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# Mechanism-of-Action Determination of GMP Synthase Inhibitors and Target Validation in *Candida albicans* and *Aspergillus fumigatus*

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## SUMMARY

**Mechanism-of-action (MOA) studies of bioactive compounds are fundamental to drug discovery. However, in vitro studies alone may not recapitulate a compound's MOA in whole cells. Here, we apply a chemogenomics approach in *Candida albicans* to evaluate compounds affecting purine metabolism. They include the IMP dehydrogenase inhibitors mycophenolic acid and mizoribine and the previously reported GMP synthase inhibitors acivicin and 6-diazo-5-oxo-L-norleucine (DON). We report important aspects of their whole-cell activity, including their primary target, off-target activity, and drug metabolism. Further, we describe ECC1385, an inhibitor of GMP synthase, and provide biochemical and genetic evidence supporting its MOA to be distinct from acivicin or DON. Importantly, GMP synthase activity is conditionally essential in *C. albicans* and *Aspergillus fumigatus* and is required for virulence of both pathogens, thus constituting an unexpected antifungal target.**

## INTRODUCTION

Improving methods to identify bioactive small molecules and their cognate protein targets remains an important challenge. Traditionally, target-specific inhibitors have been identified by using in vitro biochemical screens and their mechanisms of action (MOAs) verified in the cellular milieu by genetic means. An expanding repertoire of new approaches to study small-molecule MOAs includes transcriptional profiling, protein-chip arrays, as well as multiple proteomics-based techniques [1–3]. More recently, small-molecule microarrays [4, 5], and a variety of phenotype-based chemical genomics strategies [6] have emerged. Typically, such approaches are developed with model organisms, thus providing generic strategies toward compound MOA determination. However, in particular instances (e.g., anti-infective drug discovery), MOA analyses could be refined by customizing such strategies so they may be performed within specific pathogens of clinical relevance.

A prominent chemical-genetics-based approach to MOA determination in *Saccharomyces cerevisiae* is termed the Fitness Test (FT) [7, 8]. This assay relies on the principle that heterozygosity of a given gene in a diploid can sensitize the resultant strain to the cognate inhibitor of the corresponding protein [9]. Assays based on this phenomenon, known as chemically induced haploinsufficiency (HI), when performed across a genome-wide collection of heterozygous deletion mutants, provide important mechanistic clues relating to the inhibitory action of small molecules [7, 8, 10]. Moreover, by introducing strain-identifying barcodes into each heterozygote mutant, the complete strain collection may be rapidly screened en masse in the presence and absence of compound, and the difference in growth of individual strains subsequently inferred by microarray analysis of all barcodes. The profiles of individual mutants that display compound-specific growth effects (i.e., altered fitness) typically correspond to the inhibitor's cellular target, metabolism, uptake, or efflux.

Recently, we have adapted the FT approach to *C. albicans* [11], the primary fungal pathogen of medical importance [12, 13]. Genes represented in the *C. albicans* Fitness Test (CaFT) include those: (1) experimentally demonstrated as essential in *S. cerevisiae* [14] and/or *C. albicans* [15], (2) broadly conserved in *A. fumigatus*, and/or (3) sharing strong homology to genes conserved in metazoans [11]. Here, we apply the CaFT assay to identify the molecular target of ECC1385, an antifungal compound, as being guanosine 5′-monophosphate synthase (GMPS, encoded by *GUA1*) and mechanistically characterize this molecule in relation to other known inhibitors affecting nucleotide metabolism. Consistent with genetic studies, in vitro biochemical data verify ECC1385 acts as a potent inhibitor of Gua1. Intriguingly, although a *gua1* loss-of-function

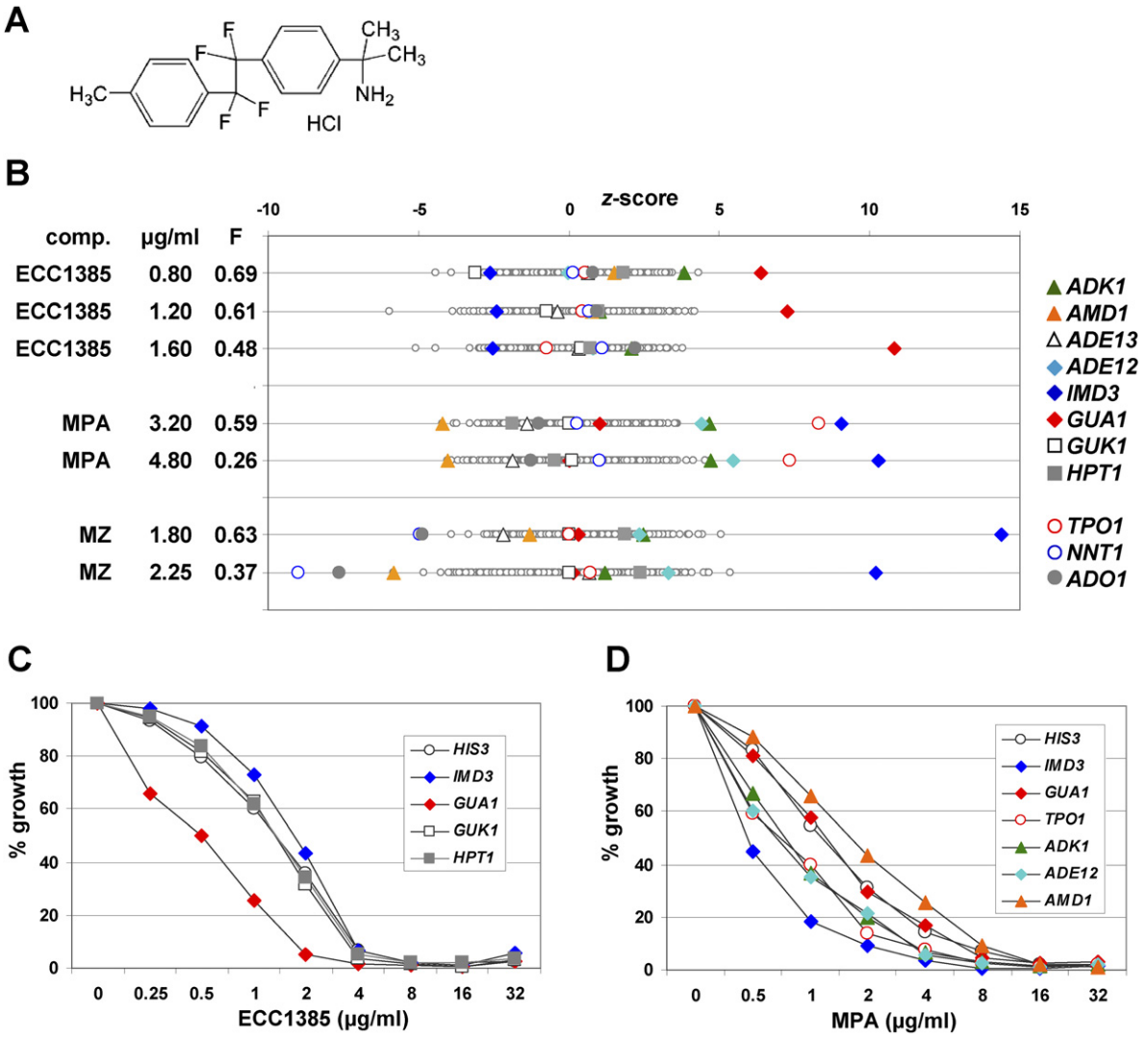


**Figure 1. CaFT Profiling of ECC1385, MPA, and MZ and Verification of Strain Sensitivities**

(A) ECC1385 structure.

(B) CaFT profiling of ECC1385, MPA, and MZ. Significant heterozygous strain depletions are noted (see text for details); small gray circles represent all other strains within the CaFT assay. Compound concentrations used and the corresponding fitness values (F) obtained are shown; the F value is the fraction of the cell growth attained in the presence of the indicated drug concentration versus untreated cells (e.g., F = 1 reflects no drug inhibitory effect on growth; F = 0.15 reflects drug treated cells reached 15% of the OD of the control culture). Positive z-score values indicate hypersensitivity to compound treatment, and negative z-score values indicate resistance. For a detailed explanation of z-score calculations, see [11].

(C) MIC determinations of ECC1385. Select heterozygote strains are indicated. The *HIS3* strain is the congenic parent of all heterozygous strains used in this study. Growth has been determined by reading culture $OD_{600}$ and comparing against the untreated culture.

(D) MIC determinations of MPA. Select heterozygote strains are indicated.

mutant is suppressed by exogenous guanine in cultures, GMPS activity is essential for the pathogenicity of *C. albicans* as well as *A. fumigatus*, the second medically significant fungal pathogen [16]. Unlike ECC1385, other structurally distinct GMPS inhibitors examined such as acivicin (ACI) and 6-diazo-5-oxo-L-norleucine (DON) do not significantly induce hypersensitivity of the *GUA1* heterozygote but likely affect alternative primary targets (Ura7 and Ade6, respectively), possessing an aminotransferase activity in common with Gua1. Collectively, these results highlight the extensive level of information achieved by monitoring chemical-induced HI across a global target set and in the context of the cellular milieu.

## RESULTS

### *C. albicans* Fitness Test MOA Analysis of ECC1385, Mycophenolic Acid, and Mizoribine

Compound ECC1385 (Figure 1A) was identified as a synthetic molecule displaying potent activity against several pathogenic fungi, including multiple *Candida spp.*, *C. neoformans*, and *A. fumigatus* (Table S1). To elucidate its

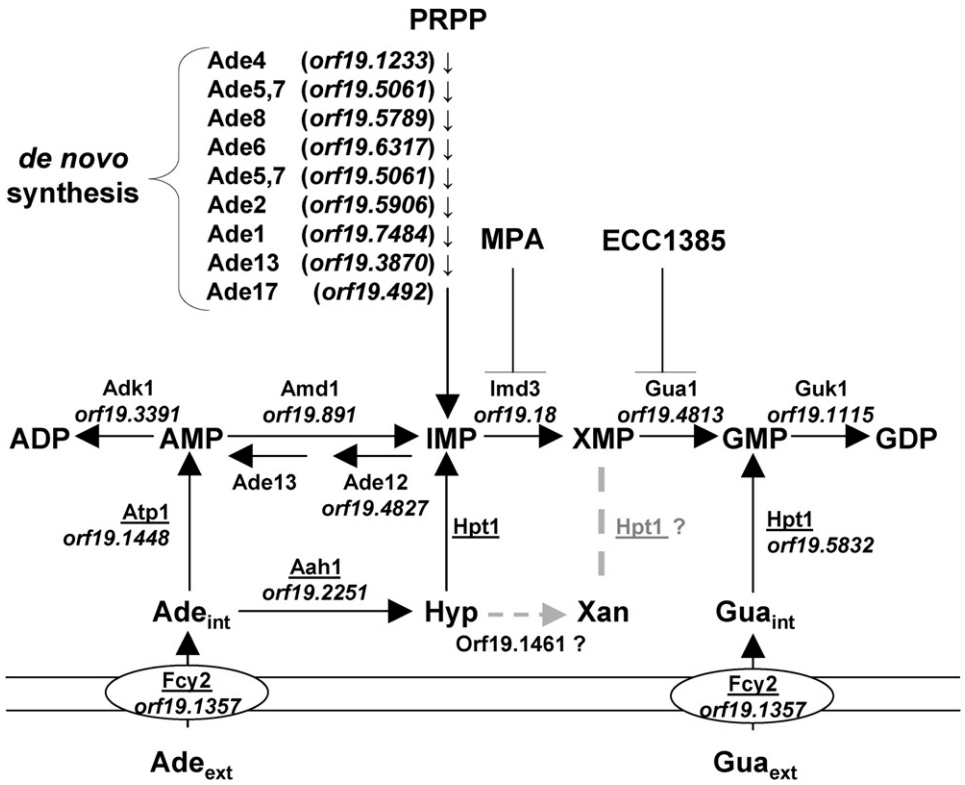


**Figure 2. Outline of the Purine Metabolism and the Salvage Pathway**

Proteins involved in the Salvage Pathway (SP) are underlined, and all *C. albicans* genes with orf19 designations are provided. MPA, mycophenolic acid; Ade, adenine; Gua, guanine; Hyp, hypoxanthine; Xan, xanthine; PRPP, 5-phosphoribosyl-1-pyrophosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; IMP, inosine 5′-monophosphate; XMP, xanthine 5′-monophosphate; GMP, guanine 5′-monophosphate; GDP, guanine 5′-diphosphate; ext., extracellular medium; int., intracellular.

MOA, compound-induced HI was surveyed in the CaFT assay, which includes 2868 heterozygous deletion strains and represents approximately 45% genome coverage [11]. CaFT analysis revealed a single heterozygote strain corresponding to *GUA1* as reproducibly hypersensitive to ECC1385 over all inhibitory concentrations tested (Figure 1B). MIC determinations of individual strains confirmed this hypersensitivity to ECC1385 (Figure 1C). *GUA1* is predicted to encode GMPS, which catalyzes the final step in the synthesis of GMP, converting XMP to GMP (Figure 2). A comparative analysis of GMPS proteins reveals that bacterial and fungal enzymes have significantly diverged from their mammalian counterparts (see Discussion and Figure S1 in the Supplemental Data available with this article online). Moreover, the ECC1385 antimicrobial potency noticeably correlates with the degree of similarity displayed by Gua1, particularly among *Candida spp.* (Table S1 and Figure S1). These observations suggest that ECC1385 targets GMPS and thus may represent a broad-spectrum antifungal compound with potential selectivity.

To test this possibility, we first examined the CaFT profiles of compounds known to affect GMP synthesis. Such studies serve to verify the robustness of the CaFT assay in determining the MOA of GMP pathway inhibitors. Mycophenolic acid (MPA) and mizoribine (MZ) are known to affect guanine nucleotide metabolism by inhibiting Imd3, an IMP dehydrogenase that catalyzes the oxidation of IMP to XMP (the substrate for Gua1) [17–19]. Indeed, the *IMD3* heterozygote was identified by CaFT analysis as the most hypersensitive strain to MPA treatment (Figure 1B). Additional MPA hypersensitive heterozygous strains identified include *TPO1*, which corresponds to an efflux pump previously reported to function in MPA detoxification [20], as well as *ADK1* and *ADE12*, while *AMD1* displayed resistance. Significantly, these genes all participate in the conversion of IMP to ADP (Figure 2); *ADK1* and *ADE12* encode adenylate kinase and adenylosuccinate synthetase, respectively, while *AMD1* encodes AMP deaminase. MIC determinations with these heterozygotes in isolation confirmed their altered sensitivity to MPA (Figure 1D). CaFT analysis also confirmed the *IMD3* heterozygote strain to be markedly hypersensitive to MZ (Figure 1B). However, two MZ resistant heterozygotes also provided insight into this compound's metabolism and import. *ADO1* encodes an adenosine kinase required to phosphorylate MZ, thereby converting the prodrug to its active form [21], while *NNT1* encodes a nucleoside transporter, which is likely responsible for the uptake of MZ (see Discussion). Therefore, CaFT profiles for known inhibitors of this

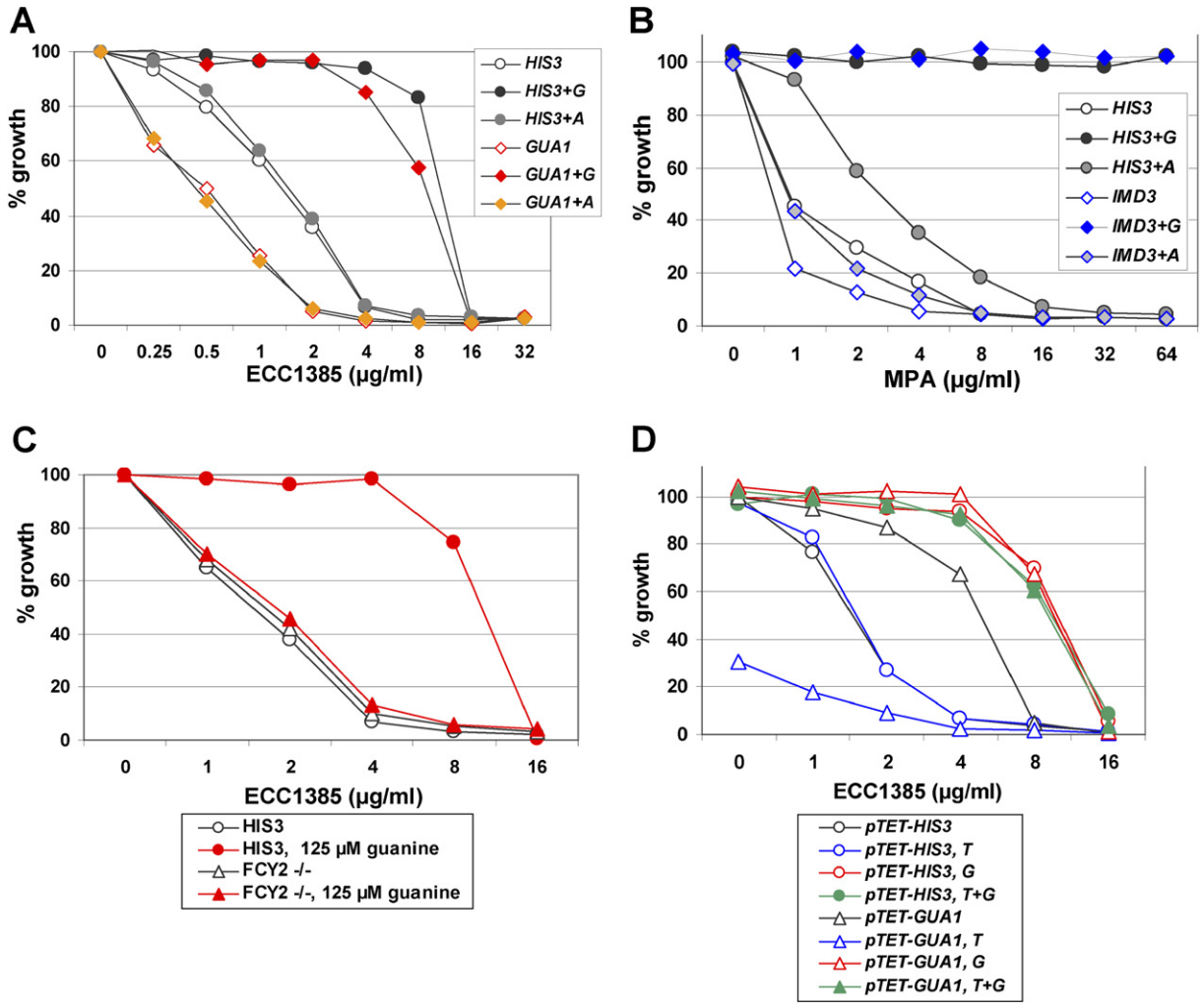


**Figure 3. Guanine Suppression of the Growth Inhibitory Effects of ECC1385 and MPA**

(A) Dose response of *GUA1* and *HIS3* heterozygous strains to ECC1385 and suppression by guanine (G, 125 μM) versus adenine (A, 1.25 mM).

(B) Dose response of *IMD3* and *HIS3* heterozygote strains to MPA and suppression by guanine (125 μM) versus adenine (1.25 mM).

(C) Dose response of the *HIS3* heterozygote control and a homozygous deletion *fcy2*$^{-/-}$ strain to ECC1385 and effect of guanine (125 μM).

(D) Dose response of pTet-*GUA1* and pTet-*HIS3* conditional mutants to ECC1385. Strains were examined under inducing (no tetracycline) or repressing conditions (50 μg/ml tetracycline) either in the presence or absence of guanine (125 μM).

pathway faithfully reflect their known MOA and support the utility of using this approach to suggest a MOA for ECC1385.

## Exogenous Guanine Suppresses ECC1385 and MPA

CaFT profiles suggest MPA and MZ are mechanistically distinct from ECC1385, inhibiting sequential enzymes in the conversion of IMP to GMP, yet all three compounds inhibit a common pathway. We predicted that the growth inhibitory effects of these compounds should be significantly reversed provided: (1) cells are supplemented an exogenous source of guanine, and (2) a functional Salvage Pathway (SP) is available to uptake guanine. Exogenous guanine significantly suppressed the inhibitory activity of ECC1385 at ≤ MIC levels (<4 μg/ml) for *C. albicans* (Figure 3A), and MPA was fully suppressed up to the highest soluble concentration (≤64 μg/ml) (Figure 3B). In contrast, adenine failed to suppress the inhibitory activity of either ECC1385 or MPA (Figures 3A and 3B).

We next examined whether the guanine-specific suppression of ECC1385 requires a functional SP. The first step of this pathway involves the uptake of guanine or adenine and is mediated by the permease Fcy2 [22]. Unlike wild-type cells, an *fcy2* homozygote deletion mutant displayed no altered sensitivity to ECC1385 when provided exogenous guanine (Figure 3C), demonstrating its absolute requirement in the guanine-dependent suppression of ECC1385. Similarly, a tetracycline regulatable mutant of *HPT1* [15] that is unable to convert imported guanine to GMP was unaffected in its sensitivity to ECC1385 (or MPA), despite the presence of guanine in the medium

(data not shown). Therefore, guanine uptake and its conversion to GMP are both required to suppress the growth inhibitory activity of ECC1385.

To further examine the susceptibility of *GUA1* mutant strains to ECC1385, a *C. albicans* pTet-*GUA1* conditional expression mutant was constructed by deleting one allele and placing the second one under the control of the tetracycline regulatable promoter [15]. As expected, the addition of a suppressing concentration of tetracycline to the pTet-*GUA1* strain markedly impaired growth (Figure 3D, closed triangles), while the simultaneous supplementation with guanine fully reversed this phenotype and paralleled wild-type susceptibility to ECC1385 (Figure 3D, closed diamonds). Intriguingly, the pTet-*GUA1* strain also displayed partial resistance to ECC1385 under nonrepressing conditions (Figure 3D, open triangles), presumably due to intrinsic *GUA1* overexpression caused by the tetracycline promoter. This reduced susceptibility was enhanced by the addition of guanine (Figure 3D, open diamonds). Collectively, these results provide independent evidence that ECC1385 inhibits the Gua1-mediated step in the GMP synthesis pathway to elicit its growth inhibitory effect.

## ECC1385 Inhibits GMP Synthase Activity In Vitro

GMPS contains two functional domains, an amidotransferase domain that removes an amino group from glutamine and a synthase domain responsible for the amination of XMP to yield GMP. Alternatively, GMPS can also use free ammonia as the nitrogen donor [23], as summarized below:

$$\mathrm{XMP + ATP + Glutamine \rightarrow}$$
$$\mathrm{GMP + AMP + pyrophosphate + Glutamate,}$$

or

$$\mathrm{XMP + ATP + NH_3 \rightarrow GMP + AMP + pyrophosphate.}$$

To evaluate whether ECC1385 directly inhibits GMPS enzymatic activity, an in vitro biochemical assay was performed with extracts prepared from pTet-*GUA1* cells grown under nonrepressing conditions that are inferred to overproduce Gua1 (see above and Figure 3D). To assay GMPS activity, $^{14}$C-labeled XMP was used and the appearance of labeled GMP was monitored by thin-layer chromatography (see Experimental Procedures). Whereas mock-treated extracts converted XMP to GMP within 30 min, ECC1385 preincubation partially inhibited XMP conversion at 1 μM and completely abolished it at ≥10 μM (Figure 4A). GMPS activity was unaffected in control extracts treated with MPA (data not shown). Consistent with the functional role of Gua1, extracts prepared from the pTet-*GUA1* strain grown under repressing conditions (50 ug/ml tetracycline) in the presence of 0.75 mM guanine lacked any detectable GMPS activity (data not shown). Therefore, ECC1385 inhibition of GMPS is a phenocopy of the genetic depletion of Gua1.

GMPS inhibition by ECC1385 was next compared to two previously described GMPS inhibitors, acivicin (ACI) and 6-diazo-5-oxo-L-norleucine (DON). Both inhibitors have been classified as glutamine antagonists that inhibit glutamine-dependent amidotransferases [24–26]. When *C. albicans* GMPS activity was assayed after preincubating the protein extract in the presence of each inhibitor with XMP, ATP, and $Mg^{2+}$ (reaction was started with the addition of glutamine as the amino donor), ECC1385 (in vitro MIC of ~10 μM) (Figure 4A, lane 8) showed a 3-fold greater potency than ACI or DON (MIC ~30 μM for both) (Figure 4A, lanes 14 and 19, respectively). However, when the GMPS assay was carried out using ammonium sulfate (AS) as the amino donor, ACI was unable to

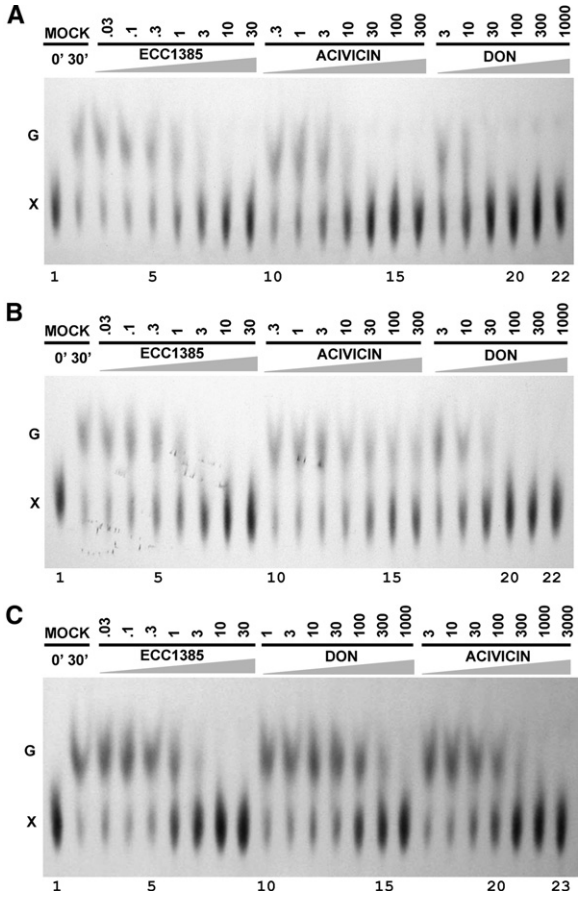


**Figure 4. ECC1385, ACI, and DON Inhibit *C. albicans* In Vitro GMP Synthase Activity**

(A) Effect of preincubation with ECC1385, ACI, or DON, on the glutamine-dependent GMPS activity. All components of the reaction mix (with the exception of glutamine) were preincubated for 10 min at 21°C and the reaction started by the addition of 1 μl of 125 mM glutamine.

(B) Effect of preincubation with ECC1385, ACI, or DON, on the ammonia-dependent GMPS activity. Reaction conditions were similar to (A), with the exception that catalysis was started by the addition of 1 μl of 1 M $(H_4N)_2SO_4$ (pH 7.5).

(C) Effect of glutamine on ECC1385, ACI, or DON, inhibitory capacity of GMPS. All components of the reaction mix (with the exception of XMP) were preincubated for 10 min at 21°C, and the reaction started by the addition of radiolabeled XMP. Standards: X, XMP; G, GMP (purified $^{14}$C-labeled nucleotides). Inhibitors were tested at the concentrations indicated at the top of each panel (in μM).

inhibit the enzymatic activity completely, even at concentrations as high as 300 μM (Figure 4B, lanes 10–16); consistent with this, ACI does not inhibit the AS-dependent human GMPS activity [27]. ECC1385 inhibited GMP synthesis to the same extent in the glutamine- and AS-dependent catalytic reactions (Figures 4A and 4B, lanes 3–9), while DON was slightly less potent in the AS-dependent reaction (MIC ~100 μM) (Figure 4B, lanes 17–22). To further examine possible mechanistic differences between these compounds, the GMPS assay was adjusted to include glutamine during preincubation and the reaction started by the addition of labeled XMP. Since glutamine effectively competes with its antagonists, thereby decreasing their potency, we reasoned that if ECC1385 does not act as a glutamine antagonist, its potency should not be diminished under these conditions. Indeed, the ECC1385 MIC against GMPS was invariant (~10 μM) (Figure 4C, lane 8), while DON and ACI MICs were approximately 30 times greater (~1000 μM) (Figure 4C, lanes 16 and 22, respectively). These results suggest that ECC1385 does not inhibit *C. albicans* GMPS activity in a manner analogous to glutamine antagonists.

## ECC1385, ACI, and DON Display Distinct MOAs in Whole Cells

ECC1385, ACI, and DON all inhibit *C. albicans* GMPS activity in vitro, yet display distinct substrate-dependent activity. Moreover, ACI and DON are reported to inhibit in vitro a family of enzymes that share a class I amidotransferase activity that is also common to GMPS [28], including carbamyl phosphate synthetase, CTP synthetase, and formyl-glycinamide ribonucleotide synthetase (FGAM synthase) [24, 29]. Therefore, CaFT profiling was performed to examine whether ACI and/or DON may have a distinct MOA in whole cells versus ECC1385. Interestingly, the CaFT profile of DON revealed the *ADE6* heterozygote strain to be markedly hypersensitive to DON, whereas the *GUA1* heterozygote was not differentially sensitive. A second unique aspect of the DON CaFT profile was the increased resistance of the *orf19.7506* heterozygote, which corresponds to an uncharacterized gene exclusive to *Candida spp.*, but whose role in the MOA of DON is unknown. MIC determinations of *ADE6* and *orf19.7506* heterozygotes confirmed their altered sensitivity to DON (Figure S2A). CaFT profiling of ACI also failed to detect a *GUA1* heterozygote depletion; instead, the *URA7* heterozygote was identified as strikingly hypersensitive to ACI (Figure 5, and also confirmed by MIC determinations, Figure S2B). As *ADE6* and *URA7* encode FGAM synthase and CTP synthase, respectively, and both enzymes possess class I amidotransferase activity, we speculate that in *C. albicans* whole cells, the corresponding primary targets of DON and ACI are Ade6 and Ura7, rather than GMPS.

To corroborate their alternative MOA in whole cells, suppression of DON growth inhibitory activity was first examined with nucleobase supplements. As predicted, adenine partially suppressed the inhibitory activity of DON (Figure S2C). Since DON also has inhibitory capacity in vitro on other transaminases involved in nucleotide synthesis (e.g., GMP synthase), the simultaneous addition of guanine and adenine was tested but no improved suppression was observed (data not shown). Similar tests involving cytosine, cytidine, or CTP supplements to suppress the effect of ACI were not feasible as the *C. albicans* pTet-*URA7* strain grown under repressing conditions was not suppressed under such conditions (Figure 6A), due either to the inability of fungal cells to take up CTP or convert cytosine to CTP [30].

Partial suppression of DON by adenine supplementation suggests that, in addition to Ade6, DON likely inhibits a second target in whole cells. This was tested by reexamining DON in the CaFT with adenine added to the medium. Under such conditions, instead of a noticeable *ADE6* heterozygote depletion, a dose-dependent *URA7* heterozygote hypersensitivity was observed, as well as continued *orf19.7506* heterozygote resistance (Figure 5). Therefore, although DON has been previously reported in vitro to inhibit GMPS activity [24], CaFT profiling studies that assess its effects globally and within the cellular milieu emphasize a more complex MOA involving multiple amidotransferases (Ade6 and Ura7) rather than Gua1.

## *GUA1* Is Essential for Virulence in *C. albicans* and *A. fumigatus*

*S. cerevisiae GUA1* is a conditional essential gene whose function is not required in the presence of guanine provided the cell is competent to convert extracellular guanine into GMP [31]. However, as *S. cerevisiae* is naturally nonpathogenic, it is unclear whether *gua1* loss-of-function mutants would impair virulence of pathogenic fungi. To test this possibility, we first examined the terminal phenotypes of the *C. albicans* pTet-*GUA1* strain as well as an *A. fumigatus GUA1* conditional mutant, constructed with a nitrate-regulatable (*NiiA*) promoter replacement strategy [32]. As expected, *GUA1* was required for growth of both pathogens under repressing conditions on minimal media lacking guanine but dispensable when guanine was supplemented to the medium (Figures 6A and 6B).

Importantly, phenotypes associated with pTet-*GUA1* and pNiiA-*GUA1* conditional mutants may be examined in an animal model of infection, as their respective promoters are efficiently repressed in vivo [15, 32]. In a murine model of systemic candidiasis, mice (n = 15) were infected with $10^6$ cells of the *C. albicans* pTet-*GUA1* strain, and divided into three different regimes: (1) doxycycline (an analog of tetracycline with superior pharmacokinetic properties) supplemented to drinking water three days prior to and throughout the infection time course (modeling a prophylactic treatment), (2) doxycycline supplemented to drinking water 2 days postinfection and throughout the infection time course (modeling a systemic treatment), and (3) no doxycycline added to the animal drinking water (virulence control) (Figure 6C). All mice pretreated with doxycycline from the onset of infection survived and remained healthy over 22 days, whereas all control mice not administered the repressor succumbed to infection within 11 days. Similarly, repression of *GUA1*

 

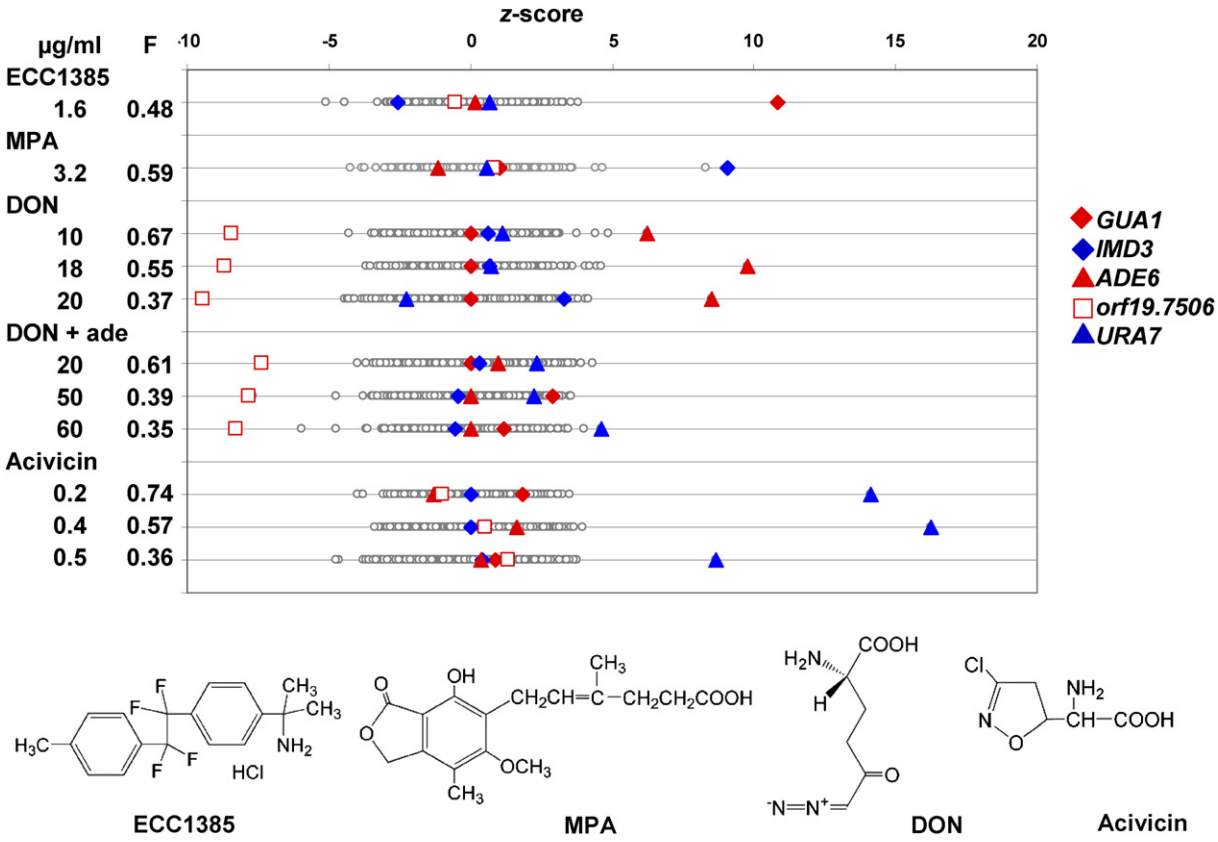


**Figure 5. CaFT Profiles of DON and ACI**

CaFT profiles identify *ADE6* and *URA7* as specifically hypersensitive heterozygotes to DON and ACI, respectively. Note that under conditions in which DON growth inhibitory activity is partially suppressed by 0.5 mM adenine supplementation, *URA7* heterozygote sensitivity is detected (see text for details). MIC determinations verify all strain sensitivities predicted in CaFT profiles (Figure S2). Representative CaFT profiles of ECC1385 and MPA are included to emphasize mechanistic differences between these compounds and DON or ACI.

starting at 2 days postinfection, resulted in no mortality or obvious morbidity. Therefore, although the *C. albicans* pTet-*GUA1* strain was fully virulent when *GUA1* was induced, its repression, even at later time points in the infection, ablated the normal virulence. All mice maintained on either doxycycline regimes remained healthy even after removing the repressor from their drinking water on day 22, until the experimental endpoint on day 36, implying that the infection was significantly cleared during the doxycycline treatment. Necropsy experiments across all treatment groups confirmed that the fungal burden in mouse kidneys was often reduced to the limit of detection (Table 1).

Although the pNiiA-regulatable promoter cannot be controlled in as elaborate a manner as the aforementioned tetracycline promoter system, it is effectively repressed in vivo due to sufficient ammonium (repressor) levels within the host and can therefore be used to study possible virulence phenotypes resulting from genetic inactivation [32]. Mice were immunocompromised with cyclophosphamide and infected with $10^5$ conidia derived from the *A. fumigatus* pNiiA-*GUA1* conditional mutant or the parental wild-type control strain CEA10. Necropsies performed at day 1 postinfection on mice treated with CEA10 and pNiiA-*GUA1* strains confirmed that both groups harbored similar infectious loads, from 3 to 7 × $10^3$ conidia/g of kidney (Table 1). CEA10 was fully virulent, with no mice surviving past day 4. In contrast, the pNiiA-*GUA1* conditional mutant appeared completely avirulent (100% survival) over the 21 day time course of the infection, with mice showing no obvious signs of lethargy or morbidity (Figure 6D). Moreover, necropsies on all surviving pNiiA-*GUA1* infected mice failed to detect *A. fumigatus* in the kidney. We conclude that, despite a demonstrated guanine-conditional essentiality of *GUA1* in both *C. albicans* and *A. fumigatus*, neither pathogen is able to scavenge sufficient guanine, and both are therefore avirulent in murine models of infection when lacking *GUA1* function.

## DISCUSSION

We have performed compound MOA studies by monitoring chemical-induced haploinsufficiency within an extensive *C. albicans* heterozygote strain set adapted for screening in a fitness test format. The robustness of this approach is demonstrated by examining known

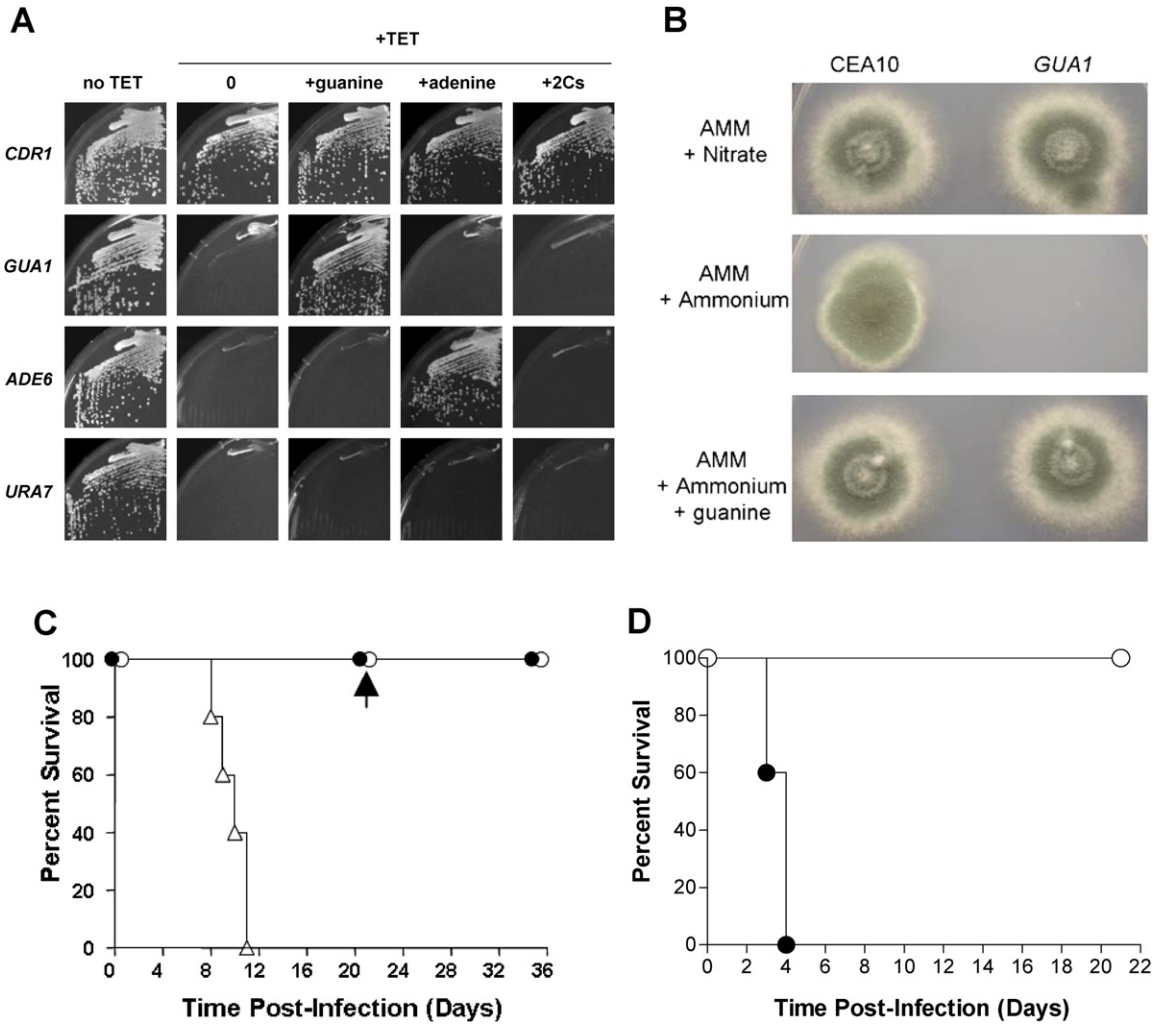


**Figure 6. *GUA1* Is a Conditional Essential Gene Required for Virulence of *C. albicans* and *A. fumigatus***

(A) Growth effects of *C. albicans* tetracycline promoter replacement mutants for *GUA1*, *CDR1* (control gene), *ADE6*, and *URA7* (see text for details) were examined on YPD at 30°C for 2 days. Growth was compared to that observed under repressing conditions (100 μg/ml tetracycline) in the absence or presence of 125 μM guanine, 1.25 mM adenine, or 1.25 mM cytosine plus 1.25 mM cytidine (2Cs).

(B) Growth effects of an *A. fumigatus NiiA* promoter replacement mutant of *GUA1* versus the wild-type strain (CEA10) were compared under inducing (AMM plus nitrate) or repressing (AMM plus ammonium) conditions at 37°C for 2 days. Exogenous guanine (400 μM) fully suppressed the growth phenotype of p*NiiA-GUA1* grown under repressing conditions.

(C) In vivo target validation of *C. albicans GUA1* in a murine model of infection. Mice were infected with $10^6$ cells of *C. albicans* pTet-*GUA1* and divided into three treatment groups: (•), doxycycline provided starting 3 days before infection; (○), doxycycline treatment provided starting 2 days after the infection; (Δ), sugar 5% (control). Twenty-one days postinfection (arrow), two mice from each group were taken for necropsy, and remaining mice switched from doxycycline to water until day 35 postinfection, when two additional mice were taken for necropsy.

(D) In vivo validation of a p*NiiA-GUA1* mutant in an immunocompromised murine model of systemic aspergillosis. Approximately $10^5$ viable conidia were injected into the tail vein of immunocompromised mice (five mice per group) and monitored for up to 22 days following infection. (○), p*NiiA-GUA1* strain; (•), wild-type strain (CEA10).

compounds classified as inhibiting various aspects of de novo purine metabolism, as well as ECC1385, which acts by inhibiting GMPS. In this approach, targets are identified empirically by screening growth inhibitory compounds of unknown MOA against a diversity of potential targets. Consequently, target validation studies are performed after chemical screening is completed and the target demonstrated to be susceptible to chemical inhibition. Applying this approach, a hypothesis for the MOA of ECC1385 was established and verified by biochemical and genetic assays consistent with GMPS being its primary target. Our work also extends the characterization of *GUA1*, which encodes GMPS. *GUA1* is a conditional essential gene, required for normal growth of both *C. albicans* and *A. fumigatus*, but dispensable in the presence of a supplementary guanine source. Importantly, depletion of *GUA1* activity is not suppressed in a host environment, as *GUA1* conditional mutants of both pathogens are

**Table 1. In Vivo Target Validation of *C. albicans* and *A. fumigatus GUA1***

Kidney Fungal Burden Postinfection with *C. albicans* pTet-*GUA1*

| Treatment | Mouse | After 21 Days (CFU/g Kidney) | Mouse | After 35 Days (CFU/g Kidney) |
|---|---|---|---|---|
| DOX-3 | A | *** | C | $6.0 \times 10^4$ |
| | B | $7.3 \times 10^4$ | D | $4.2 \times 10^4$ |
| DOX+2 | A | $1.1 \times 10^6$ | C | *** |
| | B | *** | D | $6.8 \times 10^6$ |

Necropsy Results at Day 1 Postinfection with *A. fumigatus* Strains

| Strain | Mouse | Conidia/g Kidney |
|---|---|---|
| pNii-*GUA1* | A | $4.2 \times 10^3$ |
| | B | $4.3 \times 10^3$ |
| | C | $7.1 \times 10^3$ |
| CEA10 | A | $3.6 \times 10^3$ |
| | B | $3.0 \times 10^3$ |

Triple asterisks represent less than $1 \times 10^4$. CFU, colony forming units.

avirulent under repressing conditions in murine models of infection. Thus, *GUA1* represents an unexpected antifungal therapeutic target.

Like *GUA1*, other genes required for prototrophy of intermediary metabolism function are implicated in microbial pathogenesis. In uracil biosynthesis, *URA3* (orotidine 5′-phosphate decarboxylase) is required for pathogenicity of *C. albicans* and *A. fumigatus* [33, 34]. Mutants defective in either adenine (*ade2*) or heme (*hem3*) biosynthesis are also avirulent in *C. albicans* [33] and *pabaA* mutants (defective in p-amino-benzoic acid biosynthesis) are avirulent in *A. fumigatus* [35]. In addition, *ura7* and *ade6* mutants are avirulent in a candidiasis model of infection (Figures S3A and S3B). In contrast, amino acid biosynthetic genes, including *HIS1*, *ARG4*, and *LEU2* [36], as well as *MET2*, *MET16*, *TRP5*, *HIS7*, *THR4*, and *LYS2* (our unpublished data) do not noticeably attenuate *C. albicans* pathogenesis when mutated. Presumably, these differences reflect the pathogen's proficiency to scavenge metabolites and the requirement that sufficient levels of such metabolites exist in the host environment.

A comparative analysis of *GUA1* across prokaryotes and eukaryotes supports its consideration as an antifungal target. Although Gua1 is broadly conserved, it has significantly diverged among fungi, plants, bacteria, and metazoans (Figure S1). Fungal and plant Gua1 orthologs appear most closely related, consistent with their common ancestry, whereas mammalian orthologs are most diverged. Interestingly, the fungal pathogens, including *C. albicans*, *C. glabrata*, and *A. fumigatus* all share $\geq$65% identity among one another and $\leq$38% identity to human Gua1. A similar level of divergence exists between human and fungal Erg11 (39%), the target of azole-based antifungal therapeutics [37]. Therefore, Gua1-specific inhibitory compounds could display similar fungal spectrum and selectivity. Moreover, as recent bioinformatics-based efforts to catalog broad spectrum antifungal targets [38, 39] have excluded conditional essential genes (including *GUA1*), their inclusion should broaden the complete antifungal target set if validated experimentally.

Our work demonstrates ECC1385 to be a novel Gua1 inhibitor with improved potency versus the previously reported GMPS inhibitors, ACI or DON. Moreover, unlike the latter compounds, only ECC1385 displays an in vitro potency that correlates with its growth inhibitory activity against whole cells. As ECC1385 is structurally unrelated to glutamine antagonists such as ACI and DON, its specificity and potency may result from inhibiting the GMPS activity rather than the amidotransferase activity of Gua1. In support of this possibility, unlike glutamine antagonists whose amidotransferase inhibitory activity is sensitive to whether glutamine or ammonia substrates is provided [27], no such substrate selectivity compromises ECC1385 inhibition of Gua1 (Figures 4A–4C). Inhibitors that decrease GMP levels serve as useful chemical reagents to study purine metabolism and/or as tools in molecular biology [40]. However, ECC1385 failed to exhibit efficacy at 12.5 and 25 mg/kg and displayed toxicity at 50 mg/kg in a murine model of candidiasis (data not shown). Thus, chemical improvements to ECC1385 are required to advance its therapeutic potential. Alternatively, target-based biochemical and/or whole-cell screens [38, 41, 42] could be performed to uncover new GMPS inhibitors with greater intrinsic efficacy. As both MZ and MPA are clinically used as immunomodulators, whose immunosuppression effects result from preferential GMP depletions in T cells and B cells [43], specific inhibitors of the human GMPS may also possess therapeutic potential.

Chemically induced HI, surveyed across extensive genome coverage, identifies important aspects of a compound's MOA [7, 11]. For example, although an *IMD3* heterozygote is known to be hypersensitive to MPA [20], its mechanistic significance is emphasized when compared more globally across the genome. Here, in addition to identifying Imd3 as the primary target of MPA, *ADE12* and *ADK1* heterozygote hypersensitivity and *AMD1*

heterozygote resistance to this compound reinforce the metabolic pathway targeted by the drug. Based on their phenotypes, it appears that as IMP levels increase in response to the chemical inhibition of Imd3, efficient shunting of IMP to ADP (Ade12 and Adk1) seems to partially buffer the cell from MPA, whereas conversion of AMP to IMP (Amd1) enhances its toxicity (Figure 2). Presumably, this reflects a fitness advantage for cells best able to rescue some benefit (namely ADP production) under conditions where all cells incur the same metabolic cost of accumulating IMP, which can not be converted to GMP. Imd3 was also correctly identified as the drug target of MZ, but in addition, the resistance of a *ADO1* heterozygote reflects the in vivo activation of MZ; namely, its phosphorylation by adenosine kinase to convert it from a prodrug to its active form [21]. Drug import and efflux mechanisms for both drugs were also identified. Hypersensitivity of a *TPO1* heterozygote is congruent with its reported role in the efflux of MPA [20]. *NNT1* heterozygote resistance to MZ likely reflects its reported function as a nucleoside permease [11, 44], as MZ is an imidazole-based nucleoside. Recently, it was demonstrated that *C. albicans* sensitivity to MZ is suppressed by guanine and guanosine but, unexpectedly, also by uridine (but not cytidine) [19]; the authors speculate this could be due to competition for a common transporter. Our work indicates this transporter is likely Nnt1, as it possesses transport specificity for these nucleosides and their analogs [44] and is resistant to MZ when mutated.

MPA was first identified in 1896, making it one of the earliest natural products discovered, yet its MOA and potential pharmacological properties continue to be widely investigated [43]. Recently, a study evaluated MPA effects across a collection of 4,787 yeast haploid knockout mutants [18]. Surprisingly, over 100 genes affecting MPA resistance were uncovered across a wide spectrum of cellular functions, but core aspects of IMP metabolism we identify were not reported. Although we cannot rule out species-specific MPA effects, it is more likely that this reflects differences between the two assays. We speculate that chemically induced HI combined with stringent strain-specific error models (which statistically deprioritize nonspecific drug sensitivities) effectively resolve a compound's primary MOA over more complex or indirect effects. However, potential complications or liabilities associated with this specific approach include the absence of full-genome coverage in the present assay, allelic polymorphisms and/or posttranscriptional regulation of particular genes, which may confound the haploresponsiveness of some strains, as well as the fidelity of barcodes used to monitor strain fitness in a microarray format [7–9, 11].

CaFT profiling of ECC1385 did not reveal additional aspects of its MOA other than a chemical genetic relationship with *GUA1*. Guanine-based suppression of ECC1385 supports the view that Gua1 is the drug target rather than it playing an indirect role such as metabolizing or detoxifying ECC1385. Moreover, ECC1385 inhibition of GMPS is a phenocopy of a pTet-*GUA1* conditional mutant, which similarly abolishes GMPS activity under repressing conditions. However, ECC1385 may affect an additional target(s), as suggested by the inability of guanine to fully suppress its inhibitory effects at super-MIC levels. Further analysis of ECC1385 MOA in the CaFT in the presence of guanine failed to identify any other significantly affected strain (see Supplemental Data and Figure S4A). Therefore, if ECC1385 affects a secondary target, its corresponding heterozygous strain is either absent from the screening pool or unresponsive in a haploinsufficiency-based readout.

Intriguingly, both ACI and DON inhibited *C. albicans* GMPS activity in vitro (Figure 4), yet neither compound affected the *GUA1* heterozygote in the CaFT. Instead, we hypothesize their primary targets in whole cells be Ura7 and Ade6, respectively. Consistent with this view, each enzyme possesses a common amidotransferase activity, yet only Gua1 has a GMPS activity. Moreover, ACI and DON are glutamine antagonists reported to inhibit amidotransferases by blocking their ability to use glutamine as substrate, rather than directly inhibiting GMPS activity [27]. Our work emphasizes a promiscuity of glutamine antagonists, most strikingly exemplified by the CaFT profile of DON in combination with adenine; when suppression of DON's putative primary target (*ADE6*) is achieved, a dose-dependent hypersensitivity of the *URA7* heterozygote could be observed. Consistent with our results, an analysis of the metabolic intermediates that accumulate during DON treatment in mouse leukemia cells also implicates Ade6 and Ura7 as likely targets of this drug [29]. Establishing robust correlations between a compound's MOA based on in vitro versus whole cell activity remains an important challenge; particularly in cases where the target is a member of a large functionally related protein family, such as amidotransferases or protein kinases [45]. In such cases, global haploinsufficiency-based MOA studies may provide a general means to resolving the primary target within a cellular milieu from secondary targets also inhibited in vitro but of less physiological relevance to the inhibitor's MOA.

## SIGNIFICANCE

**Identifying new drug targets and their cognate inhibitors remain a significant challenge to the development of novel antifungal therapeutics. To address these issues, we have employed a genomics-based approach applied in the principal fungal pathogen, *C. albicans*, to study the MOA of a novel antifungal compound, ECC1385. Examining its growth inhibitory effect among ~2900 *C. albicans* heterozygous deletion mutants, we identify the *GUA1* heterozygote as uniquely hypersensitive to this compound. We demonstrate by genetic and biochemical means that *GUA1* encodes GMP synthase and that ECC1385 is a potent and specific inhibitor of GMP synthase activity. Unlike the previously reported GMP synthase inhibitors acivicin and DON, only ECC1385 displays a MOA that correlates both in vitro and in whole cells. Further, by examining chemically induced haploinsufficiency produced by**

**mycophenolic acid and mizoribine, which target the preceding enzymatic step in GMP synthesis carried out by Imd3, we highlight additional MOA information pertinent to the uptake, efflux, and metabolism of these drugs. These findings illustrate the capacity to study drug MOA in the context of living cells to gain mechanistic insights that may be tested by biochemical and/or genetic means. Finally, by determining the growth inhibitory MOA of ECC1385 and genetically inactivating its target, we demonstrate *GUA1* to be essential for *C. albicans* and *A. fumigatus* virulence, thus illustrating how a chemical probe may be used to identify an unanticipated antifungal drug target.**

## EXPERIMENTAL PROCEDURES

### Chemicals and Reagents

ACI, DON, MZ, MPA, protease inhibitor cocktail for fungal extracts (PICFE), amino acids, nucleotide bases, and all buffers and chemicals, except when particularly indicated, were purchased from Sigma-Aldrich. XMP was purchased from Fluka BioChemika (cat. 95550). [8-$^{14}$C]-labeled XMP and GMP (specific activities 50–60 Ci/mol) were purchased from Moravek Biochemicals. Plastic-backed, polyethyleneimine-cellulose-covered, thin-layer chromatography plates (PEI-TLC), Polygram CEL 300 PEI (cat. #801-053) were purchased from Macherey-Nagel. ECC1385 (CAS name: α,α-dimethyl-4-[1,1,2,2-tetrafluoro-2-(4-methylphenyl)ethyl]-benzenemethanamine hydrochloride) was identified within the corporate synthetic compound library and resynthesized internally.

### *C. albicans* Fitness Test and Strain Collection

Heterozygote and tetracycline-regulatable expression strains described in this work were constructed as described in [15] and are available for noncommercial use to academic researchers following the standard Merck Material Transfer Agreement (MTA) and clearance procedures. For a complete description of genes comprising the heterozygote strain set, as well as technical aspects of the *C. albicans* Fitness Test, see [11]. Although we are unable to provide the complete heterozygote strain set at this time, we invite academic researchers to contact us regarding possible collaborations related to MOA studies of bioactive compound(s) of mutual interest. Following an MTA agreement executed by both parties, such compounds would be screened by Fitness Test analysis and results communicated back to the collaborator.

### Protein Extracts Preparation

*Candida albicans* strain pTet-*GUA1*, allowing tetracycline-repressible Gua1 expression was separately grown either in YPD (nonrepressive conditions), or YPD containing 50 ug/ml tetracycline, plus 0.75 mM guanine (repressing conditions). Cells were harvested at an $OD_{600}$ ~2 by centrifugation (10 min, 4°C, 3,000 × g), and processed at a constant temperature of 0°C to 4°C. 1,600 ODs of cells (fresh weight ~3.5 g) were washed once with 50 ml of lysis buffer (50 mM Na HEPES [pH 7.3], 10 mM 2-mercaptoethanol [2-ME], 2 mM EDTA) and collected as indicated above. Washed cells were then resuspended in 15 ml of lysis buffer containing 2 mM phenylmethylsulfonylfluoride (PMSF) plus PICFE and disrupted in a bead beater (0.5 mm glass beads). The homogenate was removed, lysis buffer was added to complete a final volume of 50 ml, and cell debris were eliminated by centrifugation (15 min, 4°C, 3,000 × g). The supernatant was kept, and the protein fraction precipitating at 80% saturation of ammonium sulfate was obtained by adding 28 g of ammonium sulfate crystals. After centrifuging for 15 min, at 3,000 × g, the pellet was resuspended in 18 ml of lysis buffer and dialysed twice against 4 l of 10 mM Na HEPES (pH 7.3), 5 mM 2-ME, 0.1 mM EDTA, at 4°C. After dialysis, antipain, pepstatin, and leupeptin were added to the dialysate to reach a final concentration of 1 ug/ml, PMSF to reach 0.2 mM, and KCl to 100 mM. After these additions, the dialysate was cleared (5 min, 4°C, 16,000 × g) and the supernatant (~27 ml) concentrated by centrifugation in Millipore Amicon Ultra 4 units, aliquoted and frozen at −80°C (protein concentration ~10 mg/ml); this fraction with <50 uM ammonium sulfate was named "AS-2D" and used as the source of GMPS activity in all the experiments performed.

### GMP Synthase Activity Assay

Protein extracts were assayed for GMPS activity at 30°C for 30 min, in a final volume of 25 μl containing: 50 mM Na HEPES (pH 8.2), 1 mM ATP, 4 mM $MgCl_2$, 0.1 mM DTT, 5 mM glutamine (replaced by 40 mM $[H_4N]_2SO_4$ when indicated), 0.1 mM EDTA, 0.3 mM $^{14}$C-XMP (13.3 Ci/mol), and 40 ug of protein (fraction AS-2D). Reactions were stopped by transferring 6 μl aliquots to 24 μl of 1.25 M formic acid, incubated on ice for 15 min, and centrifuged 10 min at 16,000 × g to pellet the denatured proteins from the soluble nucleotides. 20 μl of these supernatants were transferred into 4.8 μl of 50% triethanolamine for neutralization; 2 μl of this solution were spotted on PEI-TLC plates and run on 0.75 M Tris-HCl (pH 8.0) (solvent Ta) [46]. After chromatography, TLC plates were dried and exposed to radiographic film.

### *C. albicans* and *A. fumigatus* Virulence Studies

*C. albicans* strains were grown overnight at 30°C in YEPD, washed twice, and resuspended in water at a final concentration of $10^7$ cells/ml. Three groups of male ICR mice (five or six mice in each group) were infected by tail-vein injection ($10^6$ cells per mouse): with one group (Dox-3) receiving the doxycyline treatment 3 days prior to infection, one group (Dox +2) receiving the doxycycline 2 days after infection, and the control group, which remained on 5% sucrose without doxycycline for the duration of the experiment. Doxycycline was administered orally in the drinking water ad libitum (2 mg/ml, in a 5% sucrose solution). At 21 days postinfection, two mice from each group were taken for necropsy; remaining mice were switched to water, and survival monitored for additional 2 weeks, with additional necropsies performed at 35 days postinfection.

A pNiiA-*GUA1* conditional promoter replacement mutant of *A. fumigatus* was constructed with the nitrogen-regulatable NiiA promoter, and its virulence, and that of the wild-type CEA10, were assessed in an immunocompromised murine model for systemic infection [32]. Briefly, CD-1 mice were rendered immunocompromised by treatment with cyclophosphamide and infected by tail vein injection with $10^5$ viable *Aspergillus* conidia. Mice were kept immunocompromised and monitored for survival up to a maximum of 22 days. All experiments were performed according to the National Institutes of Health guidelines for the ethical treatment of animals.

### Supplemental Data

Supplemental Data, including four figures, one table, and a spreadsheet containing all the z scores obtained in the Fitness Test analyses, are available online at http://www.chembiol.com/cgi/content/full/14/10/1163/DC1/.

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