/mL, ~10 μM) was identical in the presence and absence of oxygen. Molecules that target the mitochondria directly typically have reduced activity against fungi cultivated anaerobically because oxidative metabolism is not required.[20] Thus, the fact that AR-12 activity is unaffected by oxygen tension suggests that it is unlikely that mitochondrial processes are its primary target. Although these results are by no means definitive, they are nonetheless consistent with the hypothesis that AR-12 interferes with processes related to carbon metabolism and, possibly, short-chain carboxylic acid homeostasis.

Chemical-Induced Haploinsufficiency Profiling Identifies Acetyl CoA Synthetase as a Potential Target of AR-12. Chemically induced haploinsufficiency-based screening (also known as haploinsufficiency profiling or HIP) provides the opportunity to identify direct molecular targets.[16] This is because heterozygous mutants are screened; therefore, the set contains essential genes. For many small molecules, the reduction of the gene dosage of proteins representing targets

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or key components of pathways affected by the molecule leads to increased susceptibility to that molecule. We therefore screened a commercially available genome-wide collection of *S. cerevisiae* strains with bar-coded heterozygous deletion mutations using methods developed by the Nislow and Gaiver laboratories.[16,21] Briefly, the pooled strains were grown for ∼20 generations in the presence or absence of AR-12 at a concentration that reduced growth by ∼20%; four independent pairs of treated/control cultures were processed and analyzed. The distribution of heterozygote mutants in the untreated and treated pools was determined by next-generation bar code sequencing. Mutants enriched or depleted in the AR-12-treated sample by 2-fold (1 $\log_2$; $P < 0.001$, t test) relative to an untreated control were identified. A total of 70 mutants met these criteria (Table S3). This type of chemical-genetic profiling identifies genes that are both directly and indirectly affected by the reduced function of the protein targeted by the small molecule.[20] Therefore, molecules that affect fundamental processes can have a large number of hits in this type of experiment due to an extensive network of indirect interactions between the target and other genes with related function. To identify candidate targets and affected pathways, we undertook a systematic analysis of the functions and processes represented by the data set of strains with altered susceptibility to AR-12.

Twelve genes in the set were dubious ORFs or were uncharacterized and were not included in subsequent analyses. GO term analysis of the remaining set of 58 genes indicated enrichment for vesicle transport (13/58, P 0.002, false discovery rate 0%) and peptidyl lysine methylation (3/58, P 0.002, false discovery rate 0%). Further analysis using both manual literature searches and the GO Slim Mapper function available on the Saccharomyces Genome Database enabled us to categorize the hits into five major groups (Figure 3A) encompassing 48 of the 58 characterized genes. These groups are carbon metabolism/mitochondrial function,[12] ER/vesicle/membrane,[12] chromatin-related process,[12] ribosomal components/assembly,[6] and stress response/drug metabolism.[6] The presence of carbon metabolism as one of the groups further supported the findings of the HOP experiments and provided additional support for the hypothesis that carbon metabolism may be related to the target of AR-12. However, it is apparent that additional genes and pathways are clearly affected by the action of AR-12.

Of the genes identified in this set, those most likely to represent a specific molecular target of AR-12 are essential genes. The abundance of deletion mutants for six essential genes (*PIK1*, *KRR1*, *ABF1*, *RSC6*, *ACS2*, and *ACC1*) was significantly reduced in AR-12-treated samples relative to that in wild type (Figure 3B). Although genes with large fitness defects frequently represent good target candidates, the deletion mutant of a target is not necessarily that with the largest fitness defect. For example, Nislow and co-workers performed HIP analysis with fluconazole under three different conditions, and its well-characterized target Erg11 was the least-fit mutant in only one of those experiments.[21] Therefore, our analysis was influenced not only by the magnitude of the fitness defect in the essential mutants but also by the results of the *C. albicans* HOP experiment. On the basis of these considerations, acetyl-CoA synthetase 2 (*ACS2*) and acetyl-CoA carboxylase 1 (*ACC1*) appeared to be of interest because they regulate acetyl-CoA homeostasis[22] and are key regulators of carboxylic acid metabolism in cells. Acs2 and Acc1 are the first two enzymes in the synthesis of fatty acids, suggesting that cells deficient in this pathway have increased susceptibility to AR-12. Also, as noted above, *ACC1* was one of the genes regulated by all three of the well-characterized transcription factors with altered AR-12 susceptibility (Figure 2B). Taken together, these data seemed to suggest that Acs2 or Acc1 might be reasonable, but certainly not definitive, candidates for targets of AR-12.

Acetyl CoA synthetase activity is essential in *S. cerevisiae* and *C. albicans* and is mediated by two isozymes in these species.[23−25] Acs1 is induced by nonglucose carbon sources but is dispensable during growth on glucose. Acs2 is constitutively expressed and localizes to both the nucleus and the cytoplasm.[26] The cellular function of Acs2 has been extensively characterized in *S. cerevisiae*, and it plays a crucial role in histone acetylation,[26,27] ribosome assembly,[28] regulation of autophagy,[29] and carbon metabolism.[22] As an initial test of the hypothesis that Acs2 may be a target for AR-12, we confirmed the chemical-induced haploinsufficiency in a *C. albicans ACS2* heterozygous mutant.[23] Consistent with the *S. cerevisiae* pooled screen, *C. albicans acs2*Δ/*ACS2* showed increased susceptibility to AR-12 relative to the reference strain (Figure 3C). The other acetyl CoA-related mutant in our set was the essential acetyl CoA carboxylase *ACC1/FAS3*. *ACC1* converts acetyl CoA to malonyl CoA and is the first committed step in the synthesis of fatty acids and sterols.[24] A key difference between Acs2 and Acc1 is that the depletion of Acs2 leads to reduced histone acetylation[26] while strains with decreased Acc1 activity have increased histone acetylation due to the accumulation of cytosolic acetyl CoA.[30]

None of the other four essential heterozygous mutants identified in the HIP screen have direct roles in carbon metabolism. However, all four genes have links to Acs1/2. First, *RSC6* is part of the chromatin remodeling machinery and binds to acetylated histones H3 and H4;[31] Acs2 is required for the acetylation of histones. Second, *ABF1* functions with RSCs in the remodeling of chromatin and in nucleosome opening, possibly by recruiting the RSC complex to evict nucleosomes.[32] In addition, *ABF1* has been shown to directly regulate the expression of *ACS1*[33] and *ACS2*[34] in yeast and thus may be required to maintain wild-type levels of acetyl CoA synthetase activity in the cell; reduction in the gene copy number of *ABF1* could, indirectly, lead to a haploinsufficient effect with respect to the overall acetyl CoA synthetase activity. Third, *KRR1* is required for 18S ribosome assembly.[28] Acs2 is also required for small ribosome biogenesis by mediating the acetylation of cytidine 1773 in the 18S rRNA.[28] Fourth, the depletion of Acs2 leads to the induction of autophagy in *S. cerevisiae*,[29] and *PIK1* is required for the induction of autophagy,[35] suggesting that autophagy maybe a compensatory response to reduced Acs2 activity.

The functions of Acs1/2 are linked directly to three of the main categories of genes identified in the AR-12 HIP screen: carbon metabolism, chromatin, and ribosome assembly. Although Asc1/2 has not been directly linked to the other main group of genes identified in our screen (secretory pathway function), genes involved in ER/Golgi secretion were identified by Takahashi et al. in a genome-wide, genetic interaction screen with a hypomorphic allele of *ACS2*.[26] Because Acs2-derived acetyl CoA is required for lipid synthesis, it is possible that alterations in lipids induced by the drug affect membrane trafficking and organelle function. In addition, Acs2 depletion triggers autophagy, a process directly related to intracellular membrane and organelle function. Although none of these data or analyses definitively established a molecular target for AR-12,

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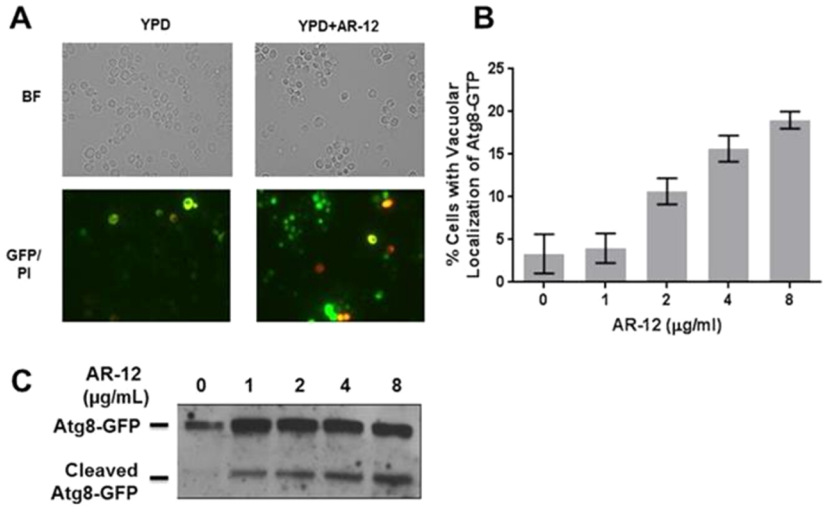

Figure 4. AR-12 induces autophagy in *S. cerevisiae.* (A) *S. cerevisiae* ATG8-GFP cells were incubated for 4 days with and without subinhibitory AR-12 (4 μg/mL) in YPD. Propidium iodide (PI) counter-staining was used to identify inviable cells. Bright field (BF) and merged red(PI)/green(GFP) channel images are shown. (B) AR-12 induces autophagy in a dose-dependent manner. Each bar is the mean from at least 2 biological replicates of at least 100 counted cells per replicate. Error bars indicate the standard deviation. MIC under these conditions is 16 μg/mL. (C) Western blot of Atg8-GFP processing from samples in (B) showing the effect of the indicated AR-12 concentration on the proteolytic cleavage of Atg8. The blot is representative of two biological replicates.

the accumulated evidence led us to test the possibility that acetyl CoA synthetase may be a potential target of AR-12 in yeast.

AR-12 Treatment Phenocopies the Depletion of Acs Activity in Yeast. To further test the hypothesis that Acs1/2 is a potential molecular target of AR-12 in yeast, we asked whether yeast exposed to AR-12 phenocopied the effects of the genetic depletion of Acs2, the essential isoform of acetyl CoA synthetase in yeast. Acs2 depletion induces autophagy in stationary-phase *S. cerevisiae* cells.[29] Atg8 is a well-established marker of autophagy and accumulates in the vacuoles of autophagocytic cells.[36] Therefore, we exposed *S. cerevisiae* cells containing Atg8p fused to GFP at its C-terminus to AR-12 and compared the amount of vacuolar Atg8-GFP to cells exposed to DMSO alone over 3 days. Consistent with literature reports,[29] DMSO-treated cultures show very few cells with vacuolar Atg8-GFP (Figure 4A). However, AR-12 exposure increased the proportion of cells with vacuolar Atg8-GFP in a dose-dependent manner (Figure 4B). Propidium iodide staining indicated that >95% of the autophagic cells were viable under these conditions. The induction of autophagy leads to proteolytic processing of Atg8-GFP, and consistent with the microscopy data, AR-12-treated cells also showed dose-dependent processing of Atg8-GFP (Figure 4C). Thus, AR-12-exposed cells phenocopy the genetic depletion of Acs2 activity under stationary-phase conditions.[29] AR-12 has also been reported to induce autophagy in human tumor and macrophage cell lines.[37]

In *S. cerevisiae*, Acs2 is localized to the nucleus and is required for histone acetylation.[26,27] In contrast, Carman et al. examined the effect of the genetic depletion of *ACS2* in *C. albicans* on histone acetylation and found none,[23] suggesting that histone acetylation may be dependent on other sources of acetyl-CoA in *C. albicans*. We therefore examined the effect of AR-12 on histone acetylation in *C. albicans*, *S. cerevisiae*, and *C. neoformans*. Cai et al. have shown that shifting stationary-phase yeast from nutrient-poor to nutrient-rich media triggers histone acetylation as the transcriptional program for cell growth is initiated.[27] Stationary-phase *S. cerevisiae*, *C. albicans*, and *C. neoformans* cells were shifted to fresh YPD in the presence or absence of subinhibitory AR-12 (growth curves were identical for treated and untreated samples). Consistent with its ability to inhibit Acs2, AR-12 reduced histone H3 acetylation in all three species. The time course for a *C. albicans* experiment is shown in Figure 5A. One hour after shifting to fresh medium, the ratio of acetylated histone H3 to total histone H3 is lower in AR-12-treated cells, and this difference persists to the 3 h time point. The quantitation for the representative experiment shown in Figure 5A is shown below the images, and that for biological replicates is shown in Figure 5B; the extent of the AR-12 effect on histone acetylation at the 1 h time point is somewhat variable, but the 2 and 3 h time points were consistent. Similar results were obtained for *S. cerevisiae* and *C. neoformans* (Figure 5C). These data are consistent with AR-12-treated cells having reduced nucleocytoplasmic acetyl-CoA synthetase activity.

AR-12 is fungicidal and leads to a loss of fungal cell integrity; this effect is demonstrated in Figure 6A, which shows that AR-12-treated *S. cerevisiae* cells stain with propidium iodide, a dye that is excluded by cells with intact cellular membranes. If Acs2 is a target of AR-12, then the depletion of Acs2 in glucose-containing media should also lead to a loss of cellular integrity and cell death. To test this, we obtained a *S. cerevisiae* strain in which the *ACS2* allele is under the control of a tetracycline-repressible promoter. The addition of doxycline (DOX) to the medium represses the expression of *ACS2*. Propidium iodide (PI) staining showed that only the DOX-treated, *tet-ACS2* cells had lost cellular integrity (Figure 6B). Specifically, 45% of the DOX-treated *tet-ACS2* cells were PI^+ (N = 119) while only 3.5% of the untreated cells were PI^+ (N = 85). We suspect that the loss of Acs2 activity leads to defects in cellular integrity because of its key role in a variety of central cellular processes.[22] These chemical genetic and chemical phenotype data are consistent with the hypothesis that AR-12 targets Acs activity in fungi.

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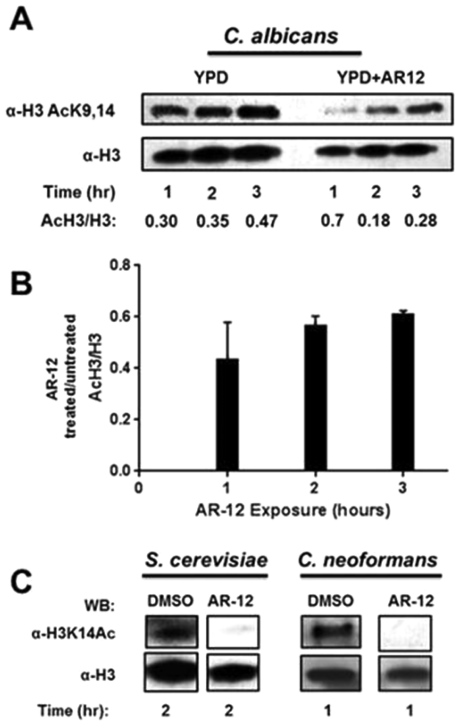

Figure 5. AR-12 inhibits histone H3 acetylation. (A) Stationary-phase *C. albicans* SC5314 was shifted to fresh YPD in the presence or absence of AR-12 (4 μg/mL) and harvested at the indicated time points. The cells were processed for Western blotting with antibodies for total histone 3 (α-H3) and histone 3 acetylated at lysines 9 and 14 (o-H3 AcK9.14). The intensity of the bands for AcH3 and total H3 were determined, and the ratio is presented as an AcH3 percentile of total H3. (B) Quantitation of replicate experiments in panel A. (C) *S. cerevisiae* and *C. neoformans* cells were treated with (AR-12:2 μg/mL for *Sc.* and 0.5 μg/mL for *Cn*), harvested at the indicated time points after the shift to fresh medium, and analyzed by Western blot with antibodies to histone H3 acetylated at lysine 14 (α-H3AcK14).

AR-12 Inhibits *S. cerevisiae* Acs1 and Human ACCS2 Acetyl CoA Synthetases in Vitro. To test the hypothesis that AR-12 inhibits acetyl CoA synthetase, we obtained purified *S. cerevisiae* Acs1 from a commercial source and examined the effect of AR-12 on the enzyme activity using a standard hydroxylamine-coupled assay.[38] We opted for this direct measure of enzyme activity as opposed to alternative coupled systems in order to avoid the possibility of AR-12 inhibiting one of the other enzymes in the system.[39,40] The assays were performed using ATP (2 mM, 2X K_m), acetate (9 mM, 10X K_m), and reduced coenzyme A (CoASH, 0.35 mM, 10X K_m) and were stopped at 30 min, a time point that was well within the linear range of the assay (Figure S2). As shown in Figure 7A, AR-12 inhibits *S. cerevisiae* Acs1 with an apparent IC_{50} of 18

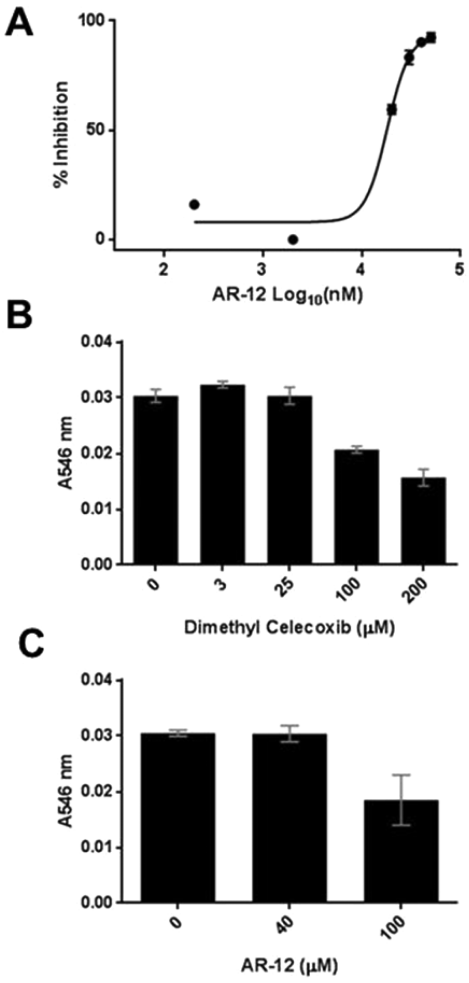

Figure 7. AR-12 inhibits acetyl-CoA synthetase in vitro. (A) AR-12 inhibits *S. cerevisiae* Acs 1 in vitro; data are expressed as % inhibition. R^2 = 0.98, and the Hill coefficient is 1.45. The curve is representative of three independent experiments. Mean IC_{50} = 18 ± 2 μM, and the maximum effect is 92 ± 3. (B) Dimethylcelecoxib inhibits Acs1 at high concentrations. (C) AR-12 is a weak inhibitor of human ACCS2. Data are means and standard deviations for two to three replicates.

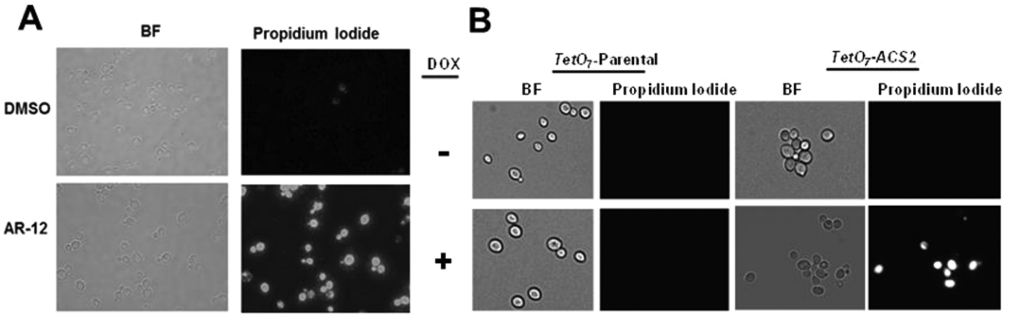

Figure 6. AR-12 and genetic depletion of Acs2 lead to a loss of cellular integrity. (A) Cells were treated with DMSO or AR-12 (8 μg/mL; MIC concentration under these conditions) for 6 h in YPD medium at 30 °C. Cells were harvested, stained with propidium iodide, and photographed under bright field and fluorescent channels. (B) Yeast strains with and without a doxycycline (*Teto*$_7$)-repressed *ACS2* allele were grown overnight in medium containing doxycycline or DMSO. The cells were processed as in panel A. See the text for quantification.

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ACS Infect. Dis. 2016, 2, 268−280

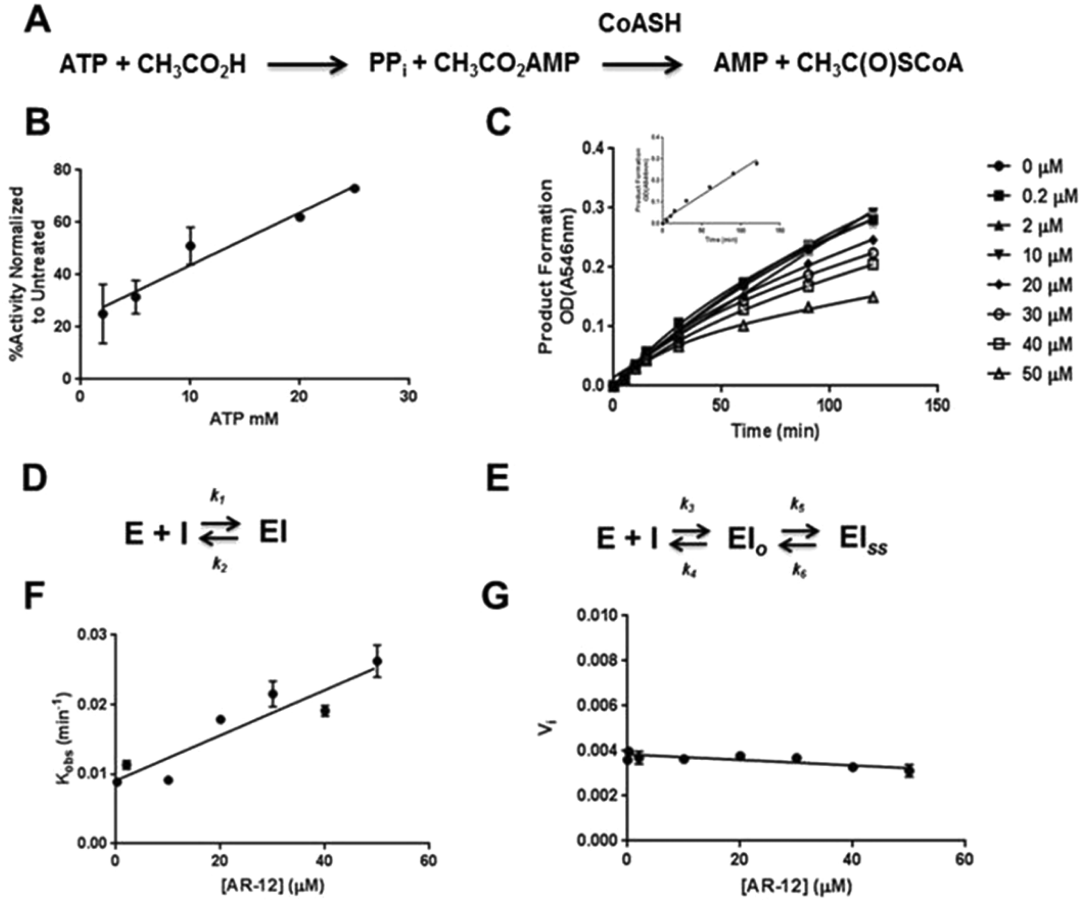

Figure 8. AR-12 is a time-dependent inhibitor of Acs1. (A) Two-step formation of acetyl CoA catalyzed by Acs1. (B) AR-12 inhibition is decreased by increasing ATP concentration. (C) Progress curves for reactions in the presence of indicated AR-12 concentration presented as raw product absorbance. This is a representative experiment with error bars (in gray) depicting the standard deviation of technical replicates. Curves are fit to the equation described in Materials and Methods. The inset shows a progress curve without AR-12 and is fit to a linear regression (R^2 = 0.90). (D) Schematic for simple, one-step, slow-binding time-dependent inhibition. (E) Schematic for two-step time-dependent inhibition. (F) Plot of k_{0Ds} values derived from the curve fit of replicate progress curves versus AR-12 concentration (R^2 = 0.83). The linear relationship is consistent with the mechanism shown in panel D. (G) Plot of initial velocity (v_i; OD A546/min) versus AR-12 concentration. The slope of zero for the plot is also consistent with the mechanism shown in panel D. Unless otherwise indicated, all data are means of replicate experiments with error bars indicating the standard error of the means.

± 3 μM (9 ± 1 μg/mL), which is similar to the MIC (10 μM) of AR-12.[5] The Hill coefficients for the fits were 1.5 ± 0.3. Dimethylcelecoxib, an analog of AR-12 and celecoxib with poor antifungal activity (MIC > 64 μg/mL), is a less potent inhibitor of Acs compared to AR-12, with a maximum inhibition of approximately 50% at its limit of solubility in the reaction mixture (Figure 7B). This correlation between Acs and antifungal activity is consistent with the hypothesis that Acs inhibition contributes to the antifungal activity of this scaffold.

AR-12 was developed as an anticancer drug,[10] but its mechanism of action for that effect also remains unclear. Recently, acetyl CoA synthetase (ACCS2) has been shown to be important for the survival of cancer cells, particularly under hypoxic conditions.[41−43] ACCS2 is expressed in a large proportion of human tumor cells and has been shown to contribute significantly to the incorporation of acetate into histones and lipids in tumor cells.[44] We therefore were interested in determining whether AR-12 inhibited ACCS2. We expressed N-terminal histidine-tagged human ACCS2 in *E. coli* and isolated it by affinity chromatography. The purified enzyme preparations were estimated to be ∼90% pure by Coomassie staining and had specific activities of 0.15−0.20 μmol/min/mg. Although AR-12 does inhibit ACCS2, it does so only at very high concentrations. Indeed, we were unable to obtain data suitable for IC_{50} determination by curve fitting due to AR-12 insolubility and poor activity. As shown in Figure 7C, 100 μM AR-12 reduces human ACCS2 activity to less than 50%. These data suggest that AR-12 is more active against yeast acetyl CoA synthetase than it is against the human ortholog.

AR-12 is a Time-Dependent, ATP-Competitive Inhibitor of Acetyl CoA Synthetase. The two-step mechanism of acetyl CoA synthetase is shown in Figure 8A.[24] In the first step, the enzyme catalyzes the formation of an acetyl-AMP intermediate and pyrophosphate from acetate and ATP. In the second reaction, CoA condenses with this intermediate to release AMP. The kinetic details of this mechanism have been extensively studied using the yeast enzyme, and it is characterized as a Bi Uni Uni Bi Ping Pong mechanism.[45] To further characterize the mechanism of Acs inhibition by AR-12, we first examined the effect of the substrate concentrations on inhibition. As shown in Figure 8B, increasing ATP concentrations led to a decrease in inhibition, consistent with AR-12 being a competitive inhibitor of ATP. These experiments were performed in the presence of saturating $MgCl_2$ (10X K_m) and

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ACS Infect. Dis. 2016, 2, 268−280

employed ATP concentrations spanning from K_m (1 mM) to 25-fold over K_m.[46] Increasing concentrations of neither CoASH nor acetate had an effect on the activity of AR-12 on Acs (data not shown). Therefore, our data are consistent with the hypothesis that AR-12 competes with ATP.

One of the characteristics of ANL enzyme inhibitors based on the AMP intermediate is time-dependent, tight-binding inhibition.[47−49] The IC_{50} values reported above were generated under conditions in which the enzyme and inhibitor were preincubated. We therefore specifically tested the effect of time on inhibition by performing time-course experiments with different concentrations of AR-12. For these experiments, AR-12 was added just before the initiation of the reaction, and all reaction components were present at 10X K_m. Under these conditions, the uninhibited reaction showed a linear progress curve over the time course of the experiment (Figure 8C, inset). The progress curves of the inhibited reactions, however, are nonlinear and show decreasing reaction velocity with increasing time as well as a steady-state rate that is significantly reduced relative to the initial rate (Figure 8C). This pattern is consistent with AR-12 showing time-dependent inhibition.[50,51] The data for Figure 8C were fitted to an equation for time-dependent inhibition (Material and Methods, $R^2 > 0.99$ for all replicates[51]). The curve fits provided k_{obs}, initial velocity (v_i), and steady-state velocity (v_{ss}) for the various inhibitor concentrations.

Two general types of time-dependent inhibition are possible:[51] a simple one-step mechanism characterized by slow, reversible binding of the inhibitor (Figure 8D) or one of several types of two-step mechanisms in which an initial EI complex converts to a second intermediate. (See Figure 8E for representative example.) To attempt to distinguish between these two general mechanisms, we analyzed the progress curve data following the general approach outlined by Copeland.[51] First, we plotted the k_{obs} values from the fitted data of two independent experiments versus inhibitor concentration, [I]. As shown in Figure 8F, this plot reveals a linear relationship between k_{obs} and [I] ($R^2 = 0.83$). This linear relationship is characteristic of a one-step slow inhibition mechanism, whereas a two-step mechanism is most commonly manifested as a hyperbolic relationship between k_{obs} and [I].[51] In some specific cases,[52] a two-step mechanism can also lead to a linear relationship between k_{obs} and [I]. However, the initial velocity (v_i) of the inhibited reactions should also be dependent on [I] if a two-step mechanism is operative.[51,52] As shown in Figure 8G, the plot of v_i versus [AR-12] yields a slope of zero, indicating that v_i is independent of [AR-12].[51] Thus, the data are most consistent with a one-step, slow-binding mechanism in which k_{obs} is dependent only on the association (k_1) and disassociation (k_2) rate constants for the formation of a single, reversible EI complex. To further confirm this mechanism, we attempted to perform rapid dilution experiments with preformed EI complexes. However, we found that the standard 100-fold rapid dilution procedure drastically reduced the activity of the enzyme. The yeast enzyme is known to be a homotrimer, and we suspect that the rapid dilution may lead to the disassociation of the enzyme.

Assuming this model of inhibition is correct, the y intercept of the plot in Figure 8F represents an estimate of k_2 or the off-rate for AR-12.[51] From this plot, K_i^{app} can also be estimated from the slope of the line. By this analysis, k_2 is 0.009 ± 0.001 min^{-1} and K_i^{app} is 30 ± 3 μM. The time course experiments were conducted at ATP concentrations 10-fold above K_m, and AR-12 appears to be competitive with ATP (Figure 8A). Therefore, we estimated K_i by application of the Cheng−Prusoff relationship to yield a K_i of ∼2.7 μM.[51] We also estimate that the upper limit for the enzyme concentration used in these experiments is approximately 50 nM based on the protein content of the commercial enzyme and its specific activity. Thus, the ratio of K_i to [E] appears to be well above that ($K_i \leq 10[E]$) generally used to define tight-binding inhibition.[51] In summary, these experiments and analyses are most consistent with AR-12 being a time-dependent inhibitor that competes with ATP via a one-step slow-binding mechanism.

DISCUSSION

Our results indicate that AR-12 inhibits acetyl CoA synthetase as part of its mechanism of antifungal activity. This conclusion is supported by four key observations: (1) *acs2*Δ/*ACS2* heterozygotes in both *S. cerevisiae* and *C. albicans* are hypersensitive to AR-12; (2) five other essential genes showing chemical-induced haploinsufficiency are also functionally connected to Acs2 or Acs1; (3) AR-12-treated yeast phenocopy cells that have decreased Acs activity or reduced acetyl CoA levels; and (4) AR-12 inhibits *S. cerevisiae* and, albeit weakly, human acetyl CoA synthetase in vitro. In mammalian systems, AR-12 has been reported to inhibit ACG kinase PDK1, a master regulator of eukaryotic cell growth.[10] Two groups have reported that they were unable to observe PDK1 inhibition with the human enzyme.[12] Consistent with these reports, we were also unable to detect a significant inhibition of human PDK1 (data not shown) or the *C. neoformans* PDK1 ortholog. Our observations indicate that Acs inhibition contributes to the antifungal activity of AR-12. Our data also suggest that AR-12 is much less active against the human enzyme in vitro. It is important to note that we have not examined the inhibition of human ACCS2 in as great a detail as the yeast enzyme. Moreover, there are other Acs isoforms in humans. Therefore, additional work will be required before definitive conclusions can be made regarding the contribution of this mechanism to the anticancer activity of AR-12.

Very few molecules have only one cellular target, and we cannot rule out the possibility that other targets contribute to the antifungal activity of AR-12. For example, Acs is part of a family of acyl-CoA ligases.[53] The fatty acyl CoA ligases are not essential in yeast, and we have not observed evidence that AR-12 inhibits their activity in whole yeast cells (data not shown), suggesting that they are unlikely to be the primary target. Other essential enzymes of the ANL adenylating family[13] are present in fungi, thus AR-12 may have activity against those as well. The chemical-genetic and chemical-phenotypic data described herein also strongly support Acs as an important target of AR-12 in yeast. Moreover, the genetic networks and phenotypes indicate that the physiology of the cell in the presence of AR-12 is consistent with a cell lacking Acs activity.

Specifically, the HOP screen identified five transcription factors that function in the regulation of carbon metabolism, a process clearly connected to Acs function. Two additional *C. albicans* transcription factors identified in the HOP screen also have connections to Acs function: *ISW2* and *UME7* (Table 1). First, *ISW2* is an ATPase involved in histone and chromatin remodeling;[54] Acs2 is required for histone acetylation and hence plays an important role in chromatin dynamics. Second, *UME7* is a *C. albicans* ortholog of *UME6* in *S. cerevisiae*. Ume6 has been shown to repress the expression of *ACS1* in *S.*

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ACS Infect. Dis. 2016, 2, 268−280

cerevisiae.[33] If *UME7* was functioning similarly in *C. albicans*, then *ACS1* expression would be expected to be increased in the homozygous *ume7*Δ/Δmutant. Generally, strains that have increased expression of the target of a small molecule are resistant to its effects; consistent with this line of reasoning, *ume7*Δ/Δ is resistant to AR-12. The network of transcription factors also shows internally consistent interactions with AR-12. Mnl1 positively regulates Hal9 and activates genes that are repressed by Nrg1.[18,19] Consistent with these relationships, the deletion of *MNL1* and *HAL9* leads to AR-12 resistance while the deletion of *NRG1* leads to a strain that is hypersensitive. On the basis of these arguments, 7 of the 12 transcription factors identified in the HOP screen have direct or indirect connections to the function of Acs in yeast.

As discussed in the Results section, the network of genes identified in the HIP screen is also consistent with Acs being an important target of AR-12 in yeast. One of the key relationships that emerged from the HIP experiments is the identification of *ACS2* and *ACC1*, two genes that code for sequential enzymes in the early stages of lipid biosynthesis. The presence of two enzymes in the same pathway in a chemical genetic screen provides good support for the notion that the particular pathway plays an important role in the activity of the drug. In principle, either *ACS2* or *ACC1* could represent a target based on the chemical genetic results. Acs2 is required for histone acetylation in yeast, and temperature-sensitive alleles of *ACS2* show decreased histone acetylation due to the depletion of nuclear acetyl CoA.[26] In contrast, analogous mutants of *ACC1* display increased levels of histone acetylation as a result of the reduced conversion of acetyl CoA to malonyl CoA and, consequently, an increase in nuclear acetyl CoA.[30] We have shown that AR-12-treated cells have reduced histone acetylation (Figure 5A), which is consistent with decreased Acs2 activity and inconsistent with decreased Acc1 activity. Taken together, our chemical genetic experiments suggested the hypothesis that Acs was targeted by AR-12. Phenotypic analysis of the effects of AR-12 on the cellular physiology and cell biology of yeast further supported that hypothesis. Subsequent biochemical assays confirmed the ability of AR-12 to inhibit yeast Acs1. This confirmation, in turn, allowed us to further rationalize both our genetic and phenotypic results. Thus, we propose that, on the basis of the accumulated data from these different approaches, AR-12 interferes with acetyl CoA synthetase activity as part of its mechanism of antifungal activity.

The mechanism of Acs inhibition by AR-12 is most consistent with a simple slow-binding, one-step, reversible process. The off-rate of AR-12 appears to be quite slow (100 min), hence AR-12 is likely to have a long residence time. As discussed by Copeland et al., a long residence time can be an advantageous property of a drug and can contribute to in vivo effectiveness.[55] Our data also indicate that AR-12 competes with ATP. AR-12 has been proposed to inhibit other ATP-dependent enzymes including PDK1 and p21-activated kinases. Indeed, molecular docking studies indicate that it may interact favorably with the ATP-binding sites of PDK1 and p21 kinases.[10,11] Therefore, it is possible that AR-12 functions as a type of ATP analog or is able to interact with ATP binding motifs, although this speculation will require additional structural studies.

Acetyl CoA synthetases are present in both eukaryotes and prokaryotes.[24] Acs plays a key role in fungal central carbon metabolism and growth through the regulation of acetyl CoA homeostasis.[22] In contrast, higher eukaryotes such as plants and mammals depend on a separate enzyme, ATP-citrate lyase, to generate the vast majority of cellular acetyl CoA.[56] ATP-citrate lyase cleaves citrate into acetyl CoA and oxaloacetate with the citrate derived from the TCA cycle. In fungi studied to date, ATP-citrate lyase is either absent (e.g., *C. albicans* and *S. cerevisiae*) or not essential (*C. neoformans*[57]), while acetyl CoA synthetase is essential. In contrast, the deletion of ATP-citrate lyase in mice is lethal,[58] while acetyl CoA synthetase knockout mice are viable.[41]

As such, acetate plays a very small role in overall cellular acetyl CoA homeostasis in humans. For example, Mashimo et al. have recently shown that acetate contributes ∼10% to the basal energy demands of normal cells, with the vast majority coming from glucose.[36] Tumor cells, on the other hand, have a dramatically increased (∼5-fold) dependence on acetate metabolism for survival. Consistent with these observations, the genetic knockdown of ACSS2 inhibited the growth of tumor cells in culture. Additionally, adult mice lacking ACSS2 exhibit a reduced tumor burden in models of hepatocellular carcinoma.[41] Intriguingly, high-ACSS2-expressing breast and brain tumors correlated with poor patient outcomes.[43] On the basis of these data and observations, a number of authors have suggested that acetyl CoA synthetase inhibitors could represent a promising approach to the development of new antitumor agents with low toxicity toward normal cells. Indeed, to our knowledge, Comerford et al. recently reported the only other non-nucleoside acetyl CoA synthetase inhibitor and showed that it inhibited Acs activity in human cell lines.[41] Our in vitro experiments indicate that AR-12 inhibits ACSS2, but this activity requires concentrations at least 5-fold higher than for EC_{50} against tumor cell lines. Thus, we cannot attribute the antitumor activity of AR-12 to Acs inhibition, although additional studies will be required to fully explore this possibility.

Nonetheless, we propose that the same considerations that support Acs as a potential antitumor target similarly apply to its role as a broad spectrum antifungal drug target with low host toxicity potential. Because Acs plays a minor role in human acetyl CoA homeostasis, acetyl CoA synthetase inhibitors with relatively low selectivity for the fungal enzyme could still be useful. AR-12 is derived from the general structure of the pyrazole class of Cox2 inhibitors[10] such as celecoxib. In addition, AR-12 has progressed as a targeted anticancer therapy and was found to be well tolerated in a phase I clinical trial. In this trial, a serum concentration of 8 μM or 3.7 μg/mL was achieved and well tolerated by subjects (S. Profoniak, Arno Pharmaceuticals, personal communication). These serum concentrations are comparable to the MIC for AR-12 against *C. albicans* and *C. neoformans*. Thus, AR-12 and related molecules have good druglike properties and toxicity profiles. The general chemical structure represented by AR-12 is a structure that is well suited for additional optimization of its antifungal properties as part of a repurposing approach, and the general scaffold may be useful for the design of other inhibitors of adenylating reactions.

MATERIALS AND METHODS

Strains and Media. The genotypes and sources for *C. albicans* and *S. cerevisiae* strains are provided in Supporting Information Table S1. The *C. albicans* transcription factor deletion mutant library was that described by Homann et al.[59] and was obtained from the Fungal Genetics Stock Center. The

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ACS Infect. Dis. 2016, 2, 268−280

S. cerevisiae tetO-ACS2 strain was obtained from ThermoScientific. The genome-wide library of *S. cerevisiae* heterozygous deletion mutants was purchased from Life Science Technologies. A yeast medium was prepared using standard recipes. All yeast incubations were done at 30 °C unless otherwise indicated.

Competitive Growth Assay with the *C. albicans* Transcription Factor Deletion Library. Reference stain SN152 (Leu^-) and each library transcription factor deletion mutant (Leu^+) were cultured overnight in YPD at 30 °C. Cells from the stationary-phase cultures were harvested and washed, and the cell density was determined by hemacytometry. A 96-well microtiter plate containing a dilution series of AR-12 (0.25−16 μg/mL) in RPMI buffered with 0.165 M MOPS (pH 7) was inoculated with 250 CFU of both SN152 and the deletion mutant. The plate was incubated at 37 °C for 24 h. The contents of the well corresponding to the lowest concentration of AR-12 that inhibited growth and the highest concentration showing growth were plated on YPD to determine the total cell density and on SDC-LEU to determine the density of the mutant strain. The fitness score (FS) was calculated as follows: $FS = [MUT_U - MUT_T/MUT_T] - [REF_U - REF_T/REF_T]$ where MUT and REF are the fractions of the mutant and reference stains in either untreated (U) or treated (T) mixed cultures. Mutants showing a FS of <−0.6 or >0.6 were defined to have significant fitness changes. This score corresponded to a 15% increase or decrease in the mutant relative to the reference strain.

Chemically Induced Haploinsufficiency Screen of the *S. cerevisiae* Heterozygous Deletion Collection. This experiment was performed following previously described protocols.[16]

Acetyl CoA Synthetase Assay. Acetyl CoA synthetase activity was measured using a $FeCl_3$/hydroxamic acid assay as described[38] with commercially available *S. cerevisiae* acetyl CoA synthetase (Sigma) and affinity purified recombinant, human ACSS2 (see below). For the IC_{50} determination, enzyme reactions contained potassium acetate (9 mM), coenzyme A (0.35 mM), magnesium chloride (4 mM), ATP (2.3 mM), DMSO (1%), or inhibitor in DMSO (1%). The buffer for the reaction was potassium phosphate (135 mM) and contained freshly prepared hydroxylamine (182 mM), reduced glutathione (9.1 mM), and potassium fluoride (45 mM). The reactions were carried out at 37 °C in water baths and quenched by the addition of an aqueous solution of $FeCl_3$ (370 mM) and trichloroaetic acid (3.3%). For each sample, a corresponding blank containing all components except enzyme was used to normalize the data. Except for the ATP concentrations, all of the components were present at 10X K_m.[33] We experimentally determined the ATP K_m of both the yeast and ACSS2 enzymes (0.9−1 mM for each). For the yeast experiments, 0.02 unit of the commercial Acs1 enzyme was used in each reaction; one unit is defined as μmoles of acetyl CoA/min at 37 °C. For reactions with ACCS2, 50 μg of an enzyme preparation with a specific activity of 0.2 U/mg was used (0.01 unit/reaction). Each data point was collected in duplicate or triplicate, and all experiments were independently performed more than three times. Progress curves were used to identify enzyme concentrations, substrate concentrations, and reaction times that were within the linear range of the reaction (progress curve in Figure S1). To determine the effect of ATP, acetate, and CoASH on inhibition, these concentrations were varied from the standard reaction. IC_{50} values were determined by curve fitting with GraphPad Prism software using the following equation: $P = P_{min} + (P_{max} - P_{min})/1 + 10^{(\log IC50 - X)\times Hill\ slope}$ where P is the product , P_{min} is the minimum product, P_{max} is the maximum product, and $X = \log_{10}[AR\text{-}12]$.

To characterize time-dependent inhibition and estimate K_i, reactions were performed as described above except that [ATP] was 10 mM (10X K_m). Progress curves were fitted to the following rate equation for time-dependent inhibition: $product = (v_s t) + (v_i - v_s/k_{obs})(1 - e^{(-k_{obs})(t)})$ where v_i is the initial reaction velocity, v_s is the steady state, t is time, and k_{obs} is the apparent rate constant.[51] The values for v_i and v_s were constrained to be positive in the curve fit. To estimate K_i^{app} and k_2, k_{obs} was plotted against [AR-12], from which the slope provides K_i^{app} and the y intercept yields k_2. To estimate K_i, the Cheng−Prusoff equation was used: $K_i = K_i^{app}(1 + [ATP]/K_m^{ATP})$.

Microscopy. Images were collected with a Nikon ES80 epifluorescence microscope equipped with a CoolSnap CCD camera using NIS-Elements software with constant exposure settings and processed equivalently in PhotoShop. ATG8-GFP localization as a reporter for autophagy was as described by Eisenberg et al.[29] The percentage of cells showing autophagy was based on counting at least 100 cells per technical replicate, and the experiment was performed on two biological replicates. Propidium iodide staining was performed as previously described.[29]

Purification of Human ACSS2. The pETb-ACSS2 construct was transformed to *E. coli* BL21(DE3)pLysS. Expression of the protein was induced with 500 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 15 h at 18 °C; expression at 30 °C leads to less active enzyme. The recombinant protein product was purified using nickel agarose affinity chromatography (Qiagen). The resulting protein solution was dialyzed overnight at 4 °C in a buffer containing 60 mM potassium phosphate (pH 7.5), 4 mM $MgCl_2$, 150 mM NaCl, and 1 mM DTT. For storage, an equal amount of 50% glycerol was added to the protein solution and stored at −20 °C. The protein was estimated to be >90% pure via SDS-PAGE analysis using Coommassie Blue visualization. The specific activity of the enzyme preparations was 0.20 ± 0.2 units/mg.

Proteolytic Processing of Atg8-GFP. The proteolytic processing of Atg8-GFP was performed as previously described.[29]

Histone Acetylation Assay. Histone acetylation was characterized using the growth conditions and protocols described previously.[26,27]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00134.

Schematic of screening assay to identify *C. albicans* transcription factor deletion mutants with altered susceptibility to AR-12; progress curve of the acetyl CoA synthetase-catalyzed reaction under conditions in which the IC_{50} value was determined; and list of strains (PDF)

List of GO terms associated with the set of *C. albicans* deletion mutants with altered AR-12 susceptibility (PDF)

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ACS Infect. Dis. 2016, 2, 268−280

List of *S. cerevisiae* heterozygous mutants with altered susceptibility to AR-12 (PDF)

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Notes

The authors declare no competing financial interest.

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