Introduction

LY 303366, an inhibitor of 1, 3-β-D-glucan synthase, was tested alone, or in co-culture with neutrophils or monocytes, for antifungal activity against *Aspergillus fumigatus* using the XTT metabolism assay. LY 303366 at 0.1 mg/L for 48 h significantly inhibited growth by conidia in a microtest plate XTT assay system. Inhibition was similar if the drug was removed after only 24 h. Microscopically, this correlated with less growth and stunted malformed hyphae. LY 303366 (0.1 mg/L) also inhibited the further growth of germlings (43%) in a 24 h assay. Antifungal activity of neutrophils against 24 h control hyphal growth was limited at an effector:target ratio of 400:1. In co-cultures of neutrophils plus drug with hyphal growth from 24 h LY 303366 cultures, the antifungal activity was additive. Neutrophils had a similar additive effect even if the drug was not present (i.e., when germinating conidia were pretreated with drug). Under conditions where monocytes did not have significant antifungal activity against hyphae, they collaborated with LY 303366 for significantly increased inhibition from 38% by LY 303366 alone to 67% by co-culture. Thus, LY 303366 has activity against germinating or germinated conidia of *Aspergillus*, human effector cells act co-operatively with LY 303366, and LY 303366 can sensitize germinating conidia for damage by host cells.

Materials and methods

A *Aspergillus fumigatus*

A patient isolate of *A. fumigatus* (96–92) was grown on Sabouraud’s dextrose agar slants at 35°C for 24 h, then allowed to form conidia at room temperature for 24–48 h. Conidia were harvested with distilled water, washed once, diluted in saline and counted. Conidia suspensions consisted primarily of single conidia (95%) or small groups of conidia with two or three conidia per group (5%). Over 95% of the conidia germinated when incubated in RPMI 1640 medium (Gibco, Grand Island, NY, USA) at 26°C or 37°C. Each study group was set up in quadruplicate cultures in every experiment.

A *Antifungal drug*

LY 303366 powder was supplied by Eli Lilly and Co., Indianapolis, IN, USA (100 mg/vial). LY 303366 was supplied by Eli Lilly and Co., Indianapolis, IN, USA (100 mg/vial).
suspended in methanol 2 g/L, diluted to 1 g/L with distilled water, sterilized by filtration and stored at 4°C. Diluent controls were prepared in identical manner but without LY 303366.

**XTT**

Inhibition of hyphal growth was measured by the colorimetric XTT–coenzyme Q method. (2,3)-Bis-(2-methoxy-4-nitro-5-sulphenyl-(2H)-tetrazolium-5-carboxanilide) sodium salt (XTT) at 0.5 g/L plus 2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q) at 0.04 g/L in phosphate-buffered saline (PBS) pH 7.4 (Sigma Chemical Co., St Louis, MO, USA) constituted the test solution. Viable cells reduce XTT to a reduced soluble form of XTT with a colour change from yellow to orange.

**LY 303366 assay**

Antifungal activity of LY 303366 alone was tested in several different configurations. LY 303366 was first tested by incubating conidia in RPMI-1640 with or without the drug in 24-well tissue culture plates, microcentrifuge tubes or wells of 96-well microtest plates at 37°C for 24 h. In a second type of test, conidia were first allowed to germinate overnight at 26°C, then germlings (conidia with germ tubes 10–20 times the diameter of a conidium in length) were incubated with or without LY 303366 for 24 h at 37°C.

**Neutrophil assay**

Polymorphonuclear neutrophils (PMN) and peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by 6% dextran-70 sedimentation followed by density gradient centrifugation of theuffy coat diluted 1:1 with RPMI-1640 on Histopaque 1077 (Sigma). The PBMC layers were harvested, washed and counted. The pelleted cells (PMN and some RBC) were collected in 0.85% NH₄Cl to lyse RBC. PMN were washed, counted and suspended in RPMI-1640 plus 10% fresh autologous human serum (referred to as complete tissue culture medium). PMN were added to microtest plate wells containing washed A. fumigatus growth from conidia incubated with RPMI-1640 with or without LY 303366 at 37°C for 24 h. Some cultures received LY 303366 again and all cultures were incubated at 37°C in an incubator (5% CO₂ + 95% air). Microtest plates were centrifuged, supernatants were aspirated and wells were washed twice with 0.2 mL of distilled water. PMN were lyzed or killed as shown by debris and deformed cells, observed by microscopy. XTT test solution (0.2 mL) was added to each well and cultures were incubated at 37°C for 1 h in a CO₂ incubator. An aliquot (0.1 mL) from each well was transferred to corresponding wells of another plate and the absorbance at 410 nm was recorded with a microtest plate reader (Dynatech M R 250, Dynatech Lab. Inc., Chantilly, VA, USA).

**Monocyte assays**

PBMC at 3 × 10⁶/mL in complete tissue culture medium were incubated for 1 or 2 h at 37°C in 60 mm plastic Petri dishes precoated with human serum (2.5 mL of cells per dish). Non-adherent cells were washed away with two washings of warm RPMI-1640 and then 2.5 mL of 0.1 M EDTA in PBS, diluted 1:1 with complete tissue culture medium was added. After 15 min at room temperature, adherent cells were collected, washed, counted and suspended in complete tissue culture medium, as previously described. These cells (monocytes) were added (4 × 10⁵ monocytes/well) to wells containing washed growth of A. fumigatus and the XTT assay was performed as above for PMN.

**Quantification of growth inhibition**

Absorbance of XTT alone at 410 nm (Shimadzu UV-160 spectrophotometer, Shimadzu Corp., K yoto, Japan) was subtracted from absorbance of culture supernatants with metabolized XTT to give the change in absorbance (ΔA) (24-well plate experiments). In 96-well plate experiments a microtest plate reader (MR 250, Dynatech) was used to determine the ΔA at 410 nm. Percent inhibition of growth was calculated by the formula: ([ΔA_control − ΔA_experimental]/ΔA_control) × 100. Since there was a linear relationship between inoculum and metabolism of XTT by respective cultures as measured by change in absorbance, to be detailed, decreased ΔA of XTT supernatants from LY 303366-treated cultures represented inhibition of growth.

**Statistical analysis**

Student’s t-test was used for statistical analysis of the data and significance set at P < 0.05. The GB-STAT program (Microsoft, Redmond, WA, USA) for Bonferroni’s adjustment to the t-test was used where appropriate.

**Results**

**Preliminary XTT assays**

Various configurations were explored for measuring growth of A. fumigatus with the XTT assay. A wide range of conidia inocula were cultured, 1 mL per well of 24-well tissue culture plates for 24 h at 37°C; growth was transferred to microcentrifuge tubes, pelleted and washed at 5000g for 10 min, and then incubated with 1 mL of XTT for 1 h at 37°C. When metabolism of XTT was measured by absorbance at 410 nm there was a linear relationship between inoculum and metabolism of XTT (Figure). The main disadvantage of this method was the difficulty in
Collaboration of phagocytes and LY 303366

completely removing sticky mycelial growth from tissue culture plate wells to the microcentrifuge tubes. Consequently the experiment was performed in 96-well microtest plates. A wide range of conidia inocula were cultured, 0.2 mL per well for 24 h at 37°C; the plates were centrifuged at 800g for 10 min, supernatants were aspirated and growth was washed with distilled water by centrifugation and observed microscopically. Washed growth was assayed in situ by the XTT method. A linear relationship similarly existed between inoculum size and metabolism of XTT over a range of 156–2500 conidia/well.

Preliminary tests of LY 303366

With the 24-well tissue culture plate XTT assay and 2 × 10^5 conidia/well, LY 303366 at 5 mg/L inhibited growth by 70% in the 24 h assay. Inhibition of growth was associated with club-like malformation of hyphal growth when observed microscopically. Similar results (40% inhibition) were obtained with LY 303366 at 5 mg/L and 2 × 10^5 conidia/tube in 1 mL cultures using microcentrifuge tubes and in-situ measurement of XTT metabolism.

In the microtest plate system, using an inoculum of 10^3 conidia/well, growth was inhibited in a concentration-dependent manner by LY 303366 where 2.5 mg/L gave 80% inhibition. If conidia were allowed to germinate at 26°C and then treated with LY 303366 at 37°C for 24 h, 0.1 mg/L inhibited growth by 43%. This indicated that LY 303366 inhibits the hyphal phase of growth as well as growth in the early germination stage. In view of the convenience and utility of the microtest plate system, it was used for co-culture experiments with PMN and monocytes with or without LY 303366.

Antifungal activity of PMN + LY 303366

After conidia and hyphae had been grown at 37°C (with or without LY 303366), cultures were washed and challenged with PMN in complete tissue culture medium with or without LY 303366 for 24 h at 37°C in a CO_2 incubator. The results are given in Table I. LY 303366 at 0.1 mg/L for 48 h significantly inhibited (57%) growth of *A. fumigatus*. Damage by LY 303366 due to exposure for 24 h persisted

![Figure](image-url)

**Figure.** Inoculum size and XTT metabolism by *A. fumigatus* in 24-well tissue culture plates. The number of conidia per well at time zero is given on the x-axis. Conidia in RPMI-1640 were incubated for 24 h at 37°C. The y-axis shows absorbance by 1 mL of metabolized XTT (mean of four samples) measured at 410 nm with a spectrophotometer (Shimadzu UV160). Standard deviation is shown (bars).

**Table I.** Activity of PMN with or without LY 303366, against *A. fumigatus*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P value</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>first 24 h</td>
<td>second 24 h</td>
<td>b</td>
</tr>
<tr>
<td>RPM I-1640</td>
<td>CTCM</td>
<td>0</td>
</tr>
<tr>
<td>LY 303366</td>
<td>LY 303366</td>
<td>57</td>
</tr>
<tr>
<td>LY 303366</td>
<td>CTCM</td>
<td>54</td>
</tr>
<tr>
<td>RPM I-1640</td>
<td>PM N</td>
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</tr>
<tr>
<td>LY 303366</td>
<td>PM N + LY 303366</td>
<td>80</td>
</tr>
<tr>
<td>LY 303366</td>
<td>PM N + CTCM</td>
<td>73</td>
</tr>
</tbody>
</table>

CTCM, complete tissue culture medium. R ef., reference value.

*Study condition: inoculum 10^3 conidia/microtest plate well. LY 303366 0.1 mg/L, PMN 4 × 10^5/well.*

*Growth in control cultures (reference value), compared with growth in experimental cultures. Inoculum = 10^3 conidia/microtest plate well.*

*Growth in LY 303366 cultures for 48 h (reference value), compared with 24 h LY 303366 growth, then PMN + LY 303366.*

*Growth in LY 303366 for 24 h then in CTCM for 24 h (reference value), compared with LY 303366 for 24 h then PMN for the second 24 h.*

*Growth in co-cultures of hyphae and PMN (reference value), compared with growth in co-cultures of PMN + hyphae continuously exposed to LY 303366 (line 5) or exposed to LY 303366 only for first 24 h (line 6).*

*NS: Not significant (P > 0.05).*
for another 24 in absence of the drug as shown by growth inhibition of 54% (Table I). Higher concentrations of LY 303366 (0.5 and 1.0 mg/L) did not significantly increase these effects.

When $4 \times 10^5$ PMN were added per well to hyphal growth (effector:target (E:T) ratio 400:1), PMN caused 29% inhibition of growth (Table I). In two experiments under these conditions, inhibition was 26 ± 4%. Lower numbers of PMN (E:T ratios of 100:1 to 10:1) caused 10–18% inhibition of growth in four experiments (data not shown); this inhibition by PMN alone could occasionally reach significance, depending on the s.d. When $4 \times 10^5$ PMN were added to hyphae that had developed in the presence of LY 303366 (0.1 mg/L), with or without more drug for 24 h, there was an additive inhibition of growth, 80% and 73% respectively (Table I). Higher concentrations of LY 303366 (0.5 or 1.0 mg/L) did not significantly alter these effects. Lower numbers of PMN (1 × 10^5 or 0.5 × 10^5) with LY 303366 at 0.1, 0.5 or 1.0 mg/L gave similar results to those shown in Table I.

Similar results were obtained when PMN from two different donors were used (results pooled). LY 303366 (0.1 mg/L) for 48 h resulted in $38 \pm 9.1\%$ inhibition ($P < 0.01$). PMN ($4 \times 10^5$) alone for 24 h caused $17 \pm 8\%$ inhibition and the combination of LY 303366 and PMN produced an additive inhibition of $51.5 \pm 0.7\%$.

A lower concentration of LY 303366 (0.01 mg/L) did not give an additive effect in this system. When the inoculum of conidia was reduced to 400/well and the number of PMN remained the same ($4 \times 10^5$/well), the collaborative effect of PMN and LY 303366 (0.1 mg/L) was additive, 63% (Table II), i.e. approximately the sum of inhibition by PMN and by LY 303366 alone.

Taken together, these results indicate that LY 303366 causes visual malformation and growth inhibition of A. fumigatus and this correlates with decreased metabolism of XTT, and that previous exposure to LY 303366 sensitizes A. fumigatus for significant damage by PMN.

A antifungal activity of monocytes plus LY 303366

Under the conditions of these assays, monocytes have less antifungal activity against hyphae than against PMN, therefore a ten-fold lower inoculum of conidia was used (100 conidia/well). LY 303366 at 0.1 mg/L for 48 h significantly inhibited growth (38%) (Table III). Monocytes (4 × 10^5/well) alone did not inhibit 24 h hyphal growth when co-cultured for 24 h. These results are specific to the method used; in other experiments (not shown) monocytes alone could be demonstrated to have anti-aspergillus activity under other study conditions (other metabolite assays, plates, times of incubation, and studies where fungal elements were added to adherent monocyte monolayers).

Under the present, stringent conditions, monocytes could collaborate with LY 303366 (0.1 mg/L) and increase inhibition from 38% to 67% (Table III). Monocytes also collaborated with LY 303366 (0.01 mg/L) by increasing inhibition from 7% to 37% in co-cultures (Table III). Similar results were found in a second identical experiment with monocytes from a different donor, e.g. monocytes (0% inhibition) collaborated with LY 303366 (0.1 mg/L) (35% inhibition) to give 50% inhibition of growth.

**Table II. A n t i f u n g a l i n t e r a c t i o n s o f P M N a n d L Y 3 0 3 3 6 6 a g a i n s t A . f u m i g a t u s h y p h a e s**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Change in absorbance at 410 nm</th>
<th>% Inhibition</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640</td>
<td>CTCM</td>
<td>0.385 ± 0.092</td>
<td>0</td>
</tr>
<tr>
<td>LY 303366</td>
<td>LY 303366</td>
<td>0.177 ± 0.021</td>
<td>54</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>PMN</td>
<td>0.354 ± 0.090</td>
<td>8</td>
</tr>
<tr>
<td>LY 303366</td>
<td>PMN + LY 303366</td>
<td>0.140 ± 0.005</td>
<td>63</td>
</tr>
</tbody>
</table>

Ref., reference value.

a Study conditions: inoculum 400 conidia/well, LY 303366 0.1 mg/L, PMN 4 = 10^5/well.

b Mean ± s.d. of change in absorbance by metabolized XTT from quadruplicate cultures.

c Growth in control cultures (reference value), compared with that in experimental cultures.

d Growth in LY 303366 cultures (reference value), compared with that in PMN plus LY 303366 cultures.

e Growth in PMN cultures (reference value), compared with that in PMN plus LY 303366 cultures.
We speculate that the altered cell wall resulting from drug exposure makes the fungal cell more susceptible to effector cell activity, including oxidative metabolites.

multiple parameters can be easily tested; it also allows for objectivity and statistical analysis.

The antifungal activity of neutrophils against hyphae of A. fumigatus in short-term 2 h challenge experiments has been measured using the MTT and XTT assays. We have used this method for measuring antifungal activity of neutrophils in long-term 24 h experiments against hyphae. We defined antifungal activity in these experiments as percentage inhibition of growth instead of hyphal damage as for 2 h challenge experiments. U under these very challenging conditions, neutrophils inhibited mycelial growth. However, even compared at the same E:T ratios, PMN activity alone was variable between experiments; aspergillus strain, PMN donor and germling size (which we found could vary after 24 h incubation) are possible factors. A t high or low levels of PMN activity, additive effects with drug were seen. On the other hand, monocytes, even against a ten-fold smaller inoculum, failed to inhibit mycelial growth.

To simulate in vivo therapeutic conditions, hyphae exposed to antifungal activity of LY 303366 were co-cultured with neutrophils and the combined antifungal activity was measured. The results showed that the antifungal activity of this combination was additive. On the other hand, monocytes co-cultured with hyphae previously exposed to LY 303366 resulted in antifungal activity greater than the sum of the individual activities. These novel findings help explain the efficacy of this class of drugs in models of infection with A. fumigatus, despite the absence of killing by drug alone in vitro. We speculate that the altered cell wall resulting from drug exposure makes the fungal cell more susceptible to effector cell activity, including oxidative metabolites.

R eferences


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