Antagonism of fluconazole and a proton pump inhibitor against Candida albicans

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Hospitalized ill patients, at risk for invasive candidiasis, often receive multiple medications including proton pump inhibitors (PPIs). The antifungal fluconazole perturbs the vacuolar proton ATPase. The PPI omeprazole antagonized *C. albicans* growth inhibition by fluconazole. A *C. albicans* codon-adapted Ca.pHluorin was generated to measure cytosolic pH. The fungal cytosol was acidified by omeprazole, and re-alkalinized by co-exposure to fluconazole. Vacuolar pH was alkalinized by fluconazole. Off-target effects of any medication on fungal pathogens may occur.

Candida species are the fourth most common cause of nosocomial bloodstream infections (18). Invasive candidiasis is a disease of ill and hospitalized patients. These patients often receive a large number of medications, whose impact on the infecting Candida cells is not usually considered.

Animals and fungi are closely related among the eukaryotes, both phyla being members of the opisthokonts. Drugs approved for various human indications have therefore been examined for antifungal activity (1, 17), since molecular targets are often conserved between humans and fungi. “Repurposed” drugs like the estrogen receptor antagonist tamoxifen (1) and the antiarrhythmic amiodarone (4) have antifungal activity alone and in combination with the antifungal fluconazole.

Fluconazole inhibits the ergosterol biosynthetic enzyme Cyp51/Erg11, consequently disrupting membrane functions. An important mechanism of its fungistatic activity is perturbation of the vacuolar proton ATPase (V-ATPase), resulting in loss of vacular acidification (19). We wondered whether perturbing V-ATPase may additionally lead to cytosolic acidification, if protons generated by
metabolic processes are not efficiently sequestered in the vacuole. If this were the case, fluconazole's antifungal activity might be potentiated by other drugs which also acidify the cytosol.

Proton pump inhibitors (PPIs), sold over the counter like omeprazole, or by prescription, like rabeprazole, are taken by millions of patients each year (6). In 2009, a PPI was prescribed at 79.4 million physician visits in the United States (5). Omeprazole has an antifungal effect and is known to inhibit the fungal plasma membrane proton ATPase Pma1 (10).

We wanted to test the idea that blocking outflow of protons from the fungal cytosol into the extracellular space with omeprazole (10), while perturbing proton pumping from the cytosol into the vacuole with fluconazole (19), may result in toxic acidification of the cytosol and antifungal synergy.

We first confirmed the inhibitory effect of omeprazole on C. albicans (Fig. 1A).

To test the effect of the drug combination, overnight-grown cells were diluted to an OD600 of 0.1 in microtiter plates containing RPMI1640 (Lifetech) 2% glucose, buffered to pH 7.0 with 165 mM MOPS, pH5 with 50 mM MES or pH 3.0 with 50 mM sodium citrate, and two-fold serial drug dilutions, or drug vehicle, DMSO. The OD600 was measured after 24 hours' incubation at 35°C. Experiments involving omeprazole, which needs to be activated to the H+,K+-ATPase-binding sulfenamide by low pH (10) (and whose active sulfenamide form is unstable at pH>4), were performed at pH 3, after confirming fluconazole activity at pH 3 (Fig. S1). Rabeprazole, whose molecular interaction with the gastric H+,K+-ATPase differs slightly from that of omeprazole, and which is activated in higher pH but is less stable at neutral pH than omeprazole (16), was tested at pH7, after pre-activation of the compound for one hour in the presence of 0.1 M HCl prior to addition to culture media. Cells were inoculated at OD600 0.1 into RPMI, which was buffered to pH3 or 7 as above, and contained two-fold decreasing omeprazole or rabeprazole concentrations. Cultures were incubated at 35°C for 24 hours,
and monitored for OD600. For growth curve experiments, wild type *C. albicans* was grown overnight on YPD agar medium at 30°C and cells were diluted to an initial OD600 0.1 in RPMI Medium 1640 (pH3). Cells were inoculated into 96-well plates with the addition of drugs as noted, incubated at 35°C and the OD600 was read automatically for 24 hours at 15 minute intervals.

Instead of synergistic or additive growth inhibition when combining fluconazole with a proton-pump inhibitor, we observed an antagonistic effect: omeprazole and its analog rabeprazole rescued cells from growth inhibition by fluconazole (Fig. 1B-D, and Fig. S2).

To directly examine the effect of these drugs on cytosolic pH, we adapted a ratiometric pHluorin protein (9) to *C. albicans* codon usage. The synthesized gene (GenScript, Piscataway, NJ) was cloned into plasmid pJK1027 (13) behind the strong *ACT1* promoter and integrated at the *ACT1* locus in strain JKC915 (15) to create JKC1559, the strain used in this study. Cytosolic pH of cells exposed to fluconazole, omeprazole, and their combination was determined as described in (3), by measuring fluorescence emission at 508nm with excitation at 405nm and 485nm, and calculating pH using a calibration curve (Fig. 2A and B). To generate the calibration curves, pHluorin-expressing, permeabilized cells were equilibrated in 6 buffers of increasing pH (pH5-8). 20μl cell suspension was added to 2ml calibration buffer and incubated at 30°C for 60 min before obtaining measurements of emission intensity at 508nm during excitation at 405 and at 485 nm. Three biological replicates, each comprising three technical replicates, were obtained for each condition. All measurements were graphed and statistically analyzed using Prism software (GraphPad Software, San Diego California), applying Student's two-tailed t-test to calculate p values.

The cytosolic pH of omeprazole-treated cells dropped, consistent with the concept that omeprazole inhibits Pma1 (Fig. 2B). Contrary to our prediction, the cytosol of fluconazole-treated cells was not
Acidified (Fig. 2B). Adding fluconazole to omeprazole normalized the cytosolic pH (Fig. 2B), antagonizing the effect of omeprazole. Low concentrations of the beta-1,3 D-glucan synthase inhibitor micafungin, as a control for general fungal cell damage, alkalinized cytosolic pH (Fig. 2B), confirming the specificity of each drug’s effect.

To test whether omeprazole antagonized alkalinization of the fungal vacuole caused by fluconazole (19), we measured vacuolar pH of cells exposed to these drugs (Fig. 2C,D), using BCECF-AM (Lifetech), a pH-sensitive fluorophore that accumulates in the yeast vacuole, as in (3). To generate calibration curves (Fig. 2C), cells pre-incubated with BCECF were permeabilized and equilibrated in 6 buffers of increasing pH. 20μl cell suspension was added to 2ml calibration buffer (pH 5-8) and incubated at 30°C for 60 min, as described in (3). Replicates and calculations were performed as for cytosolic pH. Fluconazole-exposed cells lost vacuolar acidification as expected (19) (Fig. 2D). Contrary to our expectation, the vacuolar pH of omeprazole-treated cells, and cells treated with both drugs, also showed a trend toward alkalinization (Fig. 2D). Exposure to a low concentration of micafungin strongly acidified the vacuole, confirming the specific effect of fluconazole and omeprazole on vacuolar pH. We did not identify the mechanism by which omeprazole antagonizes the fungistatic effect of fluconazole.

We examined omeprazole concentrations in the range of 10μM, as they occur in human plasma during omeprazole treatment with standard doses e.g. for gastric reflux disease (7), for the interaction studies with fluconazole. At these concentrations, omeprazole alone had no effect on C. albicans growth (Fig. 1A), but did antagonize the fungistatic effect of fluconazole (Fig. 1B-D). Only at 40-fold higher concentrations did omeprazole alone exert an inhibitory effect on C. albicans (Fig. 1A). In clinical practice, such high concentrations are unlikely to occur, so that we do not expect omeprazole alone to have utility as an antifungal. We tested rabeprazole, which is activated at slightly higher pH
than omeprazole, at pH 7. We found that in these conditions, its concentrations antagonizing fluconazole were ~32 fold higher than standard plasma levels during rabeprazole therapy (14). The pH of spaces occupied by C. albicans during invasive disease is likely to range from neutral to acidic, since inflammatory foci like abscesses can reach proton concentrations to pH 5.5 (12).

Reproducibility of antagonism between fluconazole and a second PPI, albeit in higher than clinical concentrations at neutral pH, supports the possibility of clinical antagonism of this drug class with fluconazole. Confirmation of this possibility would require in vivo studies.

Homeostatic mechanisms of cytosolic and vacuolar pH, which include cation and anion pumps and intracellular protein buffers (2), are central to the function of multiple critical enzymatic processes (8). The net effect of these multiple homeostatic mechanisms apparently needs to be experimentally determined for each pharmacological perturbation, both with respect to fungal growth, and to pH in important fungal cellular compartments. With the C. albicans-adapted pHluorin, we have generated a molecular tool to facilitate such experiments.

In conclusion, critical assessment of all drugs used in treating ill patients is necessary not only because of potential drug interactions in the patient. Apparently in patients with invasive fungal disease, drugs active on human targets may also elicit unexpected and undesirable effects on the infecting fungus. Given the very widespread usage of PPIs (6), it may not be possible to confirm this effect in a clinical study, since a sufficiently large patient group not prescribed a PPI may be difficult to recruit. Nevertheless, our results underscore the importance of critically assessing patients’ needs for long-term medications, and of avoiding unnecessary medication use (6). In addition, they underscore the importance of testing not only human pharmacokinetics (11), but also pharmacodynamics toward the fungus, of drug combinations for invasive fungal infections.
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References


Figure legends

Fig. 1. Effect of omeprazole, fluconazole and their combination on growth of C. albicans.

A. Effect of omeprazole on C. albicans growth. Results shown are representative of three biologically independent experiments, each comprising four technical replicates, and error bars represent standard deviation. B. Antagonistic effect of fluconazole and omeprazole on growth of C. albicans. Results shown are representative of three biologically independent experiments. Error bars represent standard deviations of four technical replicates. C. and D. Dose-dependent rescue of the fluconazole fungistatic effect by omeprazole, at 0.5 μg/ml (C) and 1 μg/ml (D) fluconazole. Error bars represent standard deviations of four technical replicates. Results shown are representative of three biologically independent experiments. Flu, Fluconazole; Ome, Omeprazole.

Fig. 2. Effect of omeprazole, fluconazole and their combination on pH in intracellular compartments of C. albicans.

A, B. Cytosolic pH of cells exposed to vehicle alone, fluconazole, omeprazole and their combination. (A) Calibration curve showing measured ratio of fluorescence intensity at 405nm to intensity at 485nm (I405/I485) of pHluorin-expressing, permeabilized cells, equilibrated in buffers of increasing pH. (B) Cytosolic pH of prototrophic C. albicans cells exposed to the indicated drugs. The cytosolic pH measurement was performed as described in (3) and calculated according to the calibration curve. Cells treated with 10ng/ml micafungin were used as a control. Data are reported as mean ± SD of three biologically independent experiments with three technical replicates each. Statistical comparisons were performed with Student’s two-tailed t tests (paired). “*” p=0.0025. “**” p=0.0016. “***” p=0.0021. Flu, Fluconazole; Ome, Omeprazole. C, D. The effect of omeprazole and fluconazole on the vacuolar pH. (C) Calibration curve showing measured ratio of fluorescence intensity at 490nm to intensity at 450nm (I490/I450) versus pH of cells pre-incubated with BCECF, subsequently permeabilized and equilibrated in buffers of increasing pH. (D) Vacuolar pH of
prototrophic *C. albicans* cells exposed to the indicated drugs. The vacuolar pH measurement was performed as described in (3) and calculated according to the calibration curve. Cells treated with 10 ng/ml micafungin were used as a control. Data are reported as mean ± SEM and statistical comparisons were performed with Student’s two-tailed t tests. Data are reported as mean ± SD from three biologically independent experiments with three technical replicates each. “*” *p*=0.0175. “**” *p*=0.0109. “NS” *p*=non-significant. Fku, Fluconazole; Ome, Omeprazole.