CNCSIS PN II – RU PROJECT:

TE_159/ 2010

EVALUATION OF THE ANTIFUNGAL EFFECT OF NANO CONJUGATES OF A NEW PROPICONAZOLE DERIVATIVE WITH BETA-CYCLODEXTRIN

STAGE REPORT 2012

Assessment of mortality and fungal burden reduction / gram tissue after treatment with nano-conjugates for the mice groups infected with LD_{90}.

For determination of the therapeutically efficacy of the MXP-4509 compound, different doses and administration routes for the product in two groups of mice pre inoculated with Lethal Dose 90 (LD_{90}). The mortality percentage and fungal burden per gram of renal tissue was determined in treated mice groups, for 15 days time period.

Material and Methods.

- outbreed CD1 mice, females, with 20 g average weight (Cantacuzino Institute, Bucharest);
- *Candida albicans* SC 5314 strain*;*
- MXP 4509 substance in phosphate buffer with a final concentration of 1 mg/ml active substance (propiconazole nitrate);
- For preparation of the *C. albicans* inoculum: YPD Agar dishes, inoculation loops, phosphate buffer, vortex, sterile tubes, Neubauer improved counting chamber;
- For animal manoeuvres: scrub, mask, latex gloves, rubber ring for finger
- For intravenous inoculation of the mice: contention device for mice and 25 G needle and 1 ml syringe
- For administration of the antifungal substance: gavage needle, 1 ml syringe with 25 G needle, cotton and alcohol, latex gloves, antiseptically solution;

Experimental design

Day 0 – the groups were formed and the mice were inoculated with SC 5314 strain.

The mice were divided in 3 groups (30 mice per group). Five animals were hosted in each box (boxes of 1500 U, Eurostandard IV, with 1500 cm² surface) in identical feeding conditions (granulated standard feed), temperature, relative humidity (60-70%) and light (12h light, 12h darkness).

Group I (control):

- The mice were inoculated with SC 5314 strain without any treatment;

Group II:

- The mice were inoculated with SC 5314 strain and treated intra peritoneal route with MXP 4509 in dose of 25 mg/ Kg bw;

Group III:

- The mice were inoculated with SC 5314 strain and treated oral route with MXP 4509 in dose of 50 mg/ Kg bw;

The inoculation of the mice:

- An yeast suspension is prepared in phosphate buffer and adjusted (after determination of their number in counting chamber), to a turbidity equivalent to $5x10^6$ cells/ml;
- The mice are placed in contention devices;
- The mice are heated under a infrared bulb lamp to emphasize de vein of the tail;
- The tail of the mice is fixed between the index finger and thumb from the left hand, then the needle is pointing the vein parallel with the blood vessel and 0.2 ml of suspension is inoculated;
- After inoculation, the needle is kept inside for approximately 5 seconds, thus the injected substance will be drained from the tail
- The inoculated mice are placed in the initial box where will be fed and have the same microclimate conditions for 14 days, marking daily the deceased animals.

Days 1 – 14: - to the mice from group II it was daily administered intra peritoneal 0.5 ml MXP 4509 substance and to mice from group III, 1 ml by gavage.

- The inoculated mice are daily monitored and marked down the mortality from each group;
- The dying mice are considered deceased for the following day;
- In days 3, respectively 7, from each group the kidneys are collected from a mouse, macroscopically examined and prepared a homogenised mixture with

physiologic solution using Turbomax device. From this mixture are prepared serial decimal dilutions and cultured on YPD Agar to determine the fungal burden per gram of renal tissue.

Results:

The obtained results are presented in table 1 and 2.

The comatose signs were preceded by nervous symptoms in most of the deceased mice, probably due to renal failure.

The administration of the antifungal substance has significantly improved the survival rate in animals inoculated with *Candida albicans*, the lowest mortality being registered after daily oral administration of a dose of 50 mg/kg bw active substance.

Table 1. The dynamics of mortality (deceased /day) to those 3 groups of mice

Day	Group I	Group II	Group III
3	1	-	-
4	1	-	-
5	1	-	-
6	6	3	1
7	10	2	2
8	5	2	1
9	3	-	-
10	1	-	-
11	2	-	-
Total mortality in day 14	100%	23.3%	13.3%

Table 2. The dynamics of the fungal burden (CFU/g) in renal tissue for all 3 groups of mice

Day	Group I	Group II	Group III
3	$4x10^5$	10^5	$0.3x10^5$
7	$2.2x10^5$	$2.7x10^4$	$1.7x10^4$

Concerning the fungal burden in renal tissue it is noticed a reduction for treated groups in comparison with control group, aspect correlated with the mortality dynamics.

Screening of strains from personal microorganism collection for biofilms production

The used biologic material for tests was 220 yeast strains from clinical isolates, which were previously identified as *Candida albicans* strains by germ tube test and biochemical tests. These strains were selected by molecular biology tests to accurately identify only *C. albicans* strains – known to produce *in vitro* and *in vivo* biofilms. The *C. albicans* strains were cultured in special conditions to form biofilms, in microplates with 96 wells and were treated with different concentrations of MXP-4509.

Differentiation of *Candida albicans* strains from *Candida dubliniensis* strains by duplex PCR

The accurate differentiation of *Candida albicans* strains and *Candida dubliniensis* strains, exclusively base on phenotypical features is not possible due to their inconstant results. To confirm the identification of *Candida dubliniensis* strains is preferred for its safety and speed, a technique of molecular biology where two pairs of primers are used: one specific to an intron of a gene coding the actin (DUBF / DUBR) and other for universal primers for ribosomal DNA (ITS1 / ITS4). From the two mentioned species, only *Candida dubliniensis* generates after amplification two lines, one of 288 base pairs (bp) with DUBF / DUBR set and one of approximately 600 bp, with universal primers. *Candida albicans* will generate only one line, the one of 600 bp, with universal primers.

For differentiation between *Candida albicans* strains and *Candida dubliniensis* strains by duplex PCR, the following pairs of primers were used:

- ITS 1: 5′TCCTGAGGTGAACCTGCGG3′;

- ITS 4: 5´TCCTCCGCTTATTGATATGC3´;
- DUBF: 5´GTATTTGTCGTTCCCCTTTC3´ (nucleotides 251-270);
- DUBR: 5´GTGTTGTGTGCACTAACGTC3´ (nucleotides 519-538).

Method:

The reactive quantities are calculated for the mix preparation, taking into account all the four samples that will be used (positive control 1, positive control 2, negative control, sample to be tested). The quantities of reactive substances will be added for a sample more, thus, when the mix is divided, the necessary quantities for all four samples will be enough.

The reactive substances are defrozen and maintained in ice until are used;

The mixed is prepared, the enzyme is added and mixed well;

The mix is distributed in 0.2 ml PCR tubes

It is added:

- 1 µl DNA *Candida albicans* as positive control 1; sample
- 1 µl DNA *Candida dubliniensis* as positive control 2 sample;
- 1 µl DNA from the strain that needs to be identified as testing sample;
- 1 µl of purified water as negative control sample.

The tubes are introduced inside the thermocycler and the following programme is run:

50ºC/5 minutes

95ºC/ 10 minute

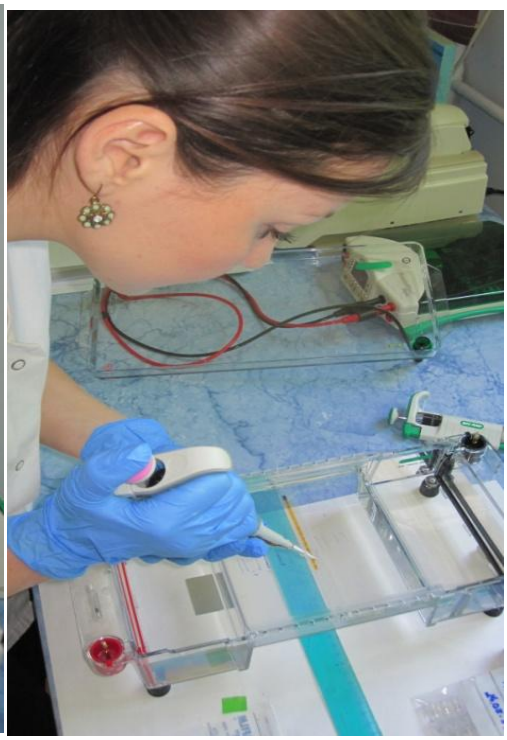
then, 94ºC/ 30 sec.

58ºC/ 30 sec. 30 cycles

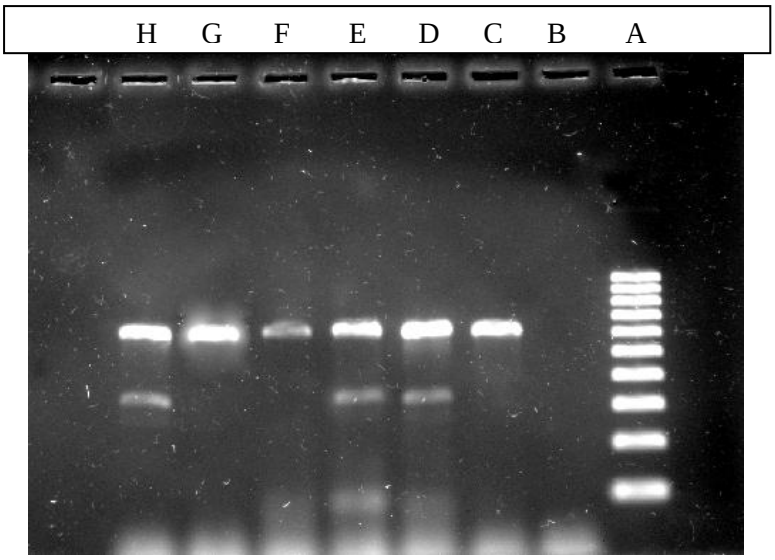
72ºC/ 30 sec.

then, 72ºC/ 10 minutes.

After the programme is ended, the samples are kept at + 4ºC until they are visualised in gel agarose.

The PCR products were resolved by horizontal gel electrophoresis in a 25 cm by 50 cm gel prepared with 1.5% (wt/vol) agarose and 1xTBE (Tris-borate-EDTA) buffer. Three microliters of loading dye and three microliters of GelRed™ (1:1000, ADN stain, Interchim) were added to three microlitres of PCR product and this mixture was loaded into wells prepared with an 8 mm by 1 mm comb tooth size, in the presence of DNA ladder 100 bp (Promega). Gels were subjected to 80 V/cm, 45 mA, 45 w for 55 minutes in 1x Tris-borate-EDTA. Gel images were captured as 8-bit TIFF images using Quantity One gel documentation software with a CCD gel documentation system.



A= 100bp DNA ladder marker, B = negative control sample, the second wells from right to left, C= *C. albicans* type strain, D= *C. dubliniensis* type strain (the one with two lines), E= *C. dubliniensis*, F= *C. albicans*, G= *C. albicans*, H= *C. dubliniensis*.

Out of 86 strains positive for germ tube test GTT (+), 80 *Candida albicans* strains were confirmed by PCR.

Tests for determination of the ability to form biofilms

For the tests to determine the ability to form biofilms were used the following materials:

- *Candida albicans* strains;
- microplates with 96 wells;
- Sabouraud Dextrose Agar (SDA);
- Yeast Nitrogen Base broth (YNB);
- Phosphate buffer saline (PBS);
- calibrated loops of 10 µl and 1 µl (Biosigma)
- Automated micropipettes (Biohit, Germania);
- Orbital mixer;
- XTT (Sigma-Aldrich Corp.);
- menadione solution;
- Microbiological hood (ABS Class II Cabinet BioQUELL, UK).

For this test were selected 80 *Candida albicans* strains. Before each experiment, the *Candida albicans* strains are aerobically cultured on Sabouraud Dextrose Agar (SDA) for 18 hours at 37°C, and then a full loop of young yeast culture is inoculated in Yeast Nitrogen Base broth (YNB) supplemented with 50 mM glucose.

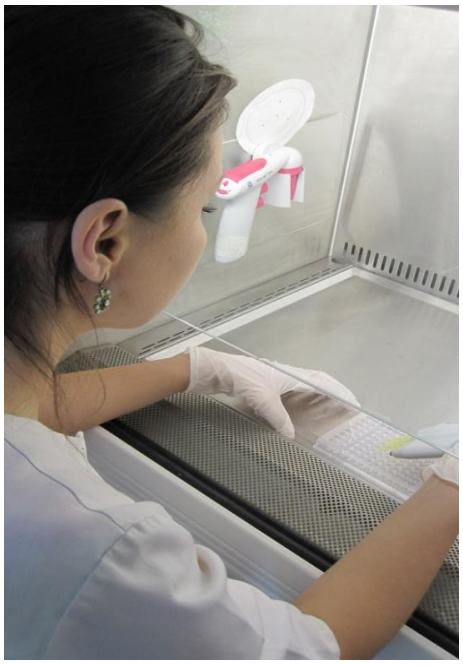
After 18 hours incubation period, the cells, at the end of exponential growth phase, are washed twice with phosphate buffer saline (PBS) (pH 7.2) and are suspended in Yeast Nitrogen Base broth (YNB) supplemented with 100 mM glucose.

The standard *Candida* suspensions are prepared from a 10^7 cells/ml concentration, adjusting the turbidity (DO) with the McFarland standards using a spectrophotometer.

Biofilms formation

The yeasts are cultured in microplates with 96 wells. Volumes of 100 µl from standard *Candida* (10^7 cellse/ml) suspension are transferred in each well and incubated for 1.5 hours (adhesion phase) at 37°C, on an orbital mixer at 75 rot/min. After adhesion phase, the cellular

suspension is slowly aspired, then each well is washed twice with PBS to remove free cells, carefully not to disturb the cells in adhesion.



The wash of wells with PBS

The incubation of the microplate – adhesion phase

In order to allow the formation of the biofilm, in each well is added 200 µl freshly prepared YNB broth supplemented with 100 mM glucose.

The microplate is incubated for 24 or 48 hours at 37 ° C at 75 rpm, on an orbital mixer. After 24 hours of incubation, the medium is aspired, biofilms are washed twice with PBS and are added 200 µl YNB broth in each wells. At various timings, the biofilms are quantified using the XTT reduction test. All tests are repeated for 6 times, in two different stages.

The test for oxidative activity

Is used XTT in PBS with 200mM glucose. XTT is dissolved in PBS at a final concentration of 1 mg/ml. The solution is sterilised by filtration and is conserved at -70°C until it needs to be used.

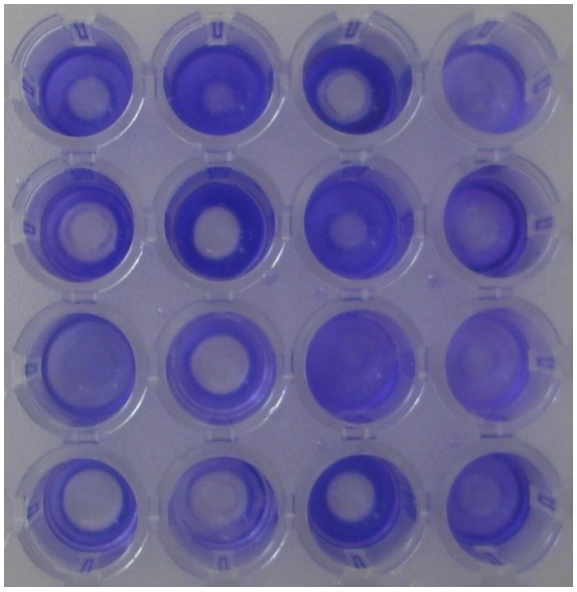
For each test, the XTT solution is defrozen on ice and is mixed with menadione solution in 20:1 concentration. The biofilms are washed twice with 200 µL PBS to eliminate the cells that are not adhering. Further, 158 µl PBS, 40 µl XTT and 2 µl menadione are transferred to each wells. The microplate is covered with aluminium and incubated at 37°C for 3 h in dark place.

Colorimetric changes are measured at 492 nm, using a microplate reader.

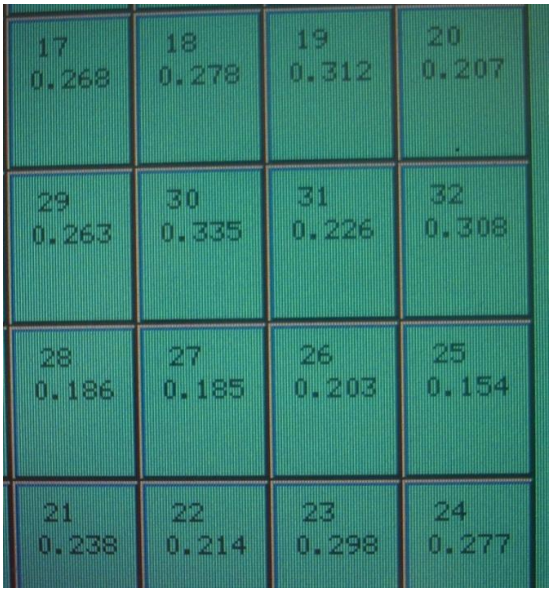
DO values for 80 *Candida albicans* strains after reading them in spectrophotometer

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B					0,162	0,257	0,217	0,294	0,153	0,308	0,283	0,186
C	0,243	0,225	0,298	0,267	0,322	0,168	0,289	0,278	0,268	0,278	0,312	0,207
D	0,328	0,269	0,214	0,189	0,256	0,247	0,306	0,217	0,236	0,335	0,226	0,308
E	0,192	0,251	0,322	0,284	0,303	0,152	0,248	0.239	0,186	0,185	0,203	0,154
F	0,285	0,323	0,285	0,166	0,273	0,216	0,332	0,264	0,238	0,214	0,298	0,277
G	0,167	0,274	0,318	0,279	0,249	0,227	0,327	0,278	0,279	0,314	0,286	0,169
H	0,327	0,275	0,172	0,286	0,259	0,332	0,296	0,207	0,279	0,182	0,315	0,296

It may be noticed that the biofilm is better formed as the colour becomes more intense and the turbidity (DO) read spectrophotometricaly, higher.



The wells with biofilms, after XTT treatment



DO values spectrophotometrically read

After reading the DO it was noticed that all *Candida albicans* strains formed biofilms, the values being between 0.154 and 0.335. From all 80 *Candida albicans* strains, 37 formed an obvious biofilm, with values over 0.250. These strains were treated with MXP-4509 substance.

The *in-vitro* tests of the antifungic effect on biofilms.

The 37 *C. Albicans* strains, highly producing of biofilms, were used to test the antifungic effect of MXP-4509 compound. The applied method was similar to the one previously described for obtaining the biofilms in the wells of microplates, the stages being as follows:

- Addition of the yeast suspension in the wells of the microplates and incubation (adhesion phase)
- Removing the inoculum and washing with PBS
- Adding a new culture medium containing different concentrations of MXP-4509 (512, 1024 and 2048 mg/l) and incubation for 24 hours
- Removing the medium containing the antifungal substance and addition of the XTT substance, followed by incubation for 180 minutes and reading of the DO at spectrophotometer.

The DO values for 37 *Candida albicans* strains after reading them on spectrophotometer (for conc. 2048 mg/l MXP-4509)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	0,034											
C	0,104	0,098	0,112	0,145	0,153	0,087	0,122	0,201	0,203	0,075	0,144	0,161
D	0,196	0,107	0,163	0,189	0,256	0,247	0,306	0,217	0,236	0,335	0,226	0,308
E	0,058	0,077	0,145	0,164	0,084	0,077	0,132	0,202	0,118	0,088	0,174	0,125
F												
G												

H												

After treating the biofilms with a substance containing 2048 mg/l MXP-4509, the optical density was significantly lower for the untreated group or for those treated with 512 or 1024 mg/l, indicating the ability of this concentration to inhibit the development of biomass in biofilms. The inhibition is not complete, but is more than 50% of the average, fact proved for other azole compounds with antifungal effect. The antifungal effect of the nano conjugates is showed on yeast biofilms due to high hydro-solubility degree of the active substance, enhanced by the beta-cyclodextrin used as carrier agent.