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Evaluation of the Etest for the assessment of synergy of antibiotic combinations against multi-resistant *Pseudomonas aeruginosa* isolates from Cystic Fibrosis patients

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Abstract

The determination of synergistic effects of antimicrobial combinations can lead to improved therapeutic options in the antibiotic treatment of cystic fibrosis patients, who are chronically infected with multi-resistant *Pseudomonas aeruginosa* isolates. In this study we evaluated the performance of the Etest in the assessment of synergy in comparison to the standard agar dilution checkerboard susceptibility test and determined the activity of two antimicrobial combinations against 163 multi-resistant *Pseudomonas aeruginosa* cystic fibrosis isolates. The agreement between the checkerboard and the Etest susceptibility test method was excellent (>90%) for both, non-mucoid and mucoid cystic fibrosis strains. The highest rate of synergy was observed for the antibiotic combination of ceftazidime and tobramycin (28.8 % of the cystic fibrosis strains) as opposed to the antibiotic combination of meropenem and tobramycin (19.0 %). However, the probability of synergy for the second antibiotic combination increased significantly when for the first antibiotic combination synergy had already been demonstrated (Fischer's exact test p=0.049). We conclude that the Etest is a valuable and practical method for routine microbiological diagnostics improving the antibiotic options in the treatment of cystic fibrosis patients chronically infected with *Pseudomonas aeruginosa*.

Introduction

Pseudomonas aeruginosa has emerged as a major opportunistic pathogen and a significant source of life-threatening nosocomial infections. It is also the most dominant bacterial pathogen that can be recovered from the chronically infected lung of cystic fibrosis (CF) patients [1-4]. Antimicrobial therapy has made an important contribution to increase the life expectancy of CF patients and is one of the cornerstones of the treatment of the disease [5,6]. Due to the frequent requirement of antibiotic therapy for treating pulmonary exacerbation and to the slow progression of lung disease, multi-resistant *Pseudomonas aeruginosa* isolates are increasingly recovered [7]. Thus, the use of antibiotic combinations exhibiting synergistic effects are a valuable addition to standard treatment regimes and may overcome treatment failures [8]. To increase bactericidal activity a combination of an aminoglycoside with a ß-lactam antibiotic is generally used. This antimicrobial combination typically exhibits synergistic activity *in vitro* [9,10] and an increased efficacy in the treatment of CF patients [11,12]. Synergism appears to be maintained even at very high MICs with drug combinations within achievable therapeutic ranges [13-16]. However, empirically chosen antibiotic combinations may not act with synergy against all multi-resistant *Pseudomonas aeruginosa* isolates [17,18] and due to the unpredictable response of *Pseudomonas aeruginosa,* a prior assessment of the activity of antimicrobial combinations is deemed necessary. *In vitro* synergism between two antibiotics is usually determined by the checkerboard titration technique or by time-kill methods. However, as the performance of these methods is very time- and material-consuming, neither is used in routine microbiological diagnostics. Recently, the Etest has been successfully used to assess the activity of antimicrobial agents in various bacterial pathogens, including *Pseudomonas* species [19-22] and it has been suggested that the Etest may provide an alternative method for the evaluation of synergy [23-25]. This study was designed to compare the reliability and reproducibility of the Etest versus the checkerboard agar dilution method for the assessment of the activity of antimicrobial

combinations in *Pseudomonas aeruginosa* CF isolates, known to exhibit phenotypes that adversely affect the performance and interpretation of standard antimicrobial susceptibility testing. A secondary objective was to define the degree of synergism of either ceftazidime or meropenem combined with tobramycin in a total of 163 ceftazidime and meropenem resistant *Pseudomonas aeruginosa* isolates that have been recovered from CF patients of two German CF centres (Munich / Hanover).

Material and methods

Bacterial strains

One hundred and sixty-three multi-resistant CF isolates of *Pseudomonas aeruginosa* were included in this study. 70 isolates were recovered from 24 patients who attended the CF outpatient clinic in Hanover, Germany within a five month period in 2002 [26]. A total of 93 clinical *Pseudomonas aeruginosa* isolates were recovered from 58 patients at the CF outpatient clinic in Munich, Germany during 1998-2004. Several isolates from one patient were included if they exhibited different phenotypes (e.g. mucoid versus non-mucoid).

The bacterial strains were identified as *Pseudomonas aeruginosa* on the basis of a positive oxidase reaction, positive catalase, growth at 42 °C and pigment production. When necessary, strains were further evaluated using the API 20 NE system (bioMérieux, Marcy-l'Etoile, France).

The strains were stored at –70 °C. After thawing the *Pseudomonas aeruginosa* isolates were subcultured twice onto Columbia blood agar (Becton Dickinson, Germany) and checked for purity before susceptibility testing was performed.

Pseudomonas aeruginosa ATCC 27853 was included as a quality control strain for all MIC determinations.

MIC determination

The MICs of tobramycin, ceftazidime and meropenem were determined for the 163 multi-resistant *Pseudomonas aeruginosa* isolates by both the agar dilution and the Etest susceptibility test method.

Susceptibility testing was performed on 18-24 h old subcultures of the clinical strains. A single inoculum adjusted to a McFarland standard of 0.5 in 0.9% NaCl (densimat; bioMérieux, France) was used. Standard agar dilution was performed according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, now CLSI) [27]. Cation-adjusted Mueller-Hinton agar plates containing a serial twofold dilution of the antimicrobial agents were prepared in-house. The plates were stored at 4 °C and used within 5 days. Inoculation was performed with an AM80 automatic inoculator (Dynatech, Zug, Switzerland) and the MIC results were recorded after an incubation time of 18-24 h at 36° C. NCCLS breakpoints were used for the assessment of interpretive category results [27]. The checkerboard method was performed the same way using two combinations of antibiotics: ceftazidime (Glaxo-Wellcome, Germany) with tobramycin (Infectopharm, Germany) and meropenem (Astra Zeneca, Germany) with tobramycin. The antibiotic concentrations ranged from 0.25 to 32 µg/ml for ceftazidime, 0.125 to 16 µg/ml for meropenem and 0.25 to 32 µg/ml for tobramycin.

For the Etest, Mueller-Hinton agar plates (Becton Dickinson, Cockeysville, Md.) were inoculated with swabs saturated with suspensions of the test organism equivalent to a 0.5 McFarland standard for non-mucoid strains and equivalent to a 1.0 McFarland for the mucoid strains. For the evaluation of the MICs of single antimicrobial agents the Etest strips (AB BIODISK, Sweden) were brought to room temperature prior to use and placed onto an agar plate. The Etest results with one-half increment were rounded up to the next highest two-fold dilution before compared to the results of the agar dilution method. The antibiotic concentration range of the Etest was 0.016 to 256 µg/ml for ceftazidime; 0.002 to 32 µg/ml for meropenem and 0.016 to 256 µg/ml for tobramycin. The results were read after 18-24 h of incubation at 36 °C. The MIC was interpreted as the value at which the inhibition zone intersected the scale on the Etest strip. For combination testing a first antibiotic strip was placed onto an agar plate at room temperature as previously described [24] and removed after

1h. Afterwards a second antibiotic strip was placed on top of the gradient of the first agent. To evaluate the effect of an antibiotic combination the fractional inhibitory concentration (FIC) index was calculated as follows:

FIC index = FIC of drug A + FIC of drug B; FIC of drug A = MIC of drug A in combination / MIC of drug A alone and FIC of drug B = MIC of drug B in combination / MIC of drug B alone. Synergism was defined as an FIC index of ≤ 0.5, additivity was defined as an FIC index of $>0.5 \leq 1$, indifference was defined as an FIC index $>1 \leq 2$ and antagonism was defined as an FIC index of >2.

Results

Strain characteristics

The Pseudomonas aeruginosa *strains tested in this study were multi-resistant isolates recovered from the respiratory tract of patients, who attended the CF outpatient clinic in Hanover or Munich. 70 strains were isolated in Hanover and 93 in Munich. The interpretive category results of the strains were determined by both the reference agar dilution and the Etest method and are listed in Table 1. Susceptibility testing by the agar dilution method was performed in Hanover and in Munich (according to their origin), whereas susceptibility testing by the Etest was performed in Hanover for all 163* Pseudomonas aeruginosa *strains. None of the 163 strains exhibited meropenem or ceftazidime MIC values in the susceptible range by either susceptibility test method (inclusion criteria of this study), whereas more than 60 % of the isolates were tobramycin susceptible.*

Reproducibility and inter-laboratory variability

The reproducibility of the MIC results was determined for both the Etest and the agar dilution susceptibility test method. Ceftazidime, meropenem and tobramycin MIC values of eight Pseudomonas aeruginosa *isolates were determined at four different points in time by both methods. The MIC results of all stains for the three antibiotic agents were within 1 log_2 dilution as determined by both susceptibility test methods indicating excellent reproducibility. The inter-laboratory variability of Etest MIC determination was also determined. MIC results of the eight* Pseudomonas aeruginosa *isolates as determined by the Etest in Munich were equivalent (within 1 log_2 dilution) to those obtained in Hanover for all three antimicrobial agents.*

Correlation of the MIC values of single antibiotics as determined by the Etest versus those determined by the agar dilution susceptibility test method

The Etest method for MIC determination was further compared to the reference agar dilution susceptibility test method and the frequency of category interpretive errors between the two test methods was determined for ceftazidime, tobramycin and meropenem. No serious errors were recorded for either antimicrobial agent (susceptible as determined by the Etest and resistant as determined by the standard agar dilution method (very major error) or resistant as determined by the Etest and susceptible as determined by the standard agar dilution method (major error)). The overall rate of minor errors (minor error 1 or minor error 2) was below 13 % (defined as a change in the interpretive category result from susceptible or intermediate to intermediate or resistant (minor error 1) or from resistant or intermediate to intermediate or susceptible (minor error 2) as compared to the reference agar dilution method).

FIC index agreement and synergy testing of 163 clinical *Pseudomonas aeruginosa* strains

A main objective of this study was the evaluation of the Etest as a method to assess synergy of antimicrobial combinations in comparison with the standard agar dilution checkerboard method. Since the MIC values of the single antibiotic testing correlated well between the Etest and the agar dilution method (as previously reported), we further determined the fractional inhibitory concentrations (FIC) indices of antimicrobial combinations for both the Etest and the checkerboard agar dilution method (Table 2). The agreement of the FIC indices - as defined as the FIC index of the same or $\pm$ 1 category - was excellent (> 93%) between the checkerboard agar dilution and the Etest method for both antibiotic combinations. A separate analysis of the agreement of the FIC indices of mucoid and non-mucoid strains (97.1 % versus 95.1% agreement) and the agreement of the FIC indices of the strains recovered in Munich and the strains recovered in Hanover revealed comparable results (91.3% agreement

for the mucoid and 96.2% agreement for the non-mucoid recovered in Munich in comparison to 87.5 % agreement for the mucoid and 96.6% agreement for the non-mucoid recovered in Hanover).

The second major aim of this study was to determine the *in vitro* efficacy of the antimicrobial combinations ceftazidime/tobramycin and meropenem/tobramycin against the 163 multiple resistant *Pseudomonas aeruginosa* clinical CF isolates. Overall, the antibiotic combination of ceftazidime and tobramycin was more effective than the antibiotic combination of meropenem and tobramycin (Table 2). More synergistic effects were observed for the antibiotic combination of ceftazidime/tobramycin and less antagonistic activities. Whereas, overall additivity was the most dominant effect for the antibiotic combination of ceftazidime and tobramycin by both test methods, indifference was most dominant as determined by the checkerboard agar dilution method and additivity as determined by the Etest for the antibiotic combination of meropenem and tobramycin. A separate analysis of synergy testing among mucoid and non-mucoid strains revealed comparable results for both phenotypes. We further determined whether the FIC indices of the two antibiotic combinations were linked. For example, synergism for both antibiotic combinations as determined by the Etest was observed in 13 out of 163 multiple resistant *Pseudomonas aeruginosa* isolates (Table 3), while the expected value would have been 8.94. Fishers exact test revealed a significant dependency ($p=0.049$) of the FIC indices for the two antibiotic combinations.

Discussion

In CF patients multi-resistant *Pseudomonas aeruginosa* strains are continuously emerging due to prolonged and frequent administration of antimicrobial therapy in these patients to manage pulmonary exacerbation. In order to benefit further from antimicrobial therapy a combination of two antipseudomonal antibiotics is usually administered and has been shown to be superior to antibiotic mono-therapy in *Pseudomonas aeruginosa* infections [11,12]. Since the traditional microbiological laboratory susceptibility results are of single antibiotics, antimicrobial combinations are usually chosen empirically. However, the response of *Pseudomonas aeruginosa* to various antimicrobial combinations has been shown to be unpredictable and thus an empirically chosen antimicrobial combination can be potentially counterproductive. Hence, to increase the therapeutic options available for the clinician a simple and reproducible technique to detect *in vitro* synergy would be desirable.

In this study we systematically analysed the performance of the Etest for synergy testing in comparison to the checkerboard agar dilution method. 163 highly resistant clinical *Pseudomonas aeruginosa* CF isolates - non-mucoid and mucoid strains - were included in this study. CF strains were anticipated to be problematic in the performance of susceptibility testing [19] and we aimed to evaluate whether the results of two methods remained comparable. We chose ceftazidime and meropenem in combination with tobramycin as two antimicrobial combinations to be evaluated in this study as both combinations are commonly used in the clinical setting and have been previously described to act synergistically [16,28]. In this study Etest MIC results of single antibiotics correlated well with those obtained by the agar dilution method and no serious errors were recorded. This is in accordance with several previous studies that report on the good correlation of the MIC results of the Etest with those of the agar dilution susceptibility testing for various antimicrobial agents [19,21-23,29]. Moreover, the reproducibility of MIC results as determined by the Etest was excellent, no serious discrepancies were recorded even by comparing the MIC values obtained at different

laboratories. Although several studies reported on the good correlation of the Etest MIC results with standard agar dilution susceptibility testing of single antibiotics, there are only very few data on the performance of the Etest detecting synergy [23-25].

In this study antimicrobial combinations were tested by superimposing the Etest strips on the same agar plate. A comparison of the results with those obtained by checkerboard agar dilution revealed that the overall agreement of the synergism results of the 163 clinical *Pseudomonas aeruginosa* CF isolates between the two methods was > 90%. Whereas more synergism or antagonism was observed using Etest (Table 2) the checkerboard agar dilution revealed additivity or indifference more frequently.

The second major aim of this study was to evaluate the synergistic activity of antimicrobial combinations on 163 clinical multi-resistant *Pseudomonas aeruginosa* CF isolates. The antibiotic combination of ceftazidime/tobramycin was slightly more effective than the combination of meropenem/tobramycin (t-test p=0.035). 75.5% and 63.8% of the strains were affected by synergism or additivity by the respective antimicrobial combinations which is in good agreement with the results of a previous study on the antimicrobial susceptibility of multi-resistant *Pseudomonas aeruginosa* strains [30]. Not only was the rate of synergism higher but also the rate of antagonism was lower for the combination of ceftazidime/tobramycin as compared to meropenem/tobramycin. This had been previously demonstrated in a study evaluating the synergistic activity of ceftazidime and a carbapenem in combination with tobramycin against *Pseudomonas aeruginosa* CF isolates using an automated broth microdilution checkerboard test system [31]. Another finding of our study was that the results of synergy testing as determined for the two antibiotic combinations were not independent. If for one particular *Pseudomonas aeruginosa* strain synergy had been demonstrated for one antibiotic combination there was a significantly increased rate of synergism for the second antibiotic combination (p= 0.049, Fischer's Exact test).The assessment of the *in vitro* synergy of antimicrobial combinations will provide more options

for successful antibiotic therapy in the clinical setting. The results of this study demonstrate that the Etest is a rapid, reproducible, easy to perform and flexible method for the determination of synergistic activity that could significantly contribute to the improvement of therapeutic options in the treatment of chronic *Pseudomonas aeruginosa* infections. Future clinical studies will have to elucidate whether *in vitro* synergy testing is useful in the clinical setting and whether the results of synergy testing influences the clinical outcome in CF patients colonised with multi-resistant *Pseudomonas aeruginosa* strains.

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Table 1 Interpretive category results generated after 24 h for mucoid and non-mucoid CF isolates

Susceptibility test method	Antimicrobial agent	All strains 163 strains			Non-mucoid *Pseudomonas aeruginosa* 111 strains			Mucoid *Pseudomonas ae* 52 strains	
		S[a] (%)	I[a] (%)	R[a] (%)	S[a] (%)	I[a] (%)	R[a] (%)	S[a] (%)	I[a] (%)
Agar dilution	Ceftazidime	0 (0.0)	30 (18.4)	133 (81.6)	0 (0.0)	19 (17.1)	92 (82.9)	0 (0.0)	11 (21
	Meropenem	0 (0.0)	37 (22.7)	126 (77.3)	0 (0.0)	29 (26.1)	82 (73.9)	0 (0.0)	8 (15.4
	Tobramycin	100 (61.3)	31 (19.0)	32 (19.6)	66 (59.5)	18 (16.2)	27 (24.3)	34 (65.4)	13 (25
Etest	Ceftazidime	0 (0.0)	27 (16.6)	136 (83.4)	0 (0.0)	16 (14.4)	95 (85.6)	0 (0.0)	11 (21
	Meropenem	0 (0.0)	33 (20.2)	130 (79.8)	0 (0.0)	21 (18.9)	90 (81.1)	0 (0.0)	12 (23
	Tobramycin	115 (70.6)	38 (23.3)	10 (6.1)	73 (65.8)	28 (25.2)	10 (9.0)	42 (80.8)	10 (19

[a] susceptible (S), intermediate (I) and resistant (R) interpretive category result

Table 2 FIC indices for the antibiotic combination of ceftazidime/tobramycin (A) and meropenem/tobramycin (B) as determined by the agar dilution method compared to the Etest.

A

Ceftazidime/ Tobramycin		FIC[a] index agar-dilution s[b]	ad[b]	i[b]	a[b]	Total
FIC[a] index	s[b]	32	14	1	0	47
Etest	ad[b]	15	56	3	2	76
	i[b]	0	14	12	2	28
	a[b]	0	7	5	0	12
Total		47	91	21	4	163

[a]Fractional inhibitory concentration (FIC)
[b] synergism (s), additivity (ad), indifference (i), antagonism (a)

B

Meropenem/ Tobramycin		FIC[a] index agar-dilution s[b]	ad[b]	i[b]	a[b]	Total
FIC[a] index	s[b]	17	13	1	0	31
Etest	ad[b]	3	41	29	0	73
	i[b]	0	2	25	2	29
	a[b]	0	3	16	11	30
Total		20	59	71	13	163

[a]Fractional inhibitory concentration (FIC)
[b] synergism (s), additivity (ad), indifference (i), antagonism (a)

Table 3 FIC indices as determined by the Etest for the antibiotic combination of ceftazidime/tobramycin as compared to the combination of meropenem/tobramycin

		Meropenem/Tobramycin FIC [a] indices				
		s[b]	ad[b]	i[b]	a[b]	Total
Ceftazidime / Tobramycin FIC [a] indices	s[b]	13	22	7	5	47
	ad[b]	17	34	13	12	76
	i[b]	1	12	7	8	28
	a[b]	0	5	2	5	12
Total		31	73	29	30	163

[a]Fractional inhibitory concentration (FIC)
[b] synergism (s), additivity (ad), indifference (i), antagonism (a)

Phagocytosis assay based on living *Candida albicans* for the detection of effects of chemicals on macrophage function

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ABSTRACT

Phagocytosis is the first step of defence against infections from the innate immune system, as it is the process of internalization of pathogens by cells with phagocytic activity, such as macrophages, which is followed by pathogen killing and destruction. Thus, phagocytosis assays are used as assays for one function of the innate immune system. As fungal infections are of increasing relevance and phagocytic mechanisms are dependent on the pathogenic organism and its viability, we established a microtiter plate phagocytosis assay based on viable, fluorescence – labelled *Candida albicans*. The distinction between internalized yeast cells and cells attached to macrophages was done via quenching of FITC - fluorescence by trypan blue, and the remaining fluorescence was quantified and used as indicator of the phagocytosis efficiency. As a proof of principle we showed that compounds acting on the dynamics of the actin cytoskeleton of the macrophages reduced the phagocytosis efficiency in a concentration dependent manner.

INTRODUCTION

Macrophages are the cells of the immune system, in particular of the unspecific innate immune system, which are responsible for the cell-mediated defence against invading foreign

material through phagocytosis. If this phenomenon of defence is lacking or disturbed then organisms are not able to protect themselves from external organisms, such as pathogenic bacteria or fungi, which are usually eliminated by phagocytosis. Such a disturbance could happen, for example, as a consequence of treatment with immunosuppressive agents. Thus, it is important to evaluate the consequences of pharmacological treatments, i.e. the effects of drugs, also on the phagocytotic activity of cells such as macrophages.

Phagocytosis is the process by which foreign particles are internalized. Besides macrophages neutrophils and dendritic cells, the so-called phagocytes, are cells specialized for this task. It is an essential part of the innate immune system, as those cells unspecifically react with all types of foreign materials, for example pathogenic organisms, without the requirement of a previous infection. The internalization of the pathogen is followed by killing and destruction of the pathogen and presentation of antigens on the surface of the phagocytes to stimulate, together with secreted cytokines, other cells of the immune system. In all phagocytosis is a complex sequence of reactions, which involves not only signal transduction from the recognition of the pathogen by cell surface receptors to gene expression in the nucleus, but also to the regulation of the dynamics of cellular structures, such as the cytoskeleton, in particular the actin filaments (Aderem and Underhill, 1999; Strzelecka et al., 1997). Any disturbances of the phagocytic process may have adverse consequences on the elimination of pathogens and may lead to an increased susceptibility for infections.

Not only pharmacological treatment with immunosuppressive agents can lead to a reduced activity of the immune system, but also exposure to chemicals. For example a decrease of the phagocytotic activity is observed, when cytochalasin, a chemical compound, which reduces the stability of actin filaments, is present. As patients with a compromised immune system, in particular the innate immune system, increasingly suffer in particular from fungal infections (Algarra et al., 2002), the evaluation of the effects of chemicals on the phagocytosis of fungi is of special relevance.

In *in vitro* phagocytosis assays a broad range of materials to be phagocytosed was used. These range from particles, such as polystyrene or latex beads, to components of living organisms, such as zymosan, an insoluble fraction from the yeast cell wall, and whole organisms, such as bacteria or fungi. Though the general principles of phagocytosis are the same for all materials, different materials are recognized by different receptors (O´Neill 2006), and thus, molecular details of the subsequent reaction cascades depend on the phagocytosed material. Successful infections by pathogenic organisms occur, if the pathogen survives the phagocytic attack (Urban et al., 2006). Some pathogens utilize the signal transduction machinery of host cells and even multiply within the phagocytes. Other pathogens, in particular fungi, are able to escape again after internalization, e.g. by the formation of hyphae. Thus, phagocytosis assays for the evaluation of the effects of compounds on the success of phagocytosis should be as close as possible to the real infection and should include viable pathogens. However, most phagocytosis assays utilize heat-killed bacteria or yeasts or even beads as models for the foreign material.

The best established formats of i*n vitro* phagocytosis assays are based on the incubation of the phagocytes (neutrophils or macrophages) with a fluorescently labelled foreign material for a defined period of time, ranging from several minutes to several hours. Fluorescein or corresponding dye substitutes are most frequently used as labels, but other dyes are also possible (Algarra et al., 2002), of which pH-sensitive dyes could be of particular interest (Beletskii et al., 2005). Labelling of all kinds of material is achieved by its incubation with an amino-reactive derivative of the dye, such as fluorescein isothiocyanat (FITC) (Liu et al., 2000) or carboxyfluorescein succinimidyl ester (FAM-SE; Busetto et al., 2004). Only living organisms can be labelled by treatment with carboxyfluorescein diacetate succinimidyl ester (CFDA/SE or CFSE). This is a non-fluorescent compound, which easily diffuses through the cell membrane and is hydrolysed by intracellular esterases to the fluorescent carboxyfluorescein succinimidyl ester, which spontaneously couples to intracellular proteins

(Tuominen-Gustafsson et al., 2006, Vander Top et al., 2006). In inactivated organisms, e.g. in heat-killed organisms, also the esterases are inactivated and, hence, not able to form CFSE.

The amount of internalized fluorescent material correlates to the phagocytotic activity and was determined via the fluorescence of phagocytes. As a prerequisite fluorescence from particles, which were only attached or did not interact with the phagocytes at all, had to be eliminated. This distinction was achieved by extensive washing (Algarra et al., 2002; Peiser et al., 2000), treatment with lysozyme (Hrabak et al., 2006), but most frequently by quenching of fluorescence by trypan blue (Wang et al., 2006; Wan et al., 1993; Bjerknes and Bassoe, 1984). Trypan blue absorbs light in the range from 475 – 675 nm (Wang et al., 2006), which covers the wavelength of fluorescein fluorescence emission (519 nm) so that fluorescein fluorescence is efficiently quenched by trypan blue. Usually trypan blue is used to stain not-living cells, as it can permeate only damaged cell membranes. Thus, in phagocytosis assays it can interact only with those fluorescent particles, which are outside the macrophages, and not with those, which were internalized (Bjerknes and Bassoe, 1984). Consequently, fluorescein fluorescence is not quenched from particles, which were internalized by phagocytes, and from living pathogens which were stained with CFSE, as in these situations the fluorophore is protected by intact cell membranes. That is why by addition of trypan blue attached and internalized particles are distinguished, if they were stained for example with FITC, or if CFSE-stained living pathogens were killed prior to their application to the phagocytes. The resulting fluorescence of the phagocytes was detected by flow cytometry (Liu et al., 2000), fluorescence microscopy (Vander Top et al., 2006) or by fluorescent microtiterplate readers (Wan et al., 1993). If large numbers of samples are to be analysed microtiterplate readers are usually the method of choice due to the possibility of automation and the generation of quantitative data.

Due to the increasing relevance of fungal infections in immunocompromised patients, we are interested in effects of chemical compounds on the phagocytosis of fungi. To reflect these

infections as realistic as possible we decided to use not the already established *in vitro* phagocytosis assays based on beads, zytosan or heat-killed *E. coli* or *S. cerevisiae*, but to establish a microtiter plate phagocytosis assay using living *Candida albicans*, which is the most important pathogenic fungus, together with a macrophage cell line as representative phagocyte. We established the fundamentals of the phagocytosis assay using *Candida albicans* stained with fluorescein via incubation with CFSE (Behnsen et al., 2007) followed by heat-killing of the organism and quenching of fluorescence of not-phagocytosed pathogens with trypan blue. Living *Candida albicans* can only be used, if it is stained with FITC. Thus we had to modify the assay protocols accordingly. As a proof of principle we could demonstrate the decrease of phagocytosis efficiency, when cytochalasin B (Bjerknes and Bassoe, 1984), rhizopodin A (Gronewold et al., 1999) and chondramide B (Sasse et al., 1998) were present. These compounds are known to act on the stability of the actin cytoskeleton.

MATERIALS AND METHODS

Solutions

Phosphate buffer, pH 7.3 (PBS) was made from PBS tablets (Gibco). Cytochalsin B was purchased from Fluka, and a stock solution in methanol (1 mg / mL) was stored at 4 °C. Stock solutions in methanol (100 µg / mL) of rhizopodin A (732 g / mol) and chondramide B (680 g / mol) were obtained from F. Sasse, Dept. CBIO, Helmholtz Centre for Infection Research, Braunschweig, Germany.

Cultivation of macrophages

The murine macrophage cell line RAW 264.7 (American Type Culture Collection, USA) was routinely culitivated in Dulbecco´s modified Eagle´s medium (DMEM) (Gibco) supplemented

with 10 % FCS (fetal calf serum, Chambrex) at 37 °C in a 10 % CO_2 in air atmosphere. Cells were subjected to no more than 20 passages.

Handling and labeling of yeasts

C. albicans strain 1386 (DSMZ, Germany) was grown to stationary phase in YPD medium (Sigma) at 30 °C with orbital shaking at 160 rpm. For fluorescence-labeling, $1x10^8$ yeasts were harvested by centrifugation (13.000 rpm, 5 min, 24 °C), washed twice in 1 mL PBS and stained with either 1 mL carboxyfluorescein diacetate, succinimidylester (CFSE, Molecular Probes, Leiden, The Netherlands; 500 µM in PBS / 0.1 % DMSO) for 1.5 h at 37 °C (Behnsen et al., 2007) or 1 ml fluorescein isothiocyanate (FITC, Sigma; 1.25 mM in PBS / 0,5 % DMSO) at 4 °C over night. Yeasts were washed three times in PBS to remove remaining dye before use. Where necessary yeast cells were killed by heating for 2 h at 65 °C.

Phagocytosis assay

RAW 264.7 macrophages were cultivated in 125 cm^2 tissue culture flaks (Corning, Los Angeles, USA) for 3 to 4 days. After reaching approximately 80 % confluence cells were harvested by scraping and the concentration of the suspension was adjusted to the desired concentration, finally $2x10^6$ cells / mL. Subsequently cells were seeded in 96-well microtiter plates (Nunc; 100µL / well) followed by an incubation of 2 h to let the cells adhere to the plates.

Then the medium was removed and the macrophages were infected with fluorescent *C. albicans* yeasts by adding 100 µL of a yeast suspension adjusted to the desired concentration by dilution with DMEM supplemented with 10 % serum (range of $1x10^6$ cells / mL to $8x10^6$ cells / mL). After phagocytosis was allowed to proceed at 37 °C in 10 % CO_2 (time scale 15 min to 120 min), fluorescence was measured through the bottom of the plates by a fluorometric multi-well plate reader (CytoFluor® Series 4000 PerSeptive Biosystems) with

Kommentar [N1]: Für genaue Konz. müssen wir nächste Ergebisse abwarten.

the excitation wavelength Ex 485 nm and the emission wavelength Em 530 nm. Subsequently the medium was removed and 100 µL trypan blue (Fluka, 250 µl/ml in PBS) was added to quench the fluorescence of yeasts which were not internalized. After an incubation of 1 min at room temperature, the trypan blue solution was removed and the remaining fluorescence was determined.

Kommentar [N2]: Settings auch angeben?

Chemical compounds, of which the influence on phagocytosis of *C. albicans* by RAW 264.7 cells was to be investigated, were added to the medium after the adherence of the macrophages and incubated for additional 2 h. Then the medium was removed and 100 µL of a solution containing fluorescent *C. albicans* ($4x10^6$ cells / mL DMEM) as well as the respective compound was added. After a phagocytosis time of 45 min the protocol of the phagocytosis assay was followed as described above.

Data analysis

Data analysis was based on the average of fluorescence values of at least 5 wells.

Background fluorescence was determined from the fluorescence of wells to which all solutions besides the macrophage suspension were added.

Maximum fluorescence was dependent on the number of *C. albicans* cells present in the respective well of the microtiter plate and on the labelling efficiency, i.e. fluorescence / yeast cell. It was determined prior to the addition of trypan blue.

Phagocytosis efficiency correlated to the amount of internalized yeast cells, i.e. to the fluorescence remaining after fluorescence quenching with trypan blue and with consideration of the background fluorescence. The effects of chemical compounds were quantified with respect to data resulting from the presence of the solvent methanol (100 %).

RESULTS

In previous investigations by fluorescence microscopy we had investigated the phagocytosis efficiency of macrophages and neutrophils for *C. albicans* stained via uptake of CFSE and hydrolysis to the fluorescent carboxyfluorescein (Behnsen et al., 2007). Fluorescence microscopy, however, does not deliver quantitative data, unless cell numbers are counted either manually or by suitable imaging software. Moreover, it is not suitable for the analysis of large sample numbers unless an automated microscope is used. Thus, we aimed at a microtiterplate assay utilizing the approach described for *E. coli* particles by Wan et al., 1993.

In Fig. 1 fluorescence signals are given, which were obtained from the incubation of different macrophage cell numbers with *C. albicans* stained with CFSE. Prior to the incubation with macrophages *C. albicans* was heated to allow quenching of the fluorescence of non-ingested cells by trypan blue. Signals increased with increasing macrophage numbers. After 30 min a maximum was achieved at $5 * 10^6$ cells / mL, which corresponded to $5 * 10^5$ cells / well. With increasing phagocytosis time signals increased further, in particular for the low macrophage cell numbers, so that even $5 * 10^5$ cells / mL ($5 * 10^4$ cells / well) led to maximum signals when phagocytosis was allowed to proceed for 2 h. This correlated well to the optimal cell number given by Wan et al. ($1 * 10^5$ cells / well). In subsequent experiments a cell number of $2 * 10^5$ cells / well was used.

To allow fluorescence quenching of living *C. albicans* by trypan blue components of the cell wall of the yeast cells had to be stained. This was achieved by incubation of *C. albicans* with FITC. Fig. 2 shows the relationship between the concentration of fluorescein-labeled *C. albicans* and the fluorescence intensity. A linear relationship was obtained in the investigated concentration range from $1 * 10^6$ cells / mL to $8 * 10^6$ cells / mL. The background fluorescence was due to components in the cell culture medium used for dilution of the cell suspension. Fluorescent *C. albicans* could be stored as usual at -20 °C. However, as fluorescein is known for its limited stability the fluorescence of each *C. albicans* suspension

was controlled before use. In Fig. 2 also the efficient fluorescence quenching by addition of trypan blue is shown. The background value of approx. 1000 RFU was the background resulting from the material of the microtiter plates. In all following experiments this background was determined with wells containing no macrophages, and data are given as difference to the background.

As shown in Fig. 3 FITC - labeled *C. albicans* was successfully used in the phagocytosis assay. Signals increased with incubation time and with yeast cell concentrations in the range from $2 * 10^6$ cells / mL to $4 * 10^6$ cells / mL. As 100 µL of the yeast suspension was used, the minimum ratio of yeast : macrophages was 1 : 1 increasing to 2 : 1. Signals increased only slightly, when $5 * 10^6$ cells / mL were applied (data not shown), and thus, $4 * 10^6$ cells / mL were used in subsequent experiments. This yeast : macrophage ratio of 2:1 is lower than in a number of publications, where the particles to be phagocytosed were used in a 10 (Algarra et al., 2002; Bkjerknes and Bassoe; 1984) – 10^3 (Wan et al., 1993) – fold excess. However, ratios of yeast : macrophages = 1 : 1 or 3 : 1 (Behnsen et al., 2007; Busetto et al., 2004) were also described.

Usually the maximum signal was obtained after approx. 60 min.. Extended incubation times between macrophages and the yeast suspension led to decreases in signals when living *C. albicans* was used, whereas signals remained almost constant or showed a slight decrease only after 120 min ($4 * 10^6$ cells / mL) when the yeast cells were inactivated by heating before they were used in the assay. The fluorescence intensity of fluorescein – solutions is pH – dependent, with the maximum being in the alkaline region and a strong decrease in acidic solutions (Babcock and Kramp, 1983; Jankowski et al., 2002). After internalization of particles by phagocytosis the phagosomes form together with lysosomes the so-called phagolysosomes, in which the pH is significantly decreased down to pH 5.5 (Jankowski et al., 2002). Thus the decreasing fluorescence intensity could be due to the acidification of the environment of the yeast cells, and the earlier onset of this decrease with living *C. albicans*

could be explained by a more efficient organelle fusion when living yeast cells were phagocytosed compared to dead yeast cells. Different efficiencies of acidification were previously observed for different phagocyte cell types (Jankowski et al., 2002).

As a proof of principle macrophages were preincubated with compounds acting on the actin cytoskeleton, such as cytochalasin B, rhizopodin A and chondramide B (Fig. 4). The inhibitory effect of cytochalasins on phagocytosis is already described in literature (e.g. Bjerknes and Bassoe, 1984: Wan et al., 1993; Busetto et al., 2004), however the degree of inhibition seems to be dependent not only on the cytochalasin concentration (Wan et al., 1993), but also on the phagocytes (macrophages or polymorphonuclear leukocytes (PMN)) and the particles to be phagocytosed (zymosan, *E. coli, C. albicans*). The combination of viable *C. albicans* with the macrophage cell line led to 30 % remaining phagocytosis (our data), which is a smaller effect than the one observed with heat – killed *C. albicans* and polymorphonuclear leukocates (PMN) (approx. 15 %; 1 µg/ mL cytochalasin) (Busetto et al., 2004).

The compounds rhizopdin A and chondramide B had been isolated as secondary metabolites from myxobacteria and were described as cytotoxic agents, which act on the actin cytoskeleton (Gronewold et al., 1999; Sasse et al., 1998). Rhizopodin was described to destabilize actin filaments (Gronewold et al., 1999), whereas chondramides accelerated actin polymerization (Sasse et al., 1998) with a different mode of action than cytochalasin. We observed that all 3 compounds inhibited phagocytosis of *C. albicans* in a concentration dependent manner, though the effects obtained with chondramide were weaker than with rhizopodin. Thus, for a reduction of the efficiency of phagocytosis it seemed to be not of major importance, by which mode of action the dynamics of the cytoskeleton was disturbed. However, subsequent investigations are required to study those correlations in more detail.

SUMMARY

We established an *in vitro* phagocytosis assay on the basis of a murine macrophage cell line and *C. albicans*. Fluorescent labeling of the yeast with FITC allowed the application of viable yeast cells, which better resembles the real infection situation than the application of heat-killed organisms or even zymosan particles.

As the assay was performed in microtiter plates it is amenable to automation and quantification. Thus, effects of chemical compounds on this function of macrophages were easily detectable and quantifiable.

It should be stated that the concentration range showing effects on phagocytosis did not correlate for all compounds with the concentrations influencing viability of the macrophages, i.e. the cytotoxicity of the compounds. In contrast to previous observations (Wan et al., 1993) we observed for cytochalasin a reduction of cell viability even in the time course of the phagocytosis assay (WST – test; data not shown), whereas for rhizopodin A and chondramide B no cytotoxic effects were observed in this time frame (data not shown).

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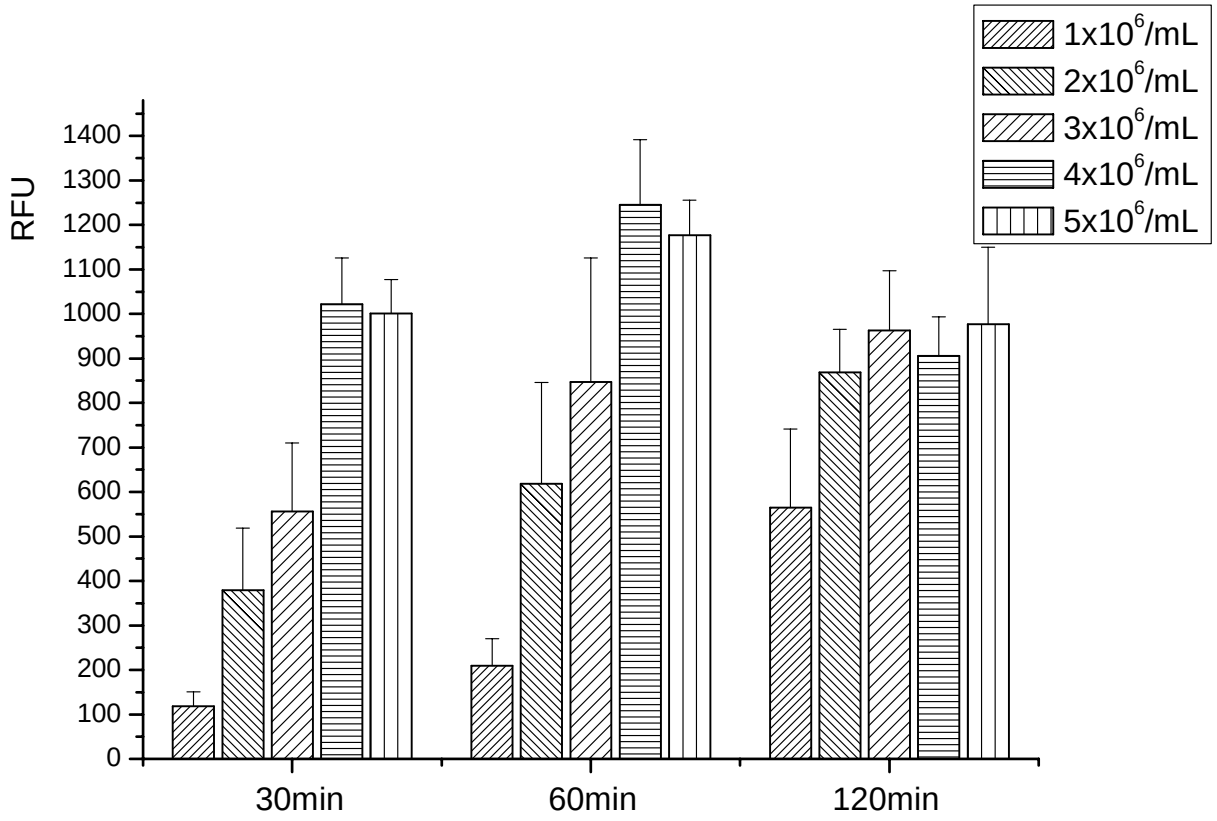
LEGENDS

Fig. 1: Influence of the macrophage cell number and the incubation time between macrophages and heat-killed CFSE-stained *C. albicans* on the fluorescence intensity determined after the addition of trypan blue with a fluorescence microtiter plate reader. 10^5 yeast cells were added in 100 µL to each well and incubated for the indicated periods of time. After removal of the yeast suspension 100 µL of the trypan blue solution was added to quench fluorescence of not-internalized cells. The fluorescence values were the difference to the background fluorescence of approx. 1000 RFU, which was determined with wells containg no macrophages. Standard deviations were obtained from 5 replicates of each sample.

Fig. 2: Relationship between the concentration of FITC – labeled *C. albicans* concentrations and fluorescence intensity. Yeast suspensions were prepared by dilution with cell culture medium. The efficiency of trypan blue quenching was shown by addition of a trypan blue solution to the adherent yeast cells.

Fig. 3: Time course of fluorescence signals resulting from phagocytosis of different concentrations of living (open symbols) and heat-killed (closed symbols) FITC-labeled *C. albicans*. Data are the difference to the background and standard deviations result from 8 replicate wells in the same microtiter plate. In each well 2 * 10^5 macrophages were seeded and allowed to adhere to the plate for 2 h before the yeast cells were added.

Fig. 4: Decrease of the phagocytotic activity of macrophages, which were pre-incubated for 2 h with test compounds before FITC-labeled viable *C. albicans.*was added. The test compounds were known to influence the stability of actin filaments and were also present during phagocytosis. Data resulted from a phagocytosis time of 45 min. and are the mean of 8 replicates. The phagocytosis activity with the solvent methanol was taken as 100 % (control measurements).


RFU
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1300
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800
700
600
500
400
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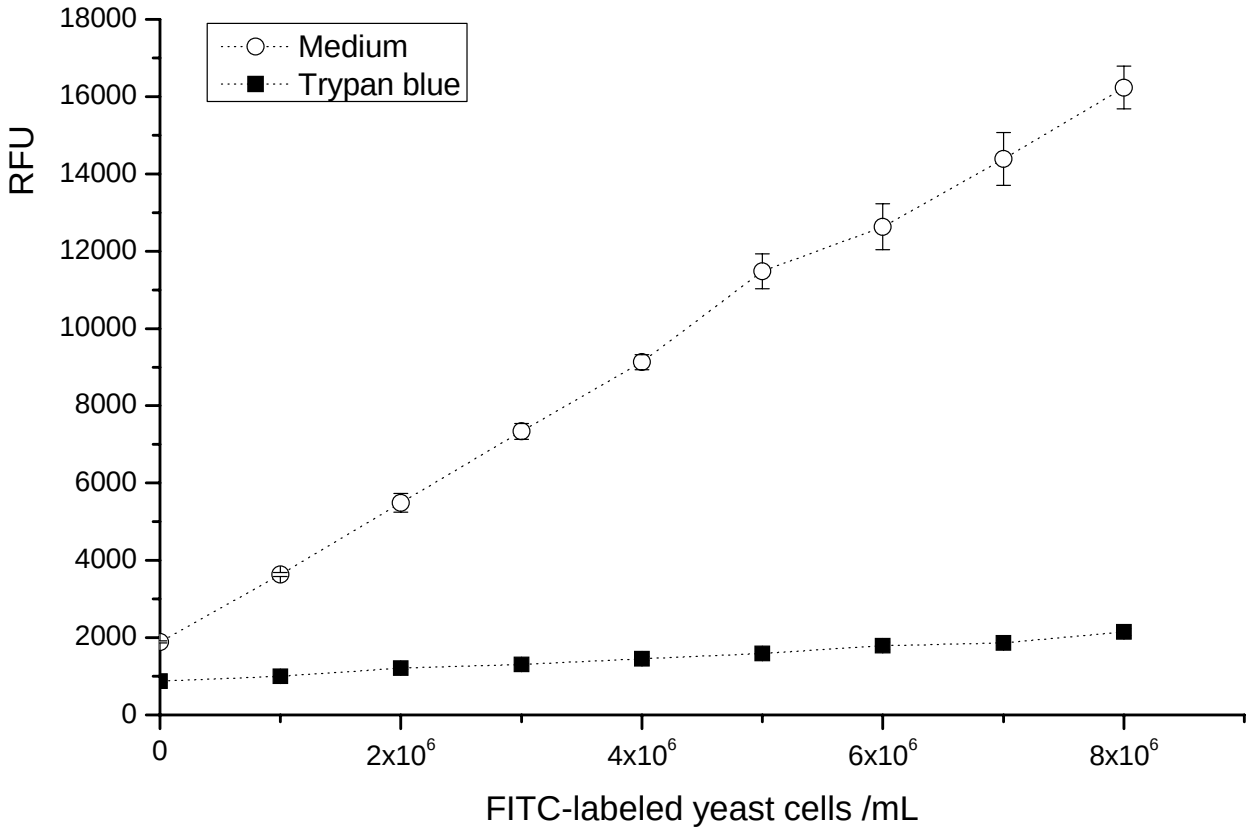
0
30min
60min
120min
1x10^6/mL
2x10^6/mL
3x10^6/mL
4x10^6/mL
5x10^6/mL

Fig. 1


Medium
Trypan blue
RFU
18000
16000
14000
12000
10000
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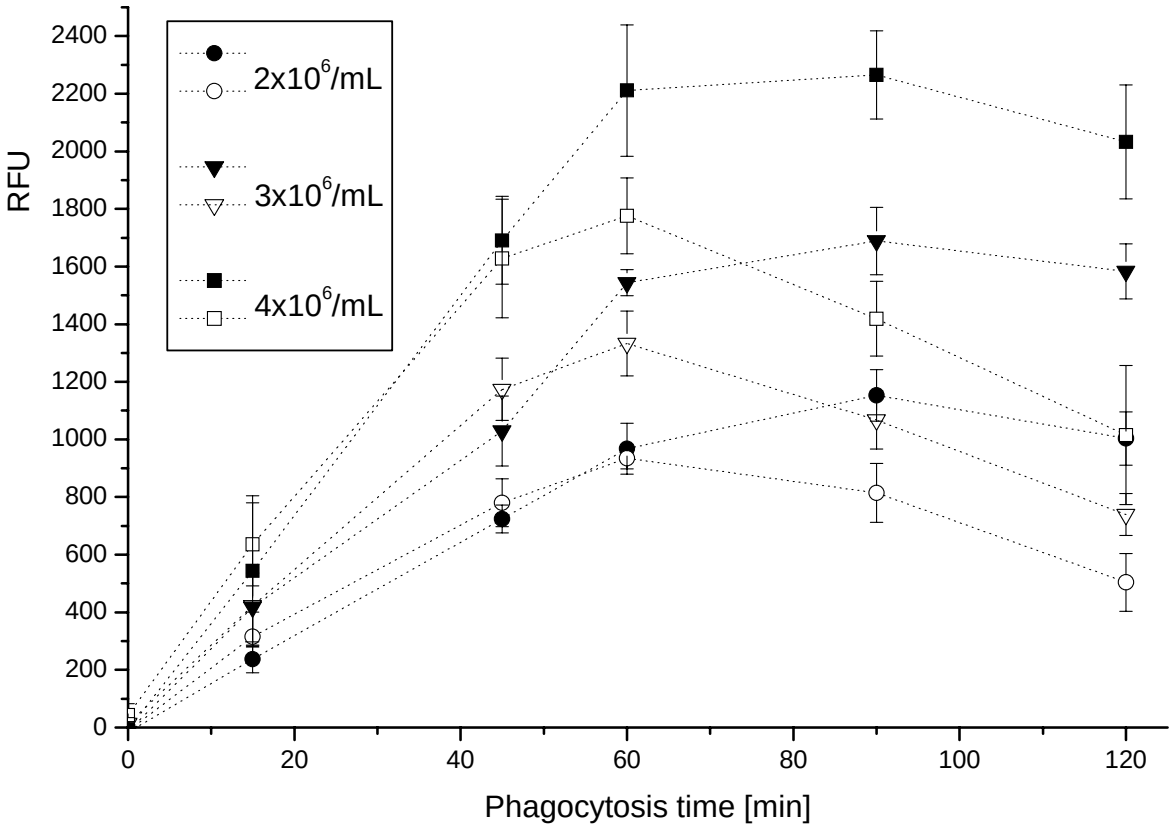
6000
4000
2000
0
0
2x10^6
4x10^6
6x10^6
8x10^6
FITC-labeled yeast cells /mL

Fig. 2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2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
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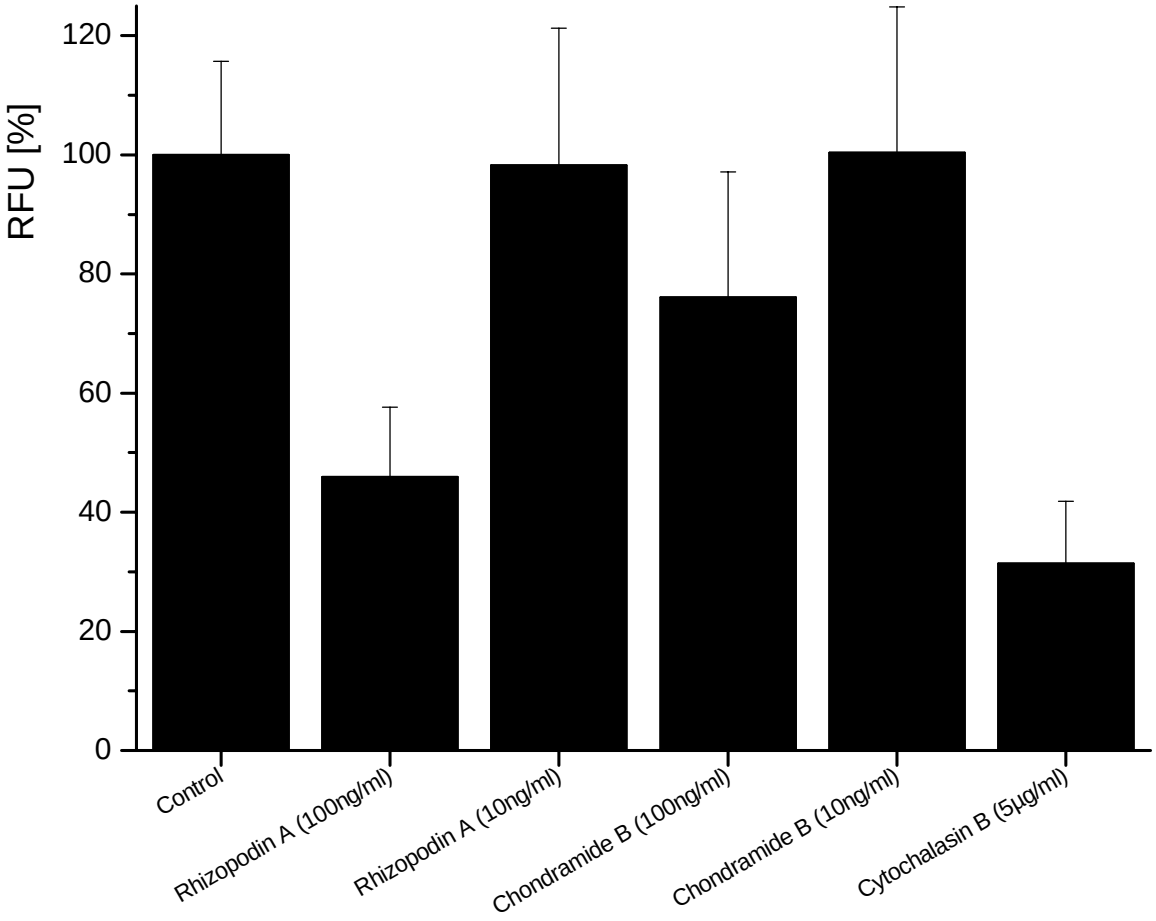
100
120
Phagocytosis time [min]
2x10^6/mL
3x10^6/mL
4x10^6/mL

Fig. 3

Fig. 4


RFU [%]
120
100
80
60
40
20
0
Control
Rhizopodin A (100ng/ml)
Rhizopodin A (10ng/ml)
Chondramide B (100ng/ml)
Chondramide B (10ng/ml)
Cytochalasin B (5µg/ml)