Efficacy of a Fixed Combination of Tetracycline, Chloramphenicol, and Colistimethate Sodium for Treatment of Candida albicans Keratitis

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PURPOSE. To evaluate the antifungal activity of a fixed antibiotic combination (AC) containing tetracycline (TET), chloramphenicol (CAF), and colistimethate sodium (CS).

METHODS. In vitro: Candida ATCC and clinical strains were used. The minimum inhibitory concentrations (MICs) of AC and of each antibiotic were determined. Fluconazole (FLC) was tested for comparison. Time-killing curves of selected strains were performed. Ex vivo keratitis: corneas were injected intrastromally with the selected strains. After the injection, corneas were divided into groups of treatments: AC, FLC, or saline. Then, the tissues were analyzed for colony-forming units per gram (CFU/g). Propidium iodide (PI) and MitoTracker (MTR) staining were used to investigate the mode of action.

RESULTS. Values of MIC required to inhibit the growth of 90% of organisms for the antibiotics alone were higher than FLC. However, their activity was enhanced when used in combination against Candida yeasts. Time-killing curves showed that at 24 hours, AC reduced the load of both strains of approximately 1 Log10 CFU/g compared with the initial inoculum (P < 0.0001). This effect was also significant versus FLC. In ex vivo, AC was effective in decreasing the loads of both strains by 4 Log10 CFU/g with respect to the control. Moreover, it showed higher activity than FLC against Candida albicans ATCC 10231 (1 Log10 CFU/g, P < 0.01 versus control). PI staining demonstrated that CS changed the membrane’s permeability, whereas MTR staining demonstrated that TET or CAF altered mitochondrial function. The cells treated with AC and stained showed both effects.

CONCLUSIONS. In this study, AC showed antifungal efficacy versus Candida spp.; this activity can be due to the synergistic effects of antibiotics in it.

Keywords: Candida, tetracycline, chloramphenicol, colistimethate sodium, fungal keratitis, mode of action

Myotic keratitis, commonly known as fungal keratitis, accounts for approximately 1% to 44% of all cases of microbial keratitis, depending on the geographic location.1,2 The genera that commonly cause infection of the cornea include Fusarium, Aspergillus, Curvularia, Bipolaris, and Candida.1–3 Among the Candida species, Candida albicans is the most common etiologic agent of keratitis.4 In this form of keratitis, one or more ocular (e.g., insufficient tear secretion, defective eyelid closure) or systemic (e.g., diabetes mellitus, immunosuppression) conditions predispose to the infection. This form of mycotic infection also may supervene on a preexisting epithelial defect due to herpes keratitis or due to abrasions caused by contaminated contact lenses.5

Management of fungal keratitis largely involves a decision on which antifungal to use and the route of administration. Most of the currently available antifungal medications have limitations, such as poor bioavailability and limited ocular penetration, especially in cases with deep-seated lesions.6–8 These factors, particularly especially in cases of severe fungal keratitis, account for the slow resolution of fungal infections, with most cases finally requiring a therapeutic penetrating keratoplasty (PKP).8

Clinically, the commercially available Natamycin 5% suspension is the initial drug of choice for fungal keratitis. If worsening of the keratitis is observed on topical Natamycin, Amphotericin B (amp B) can be substituted, although topical azoles (e.g., fluconazole [FLC] and voriconazole) are considered to be a good alternative to amp B for the treatment of Candida keratitis. They have better ocular penetration and are less toxic to the corneal epithelium, compared with amp B.9–11

The clinician must determine the length of treatment for each case based on clinical response and experience. Treatment with a systemic antifungal agent is recommended in cases of severe deep keratitis, scleritis, and endophthalmitis. Systemic antifungals are also used after PKP for fungal keratitis.9

Improvement of the antifungal arsenal is needed because existing antifungals can be associated with limited efficacy, toxicity, and resistance.12 The emergence of resistant fungal strains to current antifungals, which is exacerbated by the
necessity for long-term usage of antifungal in immunocompro-
mised individuals, causes additional difficulty in treatment. 13
Recent scientific studies have reevaluated old antibiotics,
such as chloramphenicol, tetracyclines, and polymyxins,
traditionally used for bacterial infections, for their potential
antifungal activity.14–17
Based on literature and clinical experience, in this study, we
evaluated the antifungal activity of a widely used antibacterial
ophthalmic combination (Colbiocin; SIFI SpA, Catania, Italy)
containing tetracycline (TET), chloramphenicol (CAF), and
colistimethate sodium (CS), using in vitro tests and an ex vivo
mycotic keratitis model. Specific assays were also carried out to
understand the mechanism of action.

MATERIALS AND METHODS
Antimicrobial Agent
Fixed antibiotic combination (AC) (Colbiocin; SIFI SpA)
contained CAF (4 mg/mL), TET (5 mg/mL), and CS (4.14 mg/
ML). Fluconazole (FLC) was obtained from Sigma-Aldrich,
Milan, Italy; CAF from Quimica Sintetica S.A., Madrid, Spain;
TET from Ningxia Qiyuan Pharmaceutical Co., Ningxia, China;
and CS from Xellia Pharmaceuticals APS Dalslandsградe,
Copenhagen, Denmark.

Strains
The following strains, obtained from Italian hospitals in
Messina and Catania, were used for the antimicrobial testing:
Candida albicans ATCC 2091, C. albicans ATCC 10231, and
14 clinical isolates of C. albicans (n = 7), Candida glabrata (n =
5), Candida utilis, and Candida tropicalis. The yeasts were
stored at -70°C in Microbanks vials (DID; Pro-Lab Diagnostics,
Ontario, Canada).

In Vitro Study
Antifungal Susceptibility Testing. Drug susceptibility
was determined using the Clinical and Laboratory Standards
Institute microbroth dilution protocol. 18.19 Cultures for anti-
fungal activity tests were grown in RPMI-1640 medium
supplemented with MOPS (Oxoid, Milan, Italy) containing
tetracycline (TET), chloramphenicol (CAF), and colistimethate sodium (CS), using in vitro tests and an ex vivo
mycotic keratitis model. Specific assays were also carried out to
understand the mechanism of action.

for two selected strains: C. albicans ATCC 10231 and C.
albicans n. 4 clinical isolate. FLC was tested for comparison.
Yeast suspensions were prepared to yield final inoculum of
approximately 2 x 10^6 CFU/mL. Microplates were read after
48/72 hours of incubation at 35°C. Each test was performed in
triplets. MICs were determined using the fractional
inhibitory concentration index (FICI), which was calculated
as follows: FICI = (FLC + FICB [or FICC]), where FICA = (MIC
of compound A in the presence of compound B)/(MIC
of compound A alone). Similarly, the fractional inhibitory
concentration for compound B (or compound C) was
calculated. An FICI value of ≤ 0.5 was interpreted as synergy,
whereas the FICI values between 0.5 and 1.0 were interpreted
as additive. FICI values > 4.0 were considered as antagonism
and FICI values between 1.0 and 4.0 were considered as
indifferent. 20

Time-Killing Curve. Time-killing curves for C. albicans
ATCC 10231 and C. albicans n. 4 clinical isolate were
performed at 10 times MIC values of AC and FLC. Yeast
suspending fluid were prepared to yield final inoculum of
approximately 5 x 10^5 CFU/mL. At predetermined time
points (0, 2, 4, 6, 8, 10, and 24 hours), a 0.1-mL aliquot was
removed from the control tube (drug free) and from the tube
with AC or FLC for each strain. Serial dilutions in saline were
performed. Volumes of 0.1 mL were spread onto SDA plates
and incubated at 35°C for 24 to 48 hours to determine the
critical numbers of CFU/mL. All time-kill curve studies were
conducted in triplicate.

Propidium Iodide Staining. To analyze the membrane
integrity, fraction of surviving cells of C. albicans ATCC 2091
and C. albicans n. 4 exposed to AC and each antibiotic
were stained with propidium iodide (PI) solution (Sigma-Aldrich).
Control samples of both strains were performed for compar-
ison. Briefly, treated and control cells (10^5 cells/mL) were
washed and suspended in PBS (pH 7.0). To this cell
suspension, PI solution (stock solution 1 mg/mL) was then
added to these cell suspensions that were later incubated for
10 minutes at room temperature. Cells were again washed to
remove the excess of the stain and examined under the
inverted microscope Axio Observer.Z1 with ApoTome.2 (Zeiss,
Milan, Italy).

MitoTracker Staining. To detect permeability changes of
mitochondrial membrane, fraction of surviving cells of C.
albicans ATCC 2091 and C. albicans n. 4 exposed to AC
and each antibiotic were stained with mitochondrion-specific dye
MitoTracker RedCMXRos (MTR) (Invitrogen, Fisher Scientific
Italia, Rodano-MI, Italy) according to the manufacturer’s
instructions. Control samples of both strains were performed
for comparison. Treated and control cells (10^5 cells/mL) were
collected by centrifugation and suspended in fresh medium
with the mitochondrion-specific dye MTR at a final concentra-
tion of 50 nM.18,21 Cells were incubated for 15 minutes at 35°C
in the dark. Stained cells were washed three times with PBS in
the dark and immediately observed using the above-mentioned
microscope. MTR has excitation and emission peaks at 579
and 599 nm, respectively.

Ex Vivo Study
Preparation of Inocula. The following strains were used:
C. albicans ATCC 2091 and C. albicans n. 4. The yeasts were
cultured in RPMI-1640 medium supplemented with MOPS at
35°C for 24 hours and then, a few colonies of each strain were
washed three times with PBS to reach a density of 5 x 10^6 CFU/
/mL (V-1200-VWR, Milan, Italy) and then diluted to a final
concentration of the inoculum (5 x 10^4 CFU/mL).

Rabbit Globe Harvest. Normal rabbit eyes, obtained from
a local abattoir, were enucleated immediately following
Table 1. MIC Values, μg/mL.

<table>
<thead>
<tr>
<th>Strains</th>
<th>CAF</th>
<th>TET</th>
<th>CS</th>
<th>FLC</th>
<th>AC, Dilutions</th>
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<tr>
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<td>250</td>
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<td>1:10</td>
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<td>&gt;2000</td>
<td>1.25</td>
</tr>
<tr>
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<td>250</td>
<td>&gt;2000</td>
<td>1.25</td>
<td>1:10</td>
</tr>
<tr>
<td>C. albicans 13</td>
<td>&gt;2000</td>
<td>500</td>
<td>&gt;2000</td>
<td>1.25</td>
<td>1:10</td>
</tr>
<tr>
<td>C. albicans 15</td>
<td>2000</td>
<td>500</td>
<td>&gt;2000</td>
<td>1.25</td>
<td>1:10</td>
</tr>
<tr>
<td>C. albicans 16</td>
<td>&gt;2000</td>
<td>500</td>
<td>&gt;2000</td>
<td>2.5</td>
<td>1:10</td>
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<tr>
<td>C. albicans 18</td>
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<td>500</td>
<td>&gt;2000</td>
<td>2.5</td>
<td>1:10</td>
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<tr>
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<td>500</td>
<td>&gt;2000</td>
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<td>&gt;2000</td>
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<td>500</td>
<td>&gt;2000</td>
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<tr>
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<td>&gt;2000</td>
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<td>500</td>
<td>&gt;2000</td>
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<td>&gt;2000</td>
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<tr>
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<td>1:5</td>
</tr>
</tbody>
</table>

AC: Fixed antibiotic combination containing CAF, TET, and CS, in the fixed ratio 1:1:3.

The results are expressed as means ± SDs from three experiments and statistically analyzed by a 1-way ANOVA.
(1:10 vol/vol) demonstrated similar results. The images showed only a shadow of cells lacking functional mitochondria. Moreover, the staining highlights the morphologic changes of yeast cells treated with AC and antibiotics with respect to the control cells. AC, TET, and CAF effects on mitochondrial function of \textit{C. albicans} ATCC 10231 are shown in Figure 3. The effects on mitochondrial function of \textit{C. albicans} n. 4 was similar to that of \textit{C. albicans} ATCC 10231, therefore not shown.

**Ex Vivo Study**

\textbf{C. albicans Growth Curve.} The growth curves of \textit{C. albicans} ATCC 10231 and \textit{C. albicans} n. 4 strains were superimposable. The mycotic load obtained from the corneas after intrastromal injection was $3.5 \pm 0.5 \log_{10}$ CFU/g. After 24 hours from the fungal challenge, the load increased approximately $4 \log_{10}$ CFU/g, remained almost unchanged for up 72 hours, and then decreased (data not shown).

\textbf{Treatment of \textit{C. albicans} Keratitis.} The AC was effective in the ex vivo rabbit keratitis experiments in decreasing the load of \textit{C. albicans} ATCC 10231 and \textit{C. albicans} n. 4. Therefore, AC significantly reduced the load of both \textit{C. albicans} strains by $4 \log_{10}$ CFU/g with respect to the control after six doses, up to 24 hours after infection ($P < 0.001$). Moreover, AC showed higher activity than FLC against \textit{C. albicans} ATCC 10231 (approximately $1 \log_{10}$ CFU/g) ($P < 0.01$). Similar efficacy against \textit{C. albicans} n. 4 was observed (Fig. 4).

**DISCUSSION**

Because fungi are eukaryotic cells, they share many pathways with human cells, thus increasing the probability of antifungal activity of “nonfungal drugs.” In the past few years, there has been an increased interest in revived antibiotics. Old drugs that have been recently revived include colistin, temocillin, fosfomycin, mecillinam, nitrofurantoin, and chloramphenicol for multidrug-resistant gram-negative bacteria and trimethoprim-sulfamethoxazole for methicillin-resistant \textit{Staphylococcus aureus}. Among these, colistin and chloramphenicol also demonstrated antifungal activity against yeasts.\textsuperscript{14,15,21,26} In this study, we found that CAF, TET, and CS used alone have weak, if any, antifungal activity against several \textit{Candida} yeasts with respect to FLC, but that this activity is highly enhanced when they were used as AC in fixed combination.

In this study, we found that CAF, TET, and CS used alone have weak, if any, antifungal activity against several \textit{Candida} yeasts with respect to FLC, but that this activity is highly enhanced when they were used as AC in fixed combination. The underlying mechanism of each antibiotic against yeasts may be explained as follows: TET and CAF promote mitonuclear protein imbalance and mitochondrial dysfunction, CS binds lipopolysaccharide and anionic phospholipids in the bacterial cell membrane, disrupting membrane integrity.\textsuperscript{16,17}

The mechanism of action for tetracycline and its derivative doxycycline is the inhibition of translation through binding to the bacterial 30S ribosomal unit. This specificity for a bacterial
component has led to an expectation that tetracycline does not affect eukaryotic cells. However, tetracycline leads to a state of so-called mitonuclear protein imbalance, which disturbs mitochondrial proteostasis and inhibits mitochondria function. The mitonuclear protein imbalance is accompanied by a strong decrease in cellular respiration, indicative for severely impaired mitochondrial activity. Moreover, tetracycline eliminates the diauxic shift. The lack of diauxic shift or the lack of a functional mitochondria alters sterol metabolism resulting in lower ergosterol levels.

Chloramphenicol is a known inhibitor of mitochondrial translation in eukaryotes, which binds to the A site and occupies the same position as the aminoacyl-tRNA (aa-tRNA), preventing protein synthesis in prokaryotes. Ribosomal similarities between bacteria and mitochondria may provide the basis for mitochondrial sensitivity to chloramphenicol-mediated inhibition of protein synthesis. Expression of the transferritin receptor seems to be the most relevant to the chloramphenicol-mitochondrion interaction. Specifically, chloramphenicol diminishes mitochondrion-based transferritin

**FIGURE 3.** AC, TET, and CAF effects on mitochondrial function by MTR staining. (A) Cells were incubated with TET at sub-MIC concentration (125 µg/mL) for 24 hours before staining. (B) Cells were incubated with CAF (1000 µg/mL) for 24 hours before staining. (C) Cells were incubated with AC (1:10 vol/vol) for 24 hours before staining. (D) Control, untreated cells at the same time. Cells were observed and photographed using inverted fluorescence microscopy.

**FIGURE 4.** Efficacy of AC treatment against *C. albicans* ATCC 10231 or *C. albicans* n. 4 clinical isolate. Mean Log_{10} CFU/g (± SD) change in *C. albicans* ATCC 10231 or *C. albicans* n. 4 loads of AC versus FLC treated or control group in corneal tissue ("P < 0.01 versus FLC, ***P < 0.001 versus control).
receptor expression, resulting in ferritin depletion in mitochondria.30

Polymyxins bind lipopolysaccharide and anionic phospholipids in the gram-negative bacterial cell membrane, disrupting membrane integrity.11 Polymyxins are cationic cyclic heptapeptides with a hydrophobic tail that interacts with the bacterial cytoplasmic membrane, therefore changing its permeability and triggering cell death.26 Weak anti-fungal activity of colistin and polymyxin B against several fungi has already been reported31–33 As hypothesized by Zhai et al.14 in activity of colistin and polymyxin B against several fungi has

stromal-components.34 as do the intracellular innate immune molecules and cellular-immune elements, the three-dimensional architecture remains, to in vivo animal testing. Although the ex vivo models lack the potential to be used as a mechanistically based alternative shown to reduce the insertion of cationic peptides into anionic sterols in the eukaryotic membrane, as sterols have been compared with bacteria could be partly due to the presence of sterols in the eukaryotic membrane, as sterols have been shown to reduce the insertion of cationic peptides into anionic mixed membranes to form pores.

Moreover, these results indicate that the combination of corneal organ culture and experimental microbial keratitis has the potential to be used as a mechanistically based alternative in vivo animal testing. Although the ex vivo models lack immune elements, the three-dimensional architecture remains, as do the intracellular innate immune molecules and cellular-stromal-components.34

In conclusion, we showed that AC containing the three antibiotics in fixed combination has a high efficacy against Candida spp., in both in vitro and in ex vivo models. The effect reported can be due to the different modes of action of the three antimicrobial agents used in combination: the CS increases the permeability of the yeast membrane allowing the subsequent penetration of TET and CAF, which alter mitochondrial function. AC has been widely used on the Italian market and other European countries as an eye drop/ointment to treat bacterial eye infections for 50 years. Based on these results, we can assume that AC has the potential to be used clinically as drug of first choice when the diagnosis of infectious keratitis caused by bacteria or fungi is unclear. Specific diagnostic tests are needed to rule out the condition and, if necessary, progress to appropriate treatment. Further studies are, however, needed on other yeast and mold strains, such as Fusarium and Aspergillus, to extend these findings.

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