[Frontiers in Bioscience 13, 6893-6904, May 1, 2008]

Isolation and cytotoxicity of low-molecular-weight metabolites of *Candida albicans*

Ivan Kosalec[1], Olivier Puel[2], Marcel Delaforge[3], Nevenka Kopjar[4], Roberto Antolovic[5], Dubravko Jelic[5], Biserka Matica[6], Pierre Galtier[2], Stjepan Pepeljnjak[1]

[1]Institute of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Schrottova 39, Zagreb, Republic of Croatia, [2]Groupe Immuno-mycotoxicologie, Laboratoire de Pharmacologie-Toxicologie, Institut National de Recherche Agronomique, Toulouse, France, [3]CNRS URA 2096, CEA Saclay DSV/DRM/SPI, Gif sur Yvette, France, [4]Institute for Medical Research and Occupational Health, Ksaverska cesta 2, Zagreb, Republic of Croatia, [5]GlaxoSmithKline Zagreb Research Center, Prilaz baruna Filipovica 29, Zagreb, Republic of Croatia, [6]Zagreb Public Health Institute, Department of Microbiology, Mirogojska cesta 16, Zagreb, Republic of Croatia

TABLE OF CONTENTS

1. ABSTRACT

In this study, the low molecular weight lypophilic metabolites of *C.albicans* and *C. dubliniensis* strains produced in a synthetic medium with the addition of fetal calf serum were identified using LC/MS and MS/MS technique and quantified. All strains investigated produce a metabolite with a UV spectra maximum at 224 and 279 nm and minimum at 243 nm. Following comparison with ESI, MS/MS spectral data of a reference compound, the metabolite was identified as 3-indoleethanol (tryptophol). The concentration of extracellular tryptophol in the biosynthesis of *C. albicans* and *C. dubliniensis* ranged from 2.45 μg/mL to 191 μg/mL, respectively. Contrary to previously published data, gliotoxin or gliotoxin-like compounds were not detected, and all investigated *C. albicans* and *C. dubliniensis* strains have the same metabolite profile. Cytotoxic effects of tryptophol and 3-indolelactic acid (precursor of tryptophol biosynthesis) were cell-line-dependent. The EC_{50} of tryptophol ranged between 2 and 7 mM, with the EC_{50} of 3-indolelactic acid approximately double (between 4 and 8 mM). Tryptophol exhibited cell-type dependent cytotoxicity in relatively high concentrations, with domination of apoptosis.

2. INTRODUCTION

Candida species are yeasts that exist as a commensal microflora of human and animals. They colonize the mucosal surfaces of the oral and vaginal cavities, the intestines and can be found on skin (1, 2). Opportunistic mycoses caused by *Candida* spp. are infrequent in immunocompetent individuals, where yeasts mainly cause superficial mycoses such as vulvovaginal and oropharingeal candidiasis or chronic mucocutaneous candidiasis. Changes in the host are generally required for opportunistic pathogens to change from harmless commensals to invasive pathogens (3). The factors affecting opportunistic candidosis include: use of antibiotics, steroids and chemotherapy, lymphoma or hematologic malignancy, allogeneic transplantation, HIV-infection and iatrogenic immunosuppression (4). *Candida* infections have increased dramatically over the past three decades in patients undergoing

immunosuppressive antineoplastic therapy or therapy associated with organ transplantation, cancer-patients, long-term catheterization, and use of broad-spectrum antibiotics, mainly in the population of immunocompromised patients (5-8).

Among the species of the genus *Candida*, polymorphic *C. albicans* is generally the most common species, causing mycoses above a range of other species including *C. tropicalis, C. glabrata, C. krusei, C. kefyr, C. parapsilosis, C. guillermondii, C. famata, C. dubliniensis*, etc. (8, 9).

The switch from harmless commensalisms into an invasive pathogenic state of *Candida* spp. depends mainly on the nature of the underlying host defect. With the exception of host factors, several virulence factors have been attributed to *Candida* spp. which contribute to change from commensalism to disease. Virulence attributes of *Candida* spp. include adhesion, as the first and essential step in the establishment of disease. *Candida* blastospores can also adhere to surfaces of medical devices and form biofilms, which results in an increase of candidemia and antifungal resistance related to catheter insertion (10-12). Polymorphism, as the ability to undergo reversible morphogenetic transitions between yeast, pseudohyphae, and hyphae and a feature unique to *C. albicans* and *C. dubliniensis* species among all the *Candida* spp., has been shown to be important for its pathogenicity in systemic infections (13-16). Secretion of extracellular hydrolytic enzymes such as aspartyl proteinases (17-19), phospholipases (20), lipases (21) and hemolysins (22-25) were described as virulence factors of *Candida* species. These factors likely contribute to pathogenicity, as some enzymes degrade proteins at lesion sites (such as albumin, hemoglobin, keratin, IgA, etc.) (33). Colonies of *C. albicans* can switch among different phenotypes, though the mechanism of switching and the involvement of this switching and virulence remains unclear (26-28).

The production of low-molecular-weight (LMW) metabolites of secondary fungal metabolism (mycotoxins) among certain filamentous fungi and its toxicity on mammals has been described, and the production of some toxic LMW during mycoses has been demonstrated (29-30). In the group of medically important yeasts of *Candida* species, the production of LMW metabolites and its toxic role on host cells have been poorly studied, and therefore this study addresses the production of LMW metabolites and its cytotoxic activity on different cell lines *in vitro*.

3. MATERIALS AND METHODS

3.1. Source of *Candida albicans* and *C. dubliniensis* strains and species identification

A total of 64 strains of *Candida albicans* and 6 strains of *C. dubliniensis* were analyzed for the presence of LMW metabolites. Half the *C. albicans* strains examined were clinical isolates collected from patients with vaginitis, urinary tract infections, or respiratory tract infections. Commensal isolates of *C. albicans* strains were isolated from the mouth or stool specimens of healthy donors undergoing regular examinations in the Zagreb Public Health Institute. *C. dubliniensis* strains were donated by Prof. Derek Sullivan (Trinity College, Dublin).

The yeasts were identified as follows: *Candida albicans* were identified using phenotypic characteristics on Sabouraud 2% (*w/V*) -glucose agar (Fluka, Germany), chlamydospore formation on rice-polisorbate 80 agar (Fluka, Germany), and "germ-tube" formation in fresh human serum (Fluka, Germany) according to Larone (31). All yeasts presumptively identified as *C. albicans* were plated on Pal's-agar (sunflower-seed agar) according to method described by Al Mosaid *et al.* (32) and incubated for 72h at 25±2°C, and on Sabouraud 2% (*w/V*)–glucose agar for 18h at 45±1°C for possible misidentified *C. dubliniensis* strains. Identification of *C. albicans* was also confirmed using Candida ID agar (bioMérieux, France) and Api Candida (bioMérieux, France).

3.2. *In vitro* biosynthesis and extraction of low-molecular-weight (LMW) metabolites

Prior to analysis, all strains of *C. albicans* and *C. dubliniensis* were maintained on potato-dextrose agar (Fluka, Germany), and then incubated for 24h at 37°C. After incubation, the inoculum was prepared by sampling 10 yeast colonies with a loop, which was then transferred to 5 mL sterile physiological solution. The inoculum was vortexed, and 1 mL was transferred to plastic Roux flasks (250 mL) with 100 mL of sterile minimal essential medium Eagle (MEM, Sigma, Germany) with 5% (*V/V*) fetal calf serum (Sigma, Germany). *C. albicans* and *C. dubliniensis* strains were incubated for six days at 37°C, in two groups: one in an incubator under normal atmospheric conditions and the other in an humified incubator with 5% CO_2.

At the end of the incubation period, the LMW metabolites from biosynthesis were extracted with 70 mL chloroform (Fischer Scientific, USA). The mixture was centrifuged for 30 minutes at 5200 rpm, and the chloroform fraction was evaporated in a vacuum at 50°C. The dry extract was dissolved in 200 µL methanol (Fischer Scientific, USA), filtrated through a 0.45 µm filter, and 20 µL was injected into a chromatographic column.

For the LMW-metabolites production kinetics, *C. albicans* MFBF* 40 (isolated from clinically manifested vulvovaginitis) was inoculated in MEM at 37ºC and the production of LMW metabolites was quantified every 18 hours (over 7 days) following extraction of biosynthesis. Production kinetics was conducted in triplicate, and mean ± standard deviation of LMW metabolites was calculated.

3.3. High-performance liquid chromatography (HPLC) and mass spectrometry (MS/MS) analysis of LMW

3.3.1. HPLC-DAD analysis

Lipophylic LMW metabolites analysis was performed by high pressure liquid chromatography with diode-array detection (Kontron Instruments, Milan, Italy) and a 150 mm x 4 mm Zorbax ODS 5 µm C_{18} column (Bischoff, Germany). HPLC analysis was performed with a linear elution gradient by using 0.2% (*V/V*) acetic acid (Fischer Scientific, USA) (solvent A) and acetonitrile

(Fischer Scientific, USA) (solvent B) according to the method described by Frisvad (33). The flow rate was set to 2 mL min^{-1}.

3.3.2. Mass spectrometry analysis

The HPLC-MS-MS instrument used was a LCQ DUO Ion Trap coupled with a HPLC system from Thermo-Finnigan (San Jose, CA, USA). HPLC was performed on a reverse phase column 150 × 2.1 mm Kromasil $5C_{18}$ column. Twenty µL of the methanol solution was injected directly into the HPLC system. Gradient chromatography (run of 33 min) was performed with 10 mM ammonium acetate in water (eluent A)-methanol (eluent B) as the mobile phases at a flow rate of 0.2 mL min^{-1}. The program initiates with 90/10 eluent A/B for 3 min, then eluent B was increased to 90% within 22 min, then held constant for 5 minutes, and finally returned to 90/10 eluent A/B for 3 min and allowed to re-equilibrate for 5 min. HPLC-MS measurements were performed using the electrospray ionization mode (ESI). ESI was performed at room temperature in a negative mode; tension was maintained at 4.5 kV with a capillary temperature of 250°C. MS and MS-MS adjustments were made with tryptophol. The collision energy was set at 35%.

3.4 Chemical standards and quantification by HPLC

Tryptophol (3-indoleethanol), DL-3-indolelactic acid and gliotoxin were purchased from Aldrich (Germany) and DL-tryptophan were purchased from Fluka (Germany). Before HPLC analysis, standards were dissolved in methanol to a final concentration 10 µg/mL and filter-sterilized through a 0.45 µm pore-size filter. Quantification of tryptophol produced in biosynthesis was performed with a one-fifth concentration mixture of tryptophol standard in methanol (from 1 mg/mL to 0.1 µg/mL), and the peak area vs. concentration at 270 nm was calculated. The concentration curve (R^2>0.99) was used for calculation of tryptophol.

4. CYTOTOXICITY SCREENING

4.1. Cell lines and culture conditions

The five different cell lines used in the cytotoxicity assay were purchased from the European collection of cell lines – ECACC (strain number in brackets): human monocyte leukemia THP 1 (ECACC 88081201), human Caucasian hepatocyte carcinoma (epithelial) Hep G2 (ECACC 86012804), Chinese hamster ovary (epithelial) CHO (ECACC-85050302), human Caucasian lung carcinoma (epithelial) A549 (ECACC 88081201) and fibroblasts of African green monkey COS-7 (ECACC 87021302). All cells were maintained in Dulbecco's modified Eagle's Medium (Sigma, Germany) with 10% (*V/V*) of fetal calf serum (Sigma, Aldrich), penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL) at 37ºC and humified with 5% CO_2, after removing cells from liquid nitrogen stock.

4.2. The MTT cytotoxicity assay

Compounds were examined for antiproliferative or cytotoxic continuous drug exposure by the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, USA), which is a non-radioactive colorimetric assay for measuring the number of viable cells in proliferation and cytotoxicity assays. Both adherent and suspension cells may be analyzed with this system. Compounds were dissolved in DMSO, and added to the microtiter plate wells in final concentrations: 20, 10, 5, 2.5, 1.25, 0.63, 0.31 and 0.16 mM (tryptophol and 3-indolelactic acid), or 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 µM (gliotoxin). Final dilution of DMSO did not exceed 1%. Test was performed on a defined set of different cell lines and each well contained from 50000 to 75000 (in the case of THP-1) cells. Cultures exposed to compounds, or 10% DMSO as a control, were incubated for 24 h. After exposure time, a 15 µL of the Dye Solution, containing the tetrazolium salt MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide), was added directly to the cells and is internalized and reduced into an insoluble formazan product by cellular metabolism (succinate dehydrogenase mitochondrial activity). Only those cells which are living at the time the dye is added will significantly reduce the MTT. The Solubilization Solution is added to lyse the cells and dissolve the formazan dye product. After 1-4 incubation hours, samples are read in a spectrophotometric 96-well plate reader (Ultra, TECAN, USA) at 490 nm (34). The intensity of the color that appears is directly proportional to the number of viable cells. Method was programmed for TECAN robotic system in GEMINI pipetting software (TECAN, USA), and the results were expressed as percentage of OD values of non-treated cells and as IC_{50} values

4.3. Assessment of cell viability, apoptosis and necrosis in human lymphocyte *ex situ*

4.3.1. Blood sampling

A blood sample was obtained from a healthy male donor (age 35 years, non-smoker) who gave his informed consent for participation in the study. The donor had not been exposed to diagnostic or therapeutic irradiations or to known genotoxic chemicals for a year before blood sampling. Venous blood (20 ml) was collected under sterile conditions in heparinised vacutainer tubes (Becton Dickinson) containing lithium heparin (Sigma, Germany) as an anticoagulant.

4.3.2. Test chemical

Tryptophol was dissolved in ethanol (40% water solution) to a final concentration of 64 mg/mL. The concentrations tested in the *in vitro* experiment corresponded to 2.00 mM, 1.00 mM, 0.50 mM and 0.25 mM.

4.3.3. Experimental procedure

Two independent experiments were performed for each concentration tested. Aliquots of freshly drawn venous blood were placed in sterile Falcon tubes and mixed with RPMI-1640 culture medium (Gibco, USA). They were incubated *in vitro* for 24h at 37°C in an humified atmosphere with 5% CO_2. Tryptophol was added to achieve final concentrations of 2.00 mM, 1.00 mM, 0.50 mM and 0.25 mM. The solvent (40% ethanol) and negative controls were incubated in parallel. Following treatment, the culture

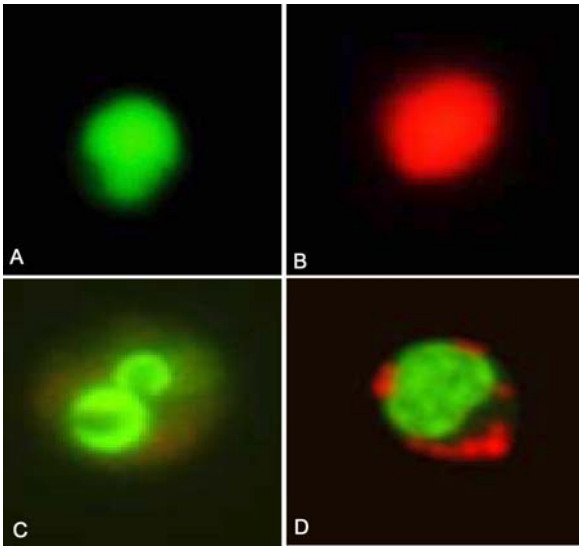

Figure 1. Appearance of lymphocyte nuclei following staining with ethidium bromide and acridine orange according to the fluorescent dye exclusion method: viable normal cells excluded ethidium bromide and their nuclei were bright green with an intact structure (a). Non-viable cells had orange to red colored chromatin with organized structure (b). Apoptotic cells (c, d) were bright green with highly condensed or fragmented nuclei.

medium containing tryptophol was carefully removed by centrifugation (10 min, 600 rpm) and the cells were washed twice in fresh RPMI-1640 medium.

4.3.4.. Lymphocyte isolation

Lymphocytes were isolated using a modification of the Ficoll-histopaque centrifugation method (35). Samples mixed with RPMI medium (1 mL) were placed in a 2.0 mL microfuge tube. Then, 500 µL of cold lymphocyte separation medium (Sigma) was layered at the bottom of the tube using a pipetman, and RPMI 1640 was added up to 2 mL. The sample was centrifuged at 4500 rpm for 3 min in a microfuge at room temperature. The lymphocytes in the upper part of the ficoll layer were pipetted out. Cells were washed twice in 0.5 mL of RPMI-1640, using centrifugation for 3 min at 4500 rpm in the microfuge. The final pellet was gently resuspended in RPMI-1640 and used in the assessment of cell viability.

4.3.5. Assessment of cell viability, apoptosis and necrosis

The dye exclusion method (36) was applied to study cell death and morphological changes in the nuclei, in which viable (intact plasma membrane) and dead (damaged plasma membrane) cells can be visualized after staining with fluorescent DNA-binding dyes (Figure 1). Ethidium bromide (Sigma, Germany) and acridine orange (Sigma, Germany) were added to the cell suspension in final concentrations of 100 µg/mL (1:1; *V/V*). Two parallel tests with aliquots of the same sample were performed and a total of 400 cells per sample were counted. Quantitative assessments were made by determination of the percentage of apoptotic and necrotic cells.

4.4. Statistical analysis

All statistical analyses were performed using GraphPad Prism Software (Sand Diego, CA, USA). The t-test was used to compare differences in tryptophol production between strains and between species, as well as between concentrations of tryptophol obtained after incubation under 5% CO_2 and under normal atmospheric conditions. For viability assessments, the χ^2 test was used to compare values obtained for cell viability, apoptosis and necrosis in tryptophol-treated and control samples. Differences were considered significant at $p < 0.05$.

5. RESULTS AND DISCUSSION

5.1. Production of LMW metabolites of *C. albicans* and *C. dubliniensis*

The representative HPLC chromatogram of *C. albicans* (N=64) and *C. dubliniensis* (N=6) strains are presented in Figure 2a. Following six days of incubation, only one compound was isolated from all the investigated strains, and peak with retention time was found around 15 minutes. The UV spectra showed maximums at 224 and 279 (Figure 2b). This UV spectrum displayed a strong similarity with the characteristic UV spectrum of indole-like compounds. Using tryptophan, 3-indoleelthanol (tryptophol), 3-indoleacetic acid, and gliotoxin, only tryptophol showed a similar retention time and UV spectrum with those of the compound isolated in *C. albicans* and *C. dubliniensis* strains culture extract. As only one compound was isolated from the biosynthesis of all 64 strains of *C. albicans* and 6 strains of *C. dubliniensis*, further identification using MS and MS/MS in the negative ESI mode were performed. The fraction with a retention time between 14.5 and 15 mm was collected using HPLC-DAD and analyzed with LC-MS. The LC-MS chromatogram displayed a unique major peak with a base peak at m/z 160 (Figure 2c). Analysis of the fraction by direct injection mass spectrometry allowed MS/MS data to be obtained (Figure 2d). These data matched the tryptophol standard MS (Figure 2e) and MS/MS spectrum (Figure 2f), since the characteristic ions m/z 142 $((M-H)-H_2O)^-$, 130 $((M-H)-CH_2O)^-$ and 116 $((M-H)- C_2H_4O)^-$ could be seen for the fraction. Loss of water moiety in molecule of tryptophol (m/z=160) corresponded with m/z 142.

Michel (37) reported similar results of MS/MS of tryptophol from infected wood, performed from the culture of the fungal species *Ophiostoma (= Ceratocystis) novo-ulmi*, which causes Dutch elm disease in trees.

It is not surprising that tryptophol, as a low-molecular-weight lypophilic alcohol, was found in the biosynthesis of polymorphic *C. albicans* and *C. dubliniesis* strains in synthetic media such as Eagle's minimal essential medium (MEM). An earlier study by Ligappa *et al.* (38) performed isolation and identification of the metabolites tryptophol, 2-phenylethanol and dimer of cyclo(proline-leucine) by means of infrared and mass spectra from chloroformic extracts from the biosynthesis of *C. albicans* in Sabouraud sucrose broth. Tryptophol and 3-indolelactic acid were also isolated from the biosynthesis of *C. albicans* Z248, *C. guillermondii* Z55, *C. krusei* Z70 and *C. tropicalis* Z56 (39) in L-tryptophan (0.25% m/V) rich medium. In a

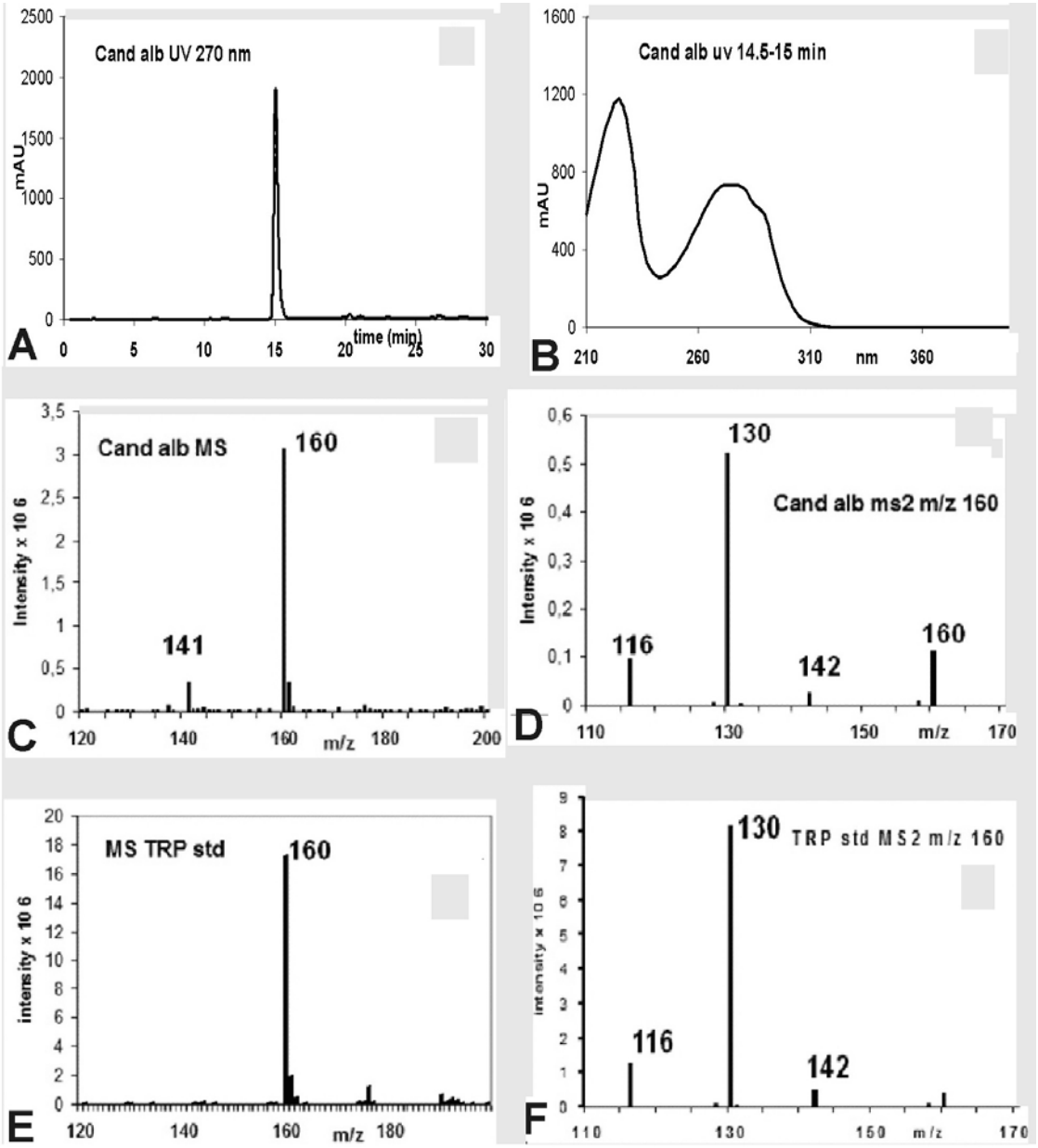

Figure 2. a) HPLC chromatogram of the extract from biosynthesis of *C. albicans* strains recorded at 270 nm, b) UV spectra of isolated compound with retention time between 14.5 and 15 minutes, c) MS spectra of LMW metabolite isolated from biosynthesis with retention time 14.5-15 minutes, d) MS/MS spectra of *m/z* 160, e) MS and f) MS/MS spectra of tryptophol standard.

later study, various amounts of tryptophan were converted into tryptophol and 3-indolelactic acid, in concentration depending on *Candida* species. However, the present study, using different *C. albicans* and *C. dubliniensis* strains (from respiratory and uro-genital tracts, as well as commensal isolates and isolates from clinically manifested mycoses), found tryptophol as the only low-molecular weight metabolite. Similar to the findings of Narayanan and Rao (39), a previous study (40) showed that *C. guillermondi, C. kefyr, C. krusei, C. parapsilosis, C. tropicalis* were found as lower tryptophol-producing species, while the production of tryptophol in the biosynthesis of *C. famata* and *C. glabrata* was not detected. *C. tropicalis* was found to be a low-producer of tryptophol (1.63±0.49 μg/mL), followed by *C. parapsilosis* (13.05±6.48 μg/mL), *C. krusei* (13.64±4.92 μg/mL), *C. kefyr* (18.34±5.11 μg/mL) and *C. guillermondii* (22.83±4.70 μg/mL), respectively (40).

Dickinson *et al.* (41) studied the conversion of the aromatic amino acids phenylalanine and tryptophan into alcohols in cultures of the yeast *Saccharomyces cerevisiae*. The pathway of tryptophan catabolism of *S. cerevisiae*

Table 1. Production of tryptophol by *Candida albicans* and *C. dubliniensis* strains

Tryptophol production	***C. albicans* N=64**	***C. dubliniensis* N=6**
Mean (μg/mL)	49.10	72.81
Median (μg/mL)	45.31	60.52
Standard deviation	34.99	52.31
Standard error	4.52	21.36

yield tryptophol as the end product, with 3-indolepyruvate and 3-indoleacetaldehyde as intermediary products. *S. cerevisiae* secretes phenylethanol (as the end-product of catabolism of phenylalanine) and tryptophol and they stimulate diploid pseudohyphal growth of *S. cerevisiae* (42). Interestingly, Chen and Fink (43) found that low concentrations of either phenylethanol or tryptophol stimulated filamentous growth of *S. cerevisiae*, and they acted synergistically. However, another aromatic alcohol – tyrosol (as the end-product of catabolism of tyrosine) did not stimulate pseudohyphal growth of *S. cerevisiae*, which indicates the structure-relationship activity of these alcohols (44). Interestingly, in a culture of *C. albicans* cells, tyrosol accelerates the morphological conversion of *C. albicans*-yeast-form cells to filaments (45). Another substance isolated from the culture of *C. albicans* acting as an autoregulatory molecule is farnesol which, contrary to tryptophol and tyrosol, suppress germ-tube formation of *C. albicans* cells (45, 46). However, the nature of dimorphic behavior of *C. albicans* cells depend not only on the presence and concentration of autoregulatory substances such as tyrosol, tryptophol and farnesol, but also on different factors, including the presence of various chemical and environmental parameters such as temperature, pH, glucose levels, nitrogen source, CO_2 levels, transition metals, chelating agents, inoculum size, and initial cell density (47, 48). The very important role of alcohols in biofilm-development has been connected with tyrosol and farnesol (48). Cells of *C. albicans* in biofilm secrete approximately 50% more tyrosol than did planctonic cells, and this action on biofilm formation is most significant during the early and intermediate stage of biofilm formation (48).

The present findings showed that tryptophol (3-indoleethanol), as the end-product of tryptophan catabolism, was the only low-weight-molecular metabolite in the biosynthesis of polymorphic *C. albicans* and *C. dubliniensis* strains in MEM with the addition of 5% (V/V) fetal calf serum. In this media, tryptophol was found to be only metabolite, though its production was not significantly different between filamentous polymorphic *C. albicans* and *C. dubliniensis* strains (Figure 3a).

However, earlier studies performed by Shah and Larsen (49) and Shah (50) suggested that clinical isolates of *C. albicans* strains secrete the potent immunosuppressive mycotoxin gliotoxin (from the class of epipoythiodioxopiperazine secondary metabolites) after seven days in synthetic media with 5% fetal calf serum. Gliotoxin was found in clinical *C. albicans* isolates from women with vulvovaganitis with a frequency of 32 gliotoxin-positive strains of 50 tested by Shah and Larsen (49). Furthermore, Shah *et al.* (51) found gliotoxin production in vaginal samples of three women with clinically manifested candidosis, while women without vaginal colonization by *C. albicans* were found to be gliotoxin-negative. After the findings of Shah *et al.* (49-51), gliotoxin was cited in articles and reviews as one of the virulence factors of *C. albicans*.

Our findings clearly show that *C. albicans* (both commensal and clinical isolates) and *C. dubliniensis* strains do not produce the immunosuppressive mycotoxin gliotoxin, which was also described by Kupfal *et al.* (52). Similar to Kupfal *et al.* (52) we did not find gliotoxin-producing *Candida* species between clinical and commensal isolates of *C. parapsilosis, C. kefyr, C. krusei, C. glabrata, C. tropicalis. C. guillermondii* and *C. famata* (53, 54).

Discrepancies can even be found in the numbers of strains between two species. Production of tryptophol was similar between strains of *C. albicans* and *C. dubliniensis* species (Table 1). The concentration of tryptophol was 49.10±34.99 μg/mL between strains of *C. albicans*, and 72.81±52.31 μg/mL between strains of *C. dubliniensis*, respectively, without significant differences in production ($p>0.05$). Tryptophol production between strains of both *Candida* species was in the high range, with a minimum at 2.45 μg/mL and maximum at 191 μg/mL. When tryptophol production was compared between *C. albicans* isolates regarding body site of isolation, no significant differences were found (Figure 3b). Similar results in tryptophol production have been noted between isolates of *C. albicans* strains isolated from clinically manifested candidosis and from mouth and stool specimens of donors without manifestation of mycoses (commensal isolates). The results are presented in Figure 3c.

Environmental conditions such as temperature and CO_2 are known to trigger the switch from yeast-like cells to the filamentous invasive form of *C. albicans* (1). Therefore we checked the influence of 5% CO_2 on tryptophol production which, together with serum in Eagle's minimal essential medium *C. albicans,* were in the filamentous form. Surprisingly, tryptophol production was found to be significantly lower in a 5% CO_2 atmosphere (Figure 3d). As presented in Figure 8, both *Candida* species produce more tryptophol under normal atmospheric conditions than under 5% CO_2.

Using production kinetics, measurable amounts of tryptophol were produced in the biosynthesis of *C. albicans* MFBF 40 after 18 hours, with an increase of tryptophol production during 162 hours (Figure 4a). Contrary to the findings of Narayanan and Rao (39), 3-indolelactic acid was not isolated from the biosynthesis of *C. albicans* MFBF 40 during the incubation period.

There is no data about the *in vivo* production of any of low-molecular-weight metabolites produced by *Candida* spp. It could be hypothesized that during candidosis, filamentous *C. albicans* and *C. dubliniensis* can secrete tryptophol extracellulary in the environment. The possible role of this metabolite upon host cells and/or commesal bacteria is unknown. Interestingly, tryptophol is a tryptophan metabolite of the blood flagellate parasite

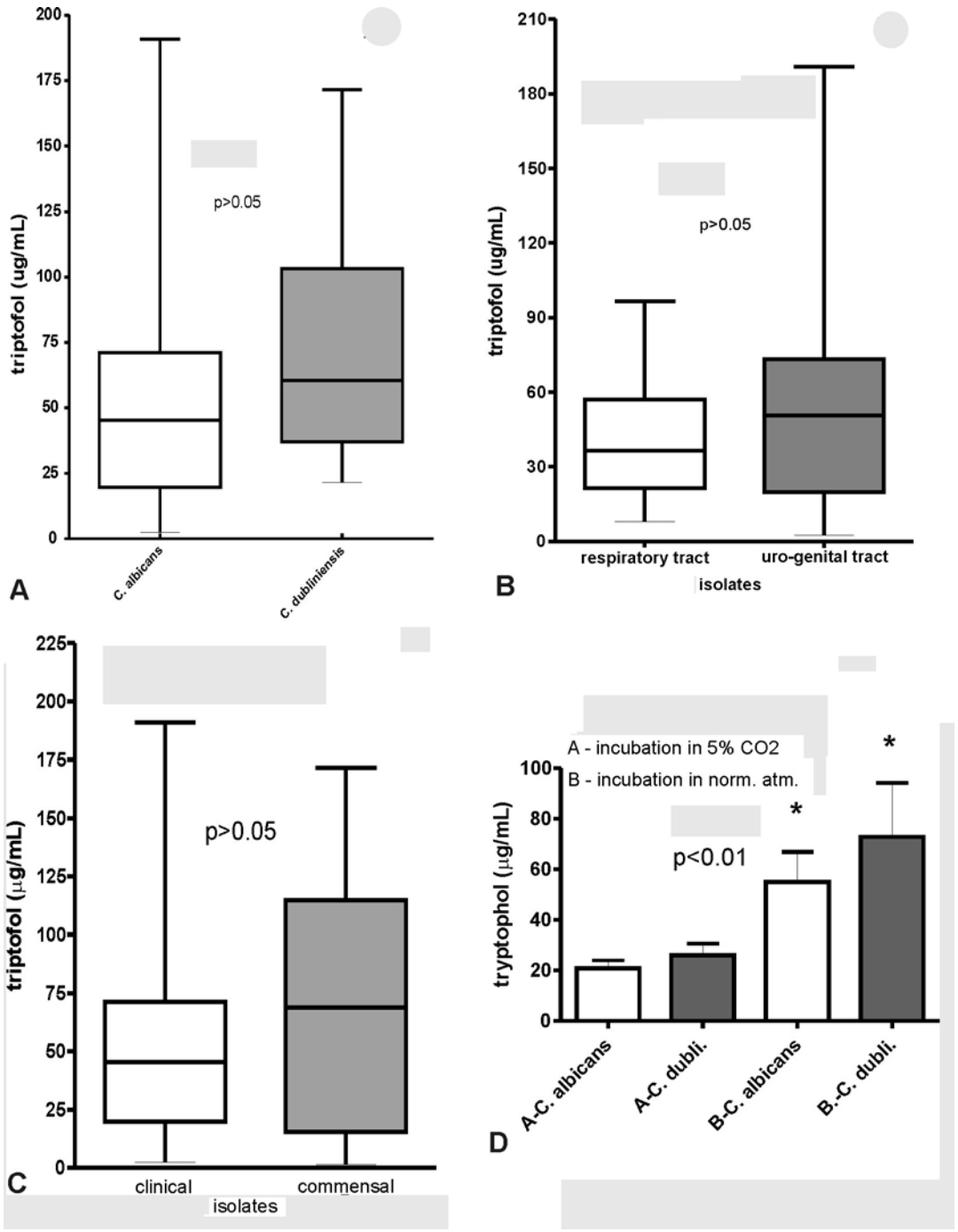

Figure 3. a) Comparison of *in vitro* tryptophol production by *C. albicans* (N=64) and *C. dubliniensis* (N=6), b) Differences in tryptophol production between isolates of *C. albicans* from two different body sites (N=30), c) Differences between tryptophol production by clinical (N=32) and commensal (N=32) *C. albicans* strains**,** d) The influence of 5% CO_2 on tryptophol production between *C. albicans* (N=6) and *C. dubliniensis* (N=6) strains.

African trypanosomes (*Trypanosoma brucei*), which produces tryptophol *in vitro*. Tryptophol is established as a hypnotic agent participating in pathophysiological mechanisms provoking a sleep-like or comatose state known as trypanosomal sleeping sickness (55-58). Moreover, when tryptophol was injected into laboratory mammals and chicks, it induced a sleep-like state and lethargy accompanied with alteration of body temperature (57, 59).

In humans, tryptophol is a minor product of tryptophan metabolism. However, its production in the

Table 2. Cytotoxicity induced by tryptophol and its precursor 3-indolelactic acid

	EC_{50} in mM / cell line				
	A549	Hep G-2	THP-1	COS-7	CHO
Tryptophol	3	4	2	6	7
3-indolelactic acid	6	7	4	8	9
Gliotoxin	0.002	0.011	0.002	0.011	0.005

The effective concentration (EC_{50}) values of tryptophol and 3-indolelactic acid following 24h exposure measured by the MTT assay using A549, Hep G-2, THP-1, COS-7 and CHO cell lines. Gliotoxin was used as control

liver significantly increased with alcohol consumption and following disulfiram treatment (57, 60). Ethanol-induced modification of the metabolism of biogenic indole amines also results in the increased production of tryptophol compounds (61). Tryptophol is a highly lypophilic compound that freely penetrates cell membranes (61). Due to the irreversible alterations of water permeability, it induces erythrocyte lysis (62). When administered to laboratory animals, it was rapidly distributed to the brain and other tissues. Tryptophol also readily penetrates into lymphoid tissues and its immunosuppressive effects have been observed in mice (63). Despite interesting biological effects, the overall toxicity profile of tryptophol has not yet been adequately explained. Recent studies concerning mutagenicity of tryptophol using the Ames test on *Salmonella enterica* serovar Typhymurium TA98 and TA100 tester strains without metabolic activation and with the S9 fraction showed no mutagenic effect in doses up to 1.6 mg/plate (64). The newest reports on tryptophol toxicity by Inagaki *et al.* (65, 66) indicate its capability to induce apoptosis in human monoblastic leukemia U937 cells, while lymphocytes were less affected by tryptophol.

5.2. Cytotoxicity screening on different cell lines and lymphocyte viability

The cytotoxicity of tryptophol, as well as its precursor 3-indolelactic acid from tryptophan catabolism, is presented in Figures 4b and 4c. Gliotoxin, known as cytotoxic mycotoxin in μM concentrations (68-70), was used as a control (Figure 4d).

Tryptophol treatment for 24 h caused cell viability to decrease in a dose-dependent manner, with effective concentrations depending on cell type. THP-1, A549 and Hep G-2 cell types were found to be the most sensitive to the cytotoxic effect of tryptophol with an EC_{50} of 2 and 3 mM, respectively (Table 2). COS-7 and CHO were most resistant, with an EC_{50} of tryptophol of 6 and 7 mM, respectively (Table 2). Gliotoxin expresses strong cytotoxic activity in cultures of all cell types tested with EC_{50} below or equal to 11 μM, which are approximately 1000 x lower concentrations than tryptophol.

3-indolelactic acid was found to be less cytotoxic than tryptophol, with an EC_{50} in double concentration for A549 and THP-1 cell types. A possible explanation of the lower cytotoxic potential of 3-indolelactic acid is carboxyl moiety in the molecule, instead of ethyl in the tryptophol molecule.

Lymphocyte viability in the control sample after 24 h of *in vitro* incubation was 89%. The percentages of viable and non-viable apoptotic or necrotic lymphocytes after 24-h *in vitro* exposure to tryptophol are shown in Figure 4e. *In vitro* treatment with tryptophol caused a dose-dependent decrease in cell viability, accompanied by increases in the percentage of apoptotic and necrotic cells. It was observed that apoptosis dominated over necrosis in all samples analyzed. Significantly reduced cell viability compared to the control was observed at each dose of tryptophol ($p<0.05$, χ^2 test) while the solvent did not significantly reduce cell viability. Inter-group comparisons showed that differences in cell viability between samples treated with 0.25 mM and 0.50 mM, as well as between samples treated with 1.00 mM and 2.00 mM were not statistically significant ($p>0.05$, χ^2 test).

Based on the results obtained in the present study, apoptosis was primary mode of lymphocyte death following *in vitro* treatment with a dose range 0.25 to 2.00 mM. Tryptophol was also recently established as an effective inducer of apoptosis. Inagaki *et al.* (66) reported the ability of tryptophol isolated from black soybean vinegar to induce apoptosis in U937 cells (human monoblastic leukemia), but not in normal lymphocytes that were studied in parallel during 24 h of treatment with 80 μg/mL (~0.5 mM) of the agent. They reported that tryptophol-induced apoptosis involved caspase-8 and -3 activation, followed by cleavage of poly (ADP-ribose) polymerase. In the present study, normal lymphocytes were used as a model system. The results obtained indicate that tryptophol was capable of inducing apoptosis in two higher concentrations (1.00 mM and 2.00 mM) than those used by Inagaki *et al.* (66). However, considering that in this study, apoptosis was detected based on morphological changes in the lymphocyte nuclei after staining with fluorescent DNA-binding dyes, further studies are necessary to clarify the exact mechanisms of lymphocyte death following treatment with tryptophol.

6. CONCLUSIONS

According to the analytical methods used and results presented in this study, it could be concluded that polymorphic *C. albicans* and *C. dubliniensis* species produce tryptophol (3-indoleethanol) as a single low-molecular-weight lipophylic metabolite *in vitro.* Its production was higher in aerobic conditions, and was detected after an 18h incubation period. In relatively high concentrations, tryptophol caused a cytotoxic effect on cultures of epithelial cells (A549, Hep G-2 and CHO) with an EC_{50} between 3 and 7 mM; in culture of fibroblast-like cells (COS-7) with EC_{50} 6 mM and in culture of monocytes (THP-1) with 2 mM. In sub-cytotoxic concentrations (less than 2 mM), tryptophol caused apoptosis as a primary mode of human lymphocyte death *ex situ*. As tryptophol induces filamentous form in yeasts, its secretion during candidoses and potential toxic effects on host cells could be expected. Contrary to previously published studies, gliotoxin was not detected in biosynthesis of clinically and commensally isolates of *C. albicans* and *C. dubliniensis* strains. The *in vivo* secretion of tryptophol as a lipophylic

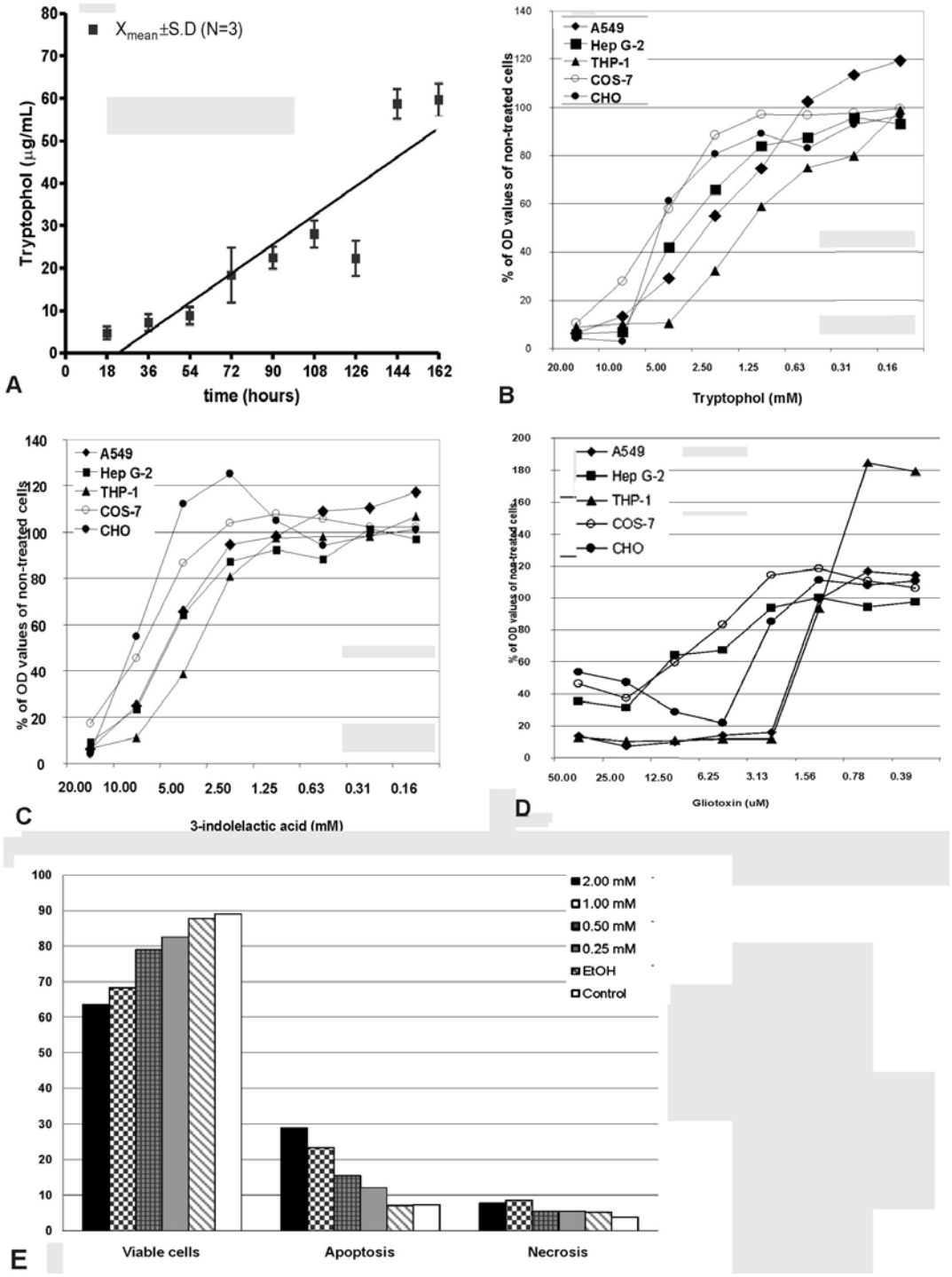

Figure 4. a) Tryptophol production kinetics (biosynthesis performed using *C. albicans* MFBF 40 at 37°C in normal atmospheric conditions with regression line), b) Cytotoxic effects of tryptophol at concentrations of 0.16–20 mM on proliferation of A549, Hep G-2, THP-1, COS-7 and CHO cell lines following 24 h exposure as determined by the MTT bioassay, c) cytotoxic effects of 3-indolelactic acid at concentrations of 0.16–20 mM on proliferation of A549, Hep G-2, THP-1, COS-7 and CHO cell lines following 24-h exposure as determined by the MTT bioassay, d) Cytotoxic effects of gliotoxin at concentrations of 0.16– 50 µM on proliferation of A549, Hep G-2, THP-1, COS-7 and CHO cell lines following 24-h exposure as determined by the MTT bioassay, e) Detection of apoptosis and necrosis of human peripheral blood lymphocytes after 24-h *in vitro* exposure to tryptophol.

low-molecular-weight metabolite, especially during candidosis, should be explored.

7. ACKNOWLEDGEMENTS

This research was supported by Federation of European Microbiological Societies (FEMS) fellowship for Ivan Kosalec. This work was supported by grants of the Ministry of Science, Education and Sport of Republic of Croatia (N° 006-0061117-1242 and 022-0222148-2137), AFSSET grant ES 2005-012 and by an ADEME Grant (N° 0275042) for Marcel Delaforge and Olivier Puel. We thank Souria Tadrist for technical assistance. We thank the Croatian Microbiological Society (Zagreb), especially to Dr. Danko Hajsig for his support, and Prof. Derek Sullivan (Trinity College, Dublin) for donation of part of the *C. dubliniensis* strains used in this study.

8. REFERENCES

1. Odds FC: *Candida* and Candidosis. Leicester: Leicester University Press (1979)

2. Fridkin SK, W. R. Jarvis: Epidemiology of nosocomial fungal infections. *Clin. Microbiol. Rev.* 9, 499-511 (1996)

3. Yang YL: Virulence factors of *Candida* species. *J. Microbiol. Immunolo. Infect*. 36, 223-228 (2003)

4. Osorio JJC, A. R. Román, J. Torre-Cisneros: Spectrum and risk factors of invasive fungal infection. *Enferm Infect Microbiol Clin.* 25, 467-76 (2007)

5. Hazen KC: New and emerging yeast pathogens. *Clin. Microbiol. Rev.* 8, 462-478 (1995)

6. Ribaud P: Fungal infections and the cancer patient, *Eur. J. Cancer* 33, S50-S54 (1997)

7. Molero G, R. Diez-Orejas, F. Navarro-Garcia, F. Monteoliva, L. Pla, C. Gil, M. Sanchez-Perez, C. Nombela: *Candida albicans*: genetics, dimorphism and pathogenicity. *Internatl Microbiol.* 1, 95-106 (1998)

8. Hobson RP: The global epidemiology of invasive *Candida* infections-is the tide turning? *J. Hosp. Infect.* 55, 159-168 (2003)

9. Sullivan D, D. Coleman: *Candida dubliniensis*: characteristics and identification. *J. Clin. Microbiol*. 36, 329-334 (1998)

10. Hawser SP, L. J. Douglas: Biofilm formation by *Candida* species on the surface of catheter materials in vitro. *Infect Immun*. 62, 915-921 (1994)

11. Sturtevan J, R. Calderone: *Candida albicans* adhensins: biochemial aspects and virulence. *Rev. Iberoam. Micol.* 14, 90-97 (1997)

12. Yang YL: Virulence factors of *Candida* species. *J. Microbiol. Immunol. Infect.* 36, 223-228 (2003)

13. Lo H J, JR Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, G. R. Fink: Nonfilamentous *C. albicans* mutants are avirulent. *Cell* 90, 939–949 (1997)

14. Gutiérrez J, P. Morales, M. A. Gonzalez, G. Quindos: *Candida dubliniensis*: a new fungal pathogen. *J. Basic Microbiol.* 42, 207-27 (2002)

15. Whiteway M, U. Oberholzer: *Candida* morphogenesis and host-pathogen interactions. *Curr. Opin. Microbiol.* 7, 350–357 (2004)

16. Li CR, R. T. Lee, Y. M. Wang, X. D. Zheng, Y. Wang: *Candida albicans* hyphal morphogenesis occurs in Sec3p-independent and Sec3p-dependent phases separated by septin ring formation. *J. Cell Sci.* 120, 1898-907 (2007)

17. Kretschmar M, B. Hube, T. Bertsch, D. Sanglard, R. Merker, M. Schröder, H. Hof, T. Nichterlein: Germ tubes and proteinase activity contribute to virulence of *Candida albicans* in murine peritonitis. *Infect. Immun.* 67, 6637-6642 (1999)

18. Naglik JR, S. J. Challacombe, B. Hube: *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol. Mol. Biol. Rev.* 67, 400-428 (2003)

19. Lian CH, W. D. Liu: Differential expression of *Candida albicans* secreted aspartyl proteinase in human vulvovaginal candidiasis. *Mycoses* 50, 383-90 (2007)

20. Ghannoumn MA: Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev.* 13, 122-143 (2000)

21. Stehr F, A. Felk , A. Gácser , M. Kretschmar , B. Mähnss , K. Neuber , B. Hube, W. Schäfer: Expression analysis of the *Candida albicans* lipase gene family during experimental infections and in patient samples *FEMS Yeast Res*. 4, 401-408 (2004)

22. Luo G, L.P. Samaranayake, J. Y. Y. Yau: *Candida* species exhibit differential in vitro hemolytic activities. *J. Clin. Microbiol.* 39, 2971-2974 (2001)

23. Luo GL, P. Samaranayake, B. P. Cheung, G .Tang: Reverse transcriptase polymerase chain reaction (RT-PCR) detection of HLP gene expression in *Candida glabrata* and its possible role in *in vitro* haemolysin production. *APMIS* 112, 283-90 (2004)

24. Kosalec I, B. Matica, N. Jarža-Davila, T. Maretić, J. Granić, S. Pepeljnjak: Haemolytic activity of *Candida albicans* and *C. dubliniensis* isolated from the oral cavity of immunocompromised patients. *Clin. Microbiol. Infect.* 12, 712 (2006)

25. Linares CE, E. S. Loreto, C. P. Silveira, P. Pozzatti, L. A. Scheid, J. M. Santurio and S. H. Alves: Enzymatic and hemolytic activities of *Candida dubliniensis* strains. *Rev. Inst. Med. Trop. Sao Paulo* 49, 203-206 (2007)

26. Slutsky B, J. Buffo, D. R. Soll: High-frequency switching of colony morphology in *Candida albicans*. *Science* 230, 666-9 (1985)

27. Vargas K, S. A. Messer, M. Pfaller, S. R. Lockhart, J. T. Stapleton, J. Hellstein and D. R. Soll: Elevated phenotypic switching and drug resistance of *Candida albicans* from human immunodeficiency virus-positive individuals prior to first thrush episode. *J. Clin. Microbiol.* 38, 3595-3607 (2000)

28. Jin Y, Y. H., Samaranayake, H. K Yip, L. P. Samaranayake: Characterization of switch phenotypes in *Candida albicans* biofilms. *Mycopathologia* 160, 191-200 (2005)

29. Lewis RE, N. P. Wiederhold, J. Chi, X.Y. Han, K. V. Komanduri, D. P. Kontoyiannis, R. A. Prince: Detection of gliotoxin in experimental and human aspergillosis. *Infect. Immun.* 73, 635-637 (2005)

30. Lewis RE, N. P. Wiederhold, M. S. Lionakis, R. A. Prince, D. P. Kontoyiannis: Frequency and species distribution of gliotoxin-producing aspergillus isolates recovered from patients at a tertiary-care cancer center. *J Clin. Microbiol.* 43, 6120–6122 (2005)

31. Larone DH: Medically Important Fungi: A Guide to Identification. Washington D.C. *ASM Press*, 4th ed. (2002)

32. Al Mosaid A, D. J. Sullivan, D. C. Coleman: Differentiation of *Candida dubliniensis* from *Candida albicans* on Pal's Agar. *J. Clin. Microbiol.* 41, 4787–4789 (2003)

33. Frisvad JC: High-performance liquid chromatography determination profiles of mycotoxins and other secondary metabolites. *J. Chromatogr*. 392, 333-347 (1987)

34. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* 65, 55-63 (1983)

35. Singh NP: Microgels for estimation of DNA strand breaks, DNA protein crosslinks and apoptosis. *Mutat. Res.* 455, 111-127 (2000)

36. Duke RC, J. J. Cohen: Morphological and biochemical assays of apoptosis. In: J. E. Coligan, A. M. Kruisbeal (eds.) Current Protocols in Immunology. *John Wiley and Sons*. New York (1992)

37. Michel A: Métabolites secondaires d'Ophiostoma novo-ulmi et de Ceratocystis fimbriata sp. platani, pathogènes de l'orme et du platane, Faculté des Sciences, Université de Neuchâtel, PhD Thesis, 2001. (http://www.unige.ch/cyberdocuments/unine/theses2001/MichelA/these.html, accessed May 2007.)

38. Lingappa BT, M. Prasad, Y. Ligappa, D. F. Hunt, K. Biemann: Phenyethanol alcohol and tryptophol: autoantibiotics produced by the fungus *Candida albicans. Science* 163, 192-193 (1969)

39. Narayanan TK, R. Rao: Beta-indoleethanol and beta-indolelactic acid production by *Candida* species: their antibacterial and autoantibiotic action. *Antimicro. Agents Chemother.* 9, 375-380 (1976)

40. Kosalec I: Toxinogenicity and mechanism of action of virulence factors of *Candida* species, PhD Thesis, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia (2006)

41. Dickinson JR, L. E. J. Salgado, M. J. E. Hewlins: The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 278, 8028-34 (2003)

42. Chen H, M. Fujita, Q. Feng, J. Clardy, G. R. Fink: Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* 101, 5048-5052 (2004)

43. Chen H, G. R. Fink: Feedback control of morphogenesis in fungi by aromatic alcohols. *Gen. Develop.* 20, 1150-1161 (2006)

44. Hornby JM, E. C. Jensen, A. D. Lisec, J. J. Tasto, B. Jahnke, R. Shoemaker, P. Dussault, K. W. Nickerson: Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl. Environ. Microbiol.* 67, 2982–2992 (2001)

45. Oh KB, H. Miyazawa, T. Naito, H. Matsuoka: Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* 98, 4664–4668 (2001)

46. Romano A: Dimorphism, In: G. C. Ainsworth and A. S. Sussman (eds.), The Fungi, vol. 2. New York: *Academic Press* (1966)

47. Nickerson KW, A. L. Atkin, J. M. Hornby: Quorum sensing in dimorphic fungi: farnesol and beyond. *Appl. Envir. Microbiol*. 72, 3805–3813 (2006)

48. Alem MAS, M. D. Y. Oteef, T. H. Flowers, L. J. Douglas: uction of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. *Eukaryot Cell* 5, 1770-1779 (2006)

49. Shah DT, B. Larsen: Identity of a *Candida albicans* toxin and its production in vaginal secretions, *Med Sci Res* 20, 353–355 (1992)

50. Shah DT: Production of gliotoxin by *Candida albicans* and its effects on human polymorphonuclear leukocytes, PhD Thesis, West Virginia University (1993)

51. Shah DT, D. D. Glover, B. Larsen: In situ mycotoxin production by *Candida albicans* in women with vaginitis. *Gynecol. Obstet. Invest.* 39, 67-69 (1995)

52. Kupfahl C, T. Ruppert, A. Dietz, G. Geginat, H. Hof: *Candida* species fail to produce the immunosuppressive secondary metabolite gliotoxin *in vitro*. *FEMS Yeast Res.* 7, 986–992 (2007)

53. Kosalec I, S. Pepeljnjak, M. Delaforge, O. Puel, P. Galtier: Possible toxicity of clinical isolates of *C. albicans*, 3rd Croatian Microbiological Congress, 4-7 October 2004, Poreč, Croatia, 115 (2004)

54. Kosalec I, S. Pepeljnjak: The toxinogenicity of clinical isolates of *Aspergillus* and *Candida* species, Scientific symposium: Mycotoxins-clinical aspects and prevention, S. Pepeljnjak (Ed.), 44-45, *a.z.p. Grafis*, Zagreb (2004)

55. Seed JR, J. Sechelski: Tryptophol levels in mice injected with pharmacological doses of tryptophol, and the effect of pyrazole and ethanol on these levels. *Life Sci.* 21, 1603-1610 (1977)

56. Tizard I, K.H. Nielsen, J.R. Seed, J. E. Hall: Biologically active products from African Trypanosomes. *Microbiol Rev*. 42, 661-81 (1978)

57. Cornford EM, W. D. Bocash, L. D. Braun, P. D. Crane, W. H. Oldendorf: Rapid distribution of tryptophol (3-indole ethanol) to the brain and other tissues. *J. Clin. Invest.* 63, 1241-128 (1979)

58. Vincendeau P, S. Lesthelle, A. Bertazzo, M. C. Okomo-Assoumou, G. Allegri, C. V. Costa: Importance of L-tryptophan metabolism in trypanosomiasis. *Adv. Exp. Med. Biol.* 467, 525-531 (1999)

59. Cornford EM, P. D. Crane, L. D. Braun, W. D. Bocash, A. M. Nyerges, W. H. Oldendorf: Reduction in brain glucose utilization rate after tryptophol (3-indole ethanol) treatment. *J. Neurochem*. 36, 1758-1765 (1981)

60. Tanaka K, B. McConnell, W. P. Niemezura, H. F. Mower: Characterization and mutagenicity of 1-nitrosotryptophol and 6-nitrotryptophol possible genotoxic substances associated with smoking and alcohol consumption. *Cancer Lett.* 44, 109-116 (1989)

61. Some M, M. O. Beck, A. Helander: Acute interaction between ethanol and serotonin metabolism in the rat. *Life Sci.* 61, 577-583 (1997)

62. Seed JR, T. M. Seed, J. Sechelski: The biological effects of tryptophol (indole-3-ethanol): hemolitic, biochemical and behavior modifying activity. *Comp. Biochem. Physio.l Part C: Comp. Pharmacol.* 60, 175-185 (1978)

63. Ackerman SB, J. R. Seed: The effects of tryptophol on immune responses and its implications towards trypanosome-induced immunosupression. *Experientia (Basel)* 15, 645-647 (1976)

64. Kosalec I, N. Kopjar, S. Ramić, V. Bačun-Družina, S. Pepeljnjak: Genotoxicity and mutagenicity of tryptophol from *Candida albicans*, Central European symposium "Power of Microbes in Industry and Environment", Zadar, 19-22 September 2007, 116 (2007)

65. Inagaki S, S. Morimura, K. Gondo, Y. Tang, H. Akutagawa, K. Kida: Isolation of tryptophol as an apoptosis-inducing component of vinegar produced from boiled extract of black soybean in human monoblastic leukemia U937 cells. *Biosci. Biotechnol. Biochem.* 71, 371-379 (2007)

66. Inagaki S, S. Morimura, Y. Tang, H. Akutagawa, K. Kida: Tryptophol induces death receptor (DR) 5-mediated apoptosis in U937 cells. *Biosci. Biotechnol. Biochem.* 71, 2065-2068 (2007)

67. Kosalec I, S. Pepeljnjak: Chemistry and biological effects of gliotoxin. *Arh. Med. Rada Toksikol.* 55, 313-320 (2004)

68. DeWitte-Orr SJ, N. C. Bols: Gliotoxin-induced cytotoxicity in three salmonid cell lines: cell death by apoptosis and necrosis. *Comp. Biochemi. Physiol. C, Toxicol. Pharmacol.* 141, 157-167 (2005)

69. Gräbsch C, G. Wichmann, N. Loffhagen, O. Herbarth, A. Müller: Cytotoxicity assessment of gliotoxin and penicillic acid in *Tetrahymena pyriformis*. *Environm Toxicol.* 21, 111-117 (2006)

Abbreviations: MFBF= *Collection of microorganisms* of the Department of Microbiology, Faculty of Pharmacy and Biochemistry of the University of Zagreb

Key Words: *Candida albicans, C. dubliniensis*, Metabolites, Tryptophol, LC, MS, Cytotoxicity, Lymphocyte, Biosynthesis, Isolation, Candidosis

Send correspondence to: Ivan Kosalec, Institute of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Schrottova 39, HR-10000 Zagreb, Republic of Croatia, Tel: 385-1-6394-492, Fax: 385-1-6394-494, E-mail: ikosalec@pharma.hr

http://www.bioscience.org/current/vol13.htm