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Link between heat shock protein 90 (Hsp90) and the mitochondrial respiratory chain in the caspofungin stress response of *Aspergillus fumigatus*

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ABSTRACT

Aspergillus fumigatus is an opportunistic mold responsible of invasive aspergillosis. Triazoles (e.g. voriconazole) represent the first-line treatment, but emerging resistance is of concern. The echinocandin drug caspofungin is used as second line treatment but has limited efficacy. The heat shock protein 90 (Hsp90) orchestrates caspofungin stress response and is the trigger of an adaptive phenomenon called the paradoxical effect (growth recovery at increasing caspofungin concentrations). The aim of this study was to elucidate Hsp90-dependent mechanisms of caspofungin stress response.

Transcriptomic profiles of the wild-type *A. fumigatus* strain (Ku80) were compared to that of a mutant strain with substitution of the native *hsp90* promoter by the *thiA* promoter (*pthiA*-hsp90), which lacks the caspofungin paradoxical effect. Caspofungin induced expression of the genes of the mitochondrial respiratory chain (MRC), in particular NADH-ubiquinone oxidoreductases (complex I), in Ku80 but not in *pthiA*-hsp90. The caspofungin paradoxical effect could be abolished by rotenone (MRC complex I inhibitor) in Ku80, supporting the role of MRC in caspofungin stress response. Fluorescent staining of active mitochondria, measurement of oxygen consumption and of ATP production confirmed the activation of the MRC in Ku80 in response to caspofungin, but this activity was impaired in *pthiA*-hsp90. Using a bioluminescent reporter for measurement of intracellular calcium, we demonstrated that inhibition of Hsp90 by geldanamycin or MRC complex I by rotenone prevented the increase of intracellular calcium, shown to be essential for caspofungin paradoxical effect.

In conclusion, our data support a role of the MRC in caspofungin stress response, which is dependent on Hsp90.

INTRODUCTION

Aspergillus fumigatus is a ubiquitous mold, which can cause a broad spectrum of diseases including the devastating invasive aspergillosis (IA) in patients with impaired immunity, such as transplant recipients or cancer patients (1, 2). The treatment of IA remains a challenge as only three drug classes are available (azoles, polyenes and echinocandins) and emergence of resistance to azoles, the first-line treatment, is increasingly reported (3). Echinocandins, such as caspofungin, micafungin and anidulafungin, can be used as second-line therapy of IA or in combination with voriconazole for refractory cases or when azole resistance is suspected (4-6). Echinocandins inhibit the synthesis of (1-3)-β-D-glucan, a major cell wall component. However, their *in vitro* activity against *A. fumigatus* is limited and only fungistatic with persistent growth above the minimal inhibitory concentration (MIC) threshold. Moreover, a “paradoxical effect”, defined as a return to growth at increasing concentrations, can be observed with caspofungin, which may have some clinical relevance (7). This phenomenon of tolerance indicates the existence of compensatory mechanisms of the cell wall, which are mediated by the heat-shock protein 90 (Hsp90) and the calcium-calcineurin pathway (7, 8). Hsp90 is a molecular chaperone playing a key role in mechanisms of stress adaptation, including the development of antifungal drug resistance or tolerance in *A. fumigatus* and other pathogenic fungi (9, 10). The essential role of Hsp90 in caspofungin stress response of *A. fumigatus* has been previously highlighted (8, 11). However, Hsp90-dependent pathways in this response remain partly unknown. We identified a yet unrevealed role of the mitochondrial respiratory chain (MRC) in caspofungin stress response, which was dependent on Hsp90.

RESULTS

Caspofungin stress results in overexpression of genes of the mitochondrial respiratory chain (MRC), which is dependent on Hsp90

Our first objective was to determine which genes are involved in caspofungin stress response in the wild-type *A. fumigatus* isolate Ku80. In order to identify which of them are

dependent on Hsp90, we used the *pthiA*-hsp90 mutant with substitution of the *hsp90* promoter by the *thiA* promoter (8). Exposure to thiamine results in *hsp90* repression and complete growth inhibition. However, in the absence of thiamine, this strain has sufficient Hsp90 levels to maintain normal basal growth, but the lack of the native *hsp90* promoter does not allow to achieve appropriate Hsp90 levels for stress adaptation when exposed to caspofungin (8). As a result, the *pthiA*-hsp90 strain has no basal growth defect, but cannot generate tolerance and paradoxical effect to caspofungin (Figure 2).

Transcriptomic analyses (RNA seq) were performed in three biological replicates of whole RNA extracts of Ku80 and *pthiA*-hsp90 (without addition of thiamine) in basal conditions and after 2h exposure to caspofungin at 2 µg/mL (i.e. concentration required to induce the paradoxical effect of caspofungin). As previously demonstrated (8), the expression of *hsp90* was significantly decreased (3.1-fold, p=0.04) in the *pthiA*-hsp90 mutant compared to Ku80 in the presence of caspofungin.

Genes for which a significant increase (fold change ≥2 and p-value ≤0.05) was observed upon caspofungin exposure in Ku80 were selected. The transcriptional response of *pthiA*-hsp90 in the absence and presence of caspofungin was analyzed for these genes (Supplementary Material S1). We found that the mitochondrial encoded genes of the mitochondrial respiratory chain (MRC) were strongly induced by caspofungin in Ku80, but not in *pthiA*-hsp90 (Figure 1A and Supplementary Material S1). Although the basal expression of some of these genes was somewhat higher in *pthiA*-hsp90 compared to Ku80, no increase was observed upon caspofungin exposure. The MRC genes of *A. fumigatus* were identified and classified in their respective complexes (I to IV) by nBlast with other fungi (*Aspergillus oryzae* and *Neurospora crassa*). A majority of the genes exhibiting the highest induction of expression in the wild-type belonged to complex I (NADH-ubiquinone oxidoreductases) (Figure 1A).

Because MRC gene expression can be influenced by fungal growth, we have looked for possible variations of the mycelial mass between Ku80 and *pthiA*-hsp90 in the presence or absence of caspofungin under the same experimental conditions (22h growth with an

additional 2h with or without caspofungin). Our data show that there was no statistically significant difference in fungal growth between Ku80 and *pthiA*-hsp90 in the presence or absence of caspofungin at the time point of the analysis (Figure 1B).

As a next step, we wanted to know if induction of MRC genes expression in Ku80 was unique to caspofungin or could result from a non-specific effect of any fungal growth inhibitory drug, using a potent antifungal drug, such as voriconazole. Transcriptomic profile of Ku80 upon 2h voriconazole exposure was analyzed and did not show any significant increase of expression of the MRC genes compared to the untreated Ku80 strain (Supplementary Material S2).

We thus hypothesized that the MRC could play a unique role in caspofungin stress response, as illustrated by the significant increase of expression of the MRC genes in Ku80 upon caspofungin exposure, but not upon voriconazole exposure. Furthermore, the lack of induction of MRC genes in *pthiA*-hsp90 suggests that MRC induction in caspofungin stress response may be dependent on Hsp90 and that this effect is not a consequence of growth defect of the *pthiA*-hsp90 mutant compared to the parental Ku80 strain.

MRC complex I activation is required for the paradoxical effect of caspofungin

In order to further investigate the potential role of the MRC in caspofungin stress response and paradoxical growth, we assessed the effect of various MRC inhibitors on Ku80 growth with increasing concentrations of caspofungin. Exposition to rotenone, a MRC complex I inhibitor, resulted in loss of paradoxical growth at increased caspofungin concentrations, an effect similar to that observed in *pthiA*-hsp90 (Figure 2). Rotenone was also able to abolish the paradoxical effect of caspofungin in other *A. fumigatus* strains, such as the wild-type AF293 strain (data not shown). However, paradoxical growth at high caspofungin concentrations was conserved in the presence of others MRC inhibitors, such as antimycin A (complex III inhibitor), oligomycin (ATPase inhibitor) and azide (complex IV inhibitor), or under hypoxic growth conditions (Supplementary Material S3). These results show that MRC complex I is important for caspofungin stress response and paradoxical effect.

Mitochondrial activity is impaired in pthiA-*hsp90 under caspofungin stress*

As a next step, we attempted to demonstrate the functional effect of *hsp90* repression and downregulation of MRC genes on the activity of the mitochondria in response to caspofungin stress. Staining of mycelia with MitoTracker™ Deep Red FM (staining all mitochondria irrespective of their activity) did not show any difference between Ku80 and *pthiA*-hsp90 (data not shown). We then used MitoTracker^{TM} Red CM-H_2XRos, which fluoresces only upon oxidative activity of mitochondria. Exposure to caspofungin (2 µg/mL) for 2h induced fluorescence in Ku80 but not in the *pthiA*-hsp90 mutant (Figure 3).

Mitochondria use oxygen to produce ATP via the MRC. In order to measure ATP production, we used a luciferase assay (CellTiter-Glo® Luminescent Cell Viability Assay), which produces a luminescent signal proportional to ATP quantity. A significant increase of ATP production was observed in Ku80 upon 2h caspofungin exposure. Although *pthiA*-hsp90 exhibited higher basal level of ATP production, no increase was observed in the presence of caspofungin (Figure 4). Finally, analyses with an oximeter showed that the consumption of oxygen by Ku80 increased upon caspofungin exposure compared to the untreated condition. However, the *pthiA*-hsp90 strain exhibited a basal defect in oxygen utilization and was unable to increase oxygen consumption under caspofungin stress (Figure 5). This impairment of oxygen consumption was not related to a growth defect of the *pthiA*-hsp90 strain, as illustrated by the comparisons of mycelial mass with the parental Ku80 strain, showing no significant differences (Figure 1B). We concluded that activation of the respiratory mitochondrial leading to ATP generation in response to caspofungin was impaired in *pthiA*-hsp90.

Both MRC and Hsp90 inhibition prevent the increase of intracellular calcium in response to caspofungin stress

Increase of intracellular calcium (Ca^{2+}) triggers the calcineurin pathway and was shown to be essential for caspofungin stress response and paradoxical growth in *A. fumigatus* (12). ATP

is required for the uptake of extracellular Ca^{2+} by ATP-channels of the cell membrane and also for release of Ca^{2+} stores from the endoplasmic reticulum (13). We hypothesized that Hsp90 and the MRC are essential for caspofungin stress response by generating the ATP required for the increase of cytoplasmic Ca^{2+}. For this purpose, we used a Ku80 strain harboring the bioluminescent Ca^{2+}-reporter aequorin ($AEQ^{\Delta akuB}$) to measure intracellular calcium (14). As previously reported (12), we observed an increase of intracellular Ca^{2+} upon caspofungin exposure (Figure 6A). However, this response was abolished in the presence of geldanamycin (Hsp90 inhibitor) or rotenone (MRC complex I inhibitor), since no Ca^{2+} increase was observed (Figure 6A). The peak of Ca^{2+} could also be abolished in the presence of the Ca^{2+} chelator BAPTA (Figure 6B), which suggests that it results from external calcium source, as previously reported (12). These results further support the link between Hsp90 and the MRC in caspofungin stress response, which could be essential for ATP production and extracellular Ca^{2+} uptake by ATP-dependent Ca^{2+} channels.

DISCUSSION

Echinocandins are gaining interest as salvage or combination therapy of IA since resistance to azoles is emerging. Their limited *in vitro* antifungal activity against *A. fumigatus* is the consequence of a compensatory stress response as illustrated by persistent growth above the minimal inhibitory concentration and the so-called “paradoxical effect” with loss of efficacy at increasing concentrations. In this study, we provide further insights in the mechanisms of this tolerance phenomenon highlighting a previously unknown role of the mitochondrial respiratory chain (MRC), which is dependent from the molecular chaperone Hsp90. Exposure of the wild-type *A. fumigatus* to caspofungin resulted in activation of the MRC with increased oxygen consumption and ATP production. This stress response seems to be unique to caspofungin, as we did not observe an increase of the expression of MRC genes in the presence of voriconazole. Compromising Hsp90 function (by loss of the native *hsp90* promoter) resulted in lack of activation of the MRC genes, in particular the NADH-ubiquinone oxidoreductases (complex I). This coincided with mitochondrial dysfunction as

illustrated by impaired use of oxygen and ATP production in the presence of caspofungin. Furthermore, inhibition of MRC complex I by rotenone resulted in similar effects compared to *hsp90* repression with abolition of the paradoxical effect of caspofungin and absence of the increase of intracellular calcium, which is known to be essential for the paradoxical effect (8, 12).

Mitochondria are important for many cellular processes in eukaryotes. In *A. fumigatus*, genes involved in mitochondrial dynamics and in the endoplasmic reticulum-mitochondria encounter structure (ERMES) were shown to play a role in virulence and antifungal resistance (15, 16). However, little is known about the role of MRC. The MRC is composed of four-electron transfer complexes (I-IV) located in the inner membrane of mitochondria. Transfer of electrons generate a proton gradient and allows ATP production by an ATPase (complex V) (17). Complex I (NADH-ubiquinone oxidoreductases) plays an important role in energy conversion and loss of its activity results in mitochondrial dysfunction (18). In the model mold *Neurospora crassa*, complex I consists of 35 subunits, of which 7 are encoded by mitochondrial DNA: ND1 to ND6 (18). An alternative pathway involved in respiratory activity exists and consists of a single polypeptide, located in the mitochondrial inner membrane, that oxidizes NADPH and is not coupled to proton pumping (18). In our transcriptomic data, only the mitochondrial-encoded genes of the MRC were overexpressed in the presence of caspofungin, while no significant changes were observed for the chromosomal-encoded genes of the MRC and the *aox* gene of the alternative pathway.

Grahl *et al*. demonstrated a role of mitochondrial respiration in oxidative stress response and virulence, which was compromised after deletion of cytochrome C (*cycA*, complex III) (17). However, we did not observe an increase of *cycA* expression in the presence of caspofungin in our study. Bromley *et al.* showed that mitochondrial complex I enzymes are involved in resistance to azoles (19). Deletion or mutation of the chromosomal gene encoding the 29.9 KD subunit of this complex (Afu2g10600) resulted in azole resistance. The same effect was achieved by pharmacological inhibition by rotenone. While the role of MRC in echinocandin resistance of *A. fumigatus* has not been previously investigated, Chamilos et al. showed that

inhibition of the MRC by antimycin A (complex III inhibitor) and BHAM (alternative pathway inhibitor) could increase the susceptibility to caspofungin in the pathogenic yeast *Candida parapsilosis* (20). In the present study, enhanced caspofungin activity against *A. fumigatus* was not observed with the addition of antimycin A or azide, but only with rotenone (complex I inhibitor). Taken together, these data suggest that the MRC may have crucial and distinct roles in modulating antifungal drug stress responses.

While our data indicate a key role of the MRC in caspofungin stress adaptation, the link between the MRC and Hsp90 is more complex to elucidate. Because both Hsp90 and the MRC are important for fungal growth and morphogenesis, an alternative hypothesis could be that the impact of Hsp90 impairment on MRC function is an epiphenomenon resulting from fungal growth defect. To minimize this effect, we used our *pthiA*-hsp90 mutant, which has sufficient Hsp90 levels to maintain basal growth, but cannot achieve appropriate Hsp90 levels under caspofungin stress. Indeed our measurements of mycelial mass under the experimental conditions of this study confirmed that there was no statistically significant differences of fungal growth between Ku80 and *pthiA*-hsp90 in the absence or presence of caspofungin. However, we observed some basal alterations of oxygen consumption, ATP production and MRC gene expression in the *pthiA*-hsp90 mutant suggesting some basal impairment of MRC function with potential impact on growth and morphogenesis, albeit not phenotypically apparent. While caspofungin has a more pronounced inhibitory effect on the rate of fungal growth over time in the *pthiA*-hsp90 mutant, this effect is manifest only after a few days (loss of paradoxical growth in *pthiA*-hsp90) but is not apparent at the early time point of this analysis (e.g. 2h caspofungin exposure). This cannot explain the drastically opposite response of MRC gene expression observed immediately after caspofungin exposure with strong MRC induction in the wild-type Ku80 and complete lack of MRC activation in the *pthiA*-hsp90 mutant. Moreover, we did not observe any increase of MRC gene expression in Ku80 with another antifungal drug such as voriconazole, which suggests that MRC activation does not simply reflect the non-specific effect of growth inhibition, but is rather a specific response to caspofungin stress.

The mechanism by which Hsp90 can influence mitochondrial activity remains unclear. We know from our previous work that Hsp90 does not move to the mitochondria upon caspofungin exposure (21). We thus hypothesize that the impact of Hsp90 on MRC function is probably indirect. As an essential molecular chaperone, Hsp90 controls the activation of multiple client proteins, including transcription factors, which may induce MRC gene expression. However, the mechanisms of regulation of mitochondrial genes remain largely unknown. The role of calcium and calcineurin pathway in caspofungin tolerance and paradoxical effect has been previously established (12, 22). Here, we demonstrate that both pharmacologic inhibition of Hsp90 and MRC resulted in a lack of increase of cytosolic Ca^{2+} in response to caspofungin. Indeed, ATP produced by the MRC may be required for the activity of the Ca^{2+} channels and Ca^{2+} homeostasis in stress responses.

We conclude that mitochondria play an important role in the mechanisms of stress response and tolerance to caspofungin in *A. fumigatus* and that appropriate Hsp90 levels are required for the activation of the mitochondrial respiratory chain in this response. These results may open perspectives for identifying novel antifungal targets in this pathway, in particular the MRC, which remains largely unexplored in *A. fumigatus*.

MATERIALS AND METHODS

Strains and growth conditions

Three *A. fumigatus* strains were used in this study: the akuB^{KU80} strain (here referred as Ku80), used as the reference strain (23) and the *pthiA*-hsp90, in which the native *hsp90* promoter was replaced by the *thiA* promoter in the Ku80 background (8). The AEQ$^{\Delta akuB}$ strain (a gift from Nick Read, Manchester, UK), with the aequorin luminescent reporter expressed in the Ku80 background, was used for measurement of intracellular calcium (14).

Cultures were performed on glucose minimal medium (GMM) with supplementation of 1.5% agar for solid plates (24).

The antifungal drugs used in this study were were obtained as powder suspensions (Sigma-Aldrich, Saint-Louis, Missouri, USA) and dissolved in sterile water (caspofungin) or DMSO (voriconazole), for a stock concentration of 5 mg/mL.

Transcriptomic analyses

Transcriptomic analyses were performed with the parental Ku80 strain and the *pthiA*-hsp90 mutant in the absence of any drug and in the presence of caspofungin or voriconazole. For untreated conditions, a suspension of about 4.10^5 spores/mL was inoculated in 250-ml GMM broth and incubated for 24h at 37°C under constant agitation (225 rpm). For the treated-conditions, the spore suspension was grown for 22h in the absence of any drug and the antifungal drug (caspofungin or voriconazole) was then added at the concentration of 2 µg/mL for an additional 2h of incubation. The mycelial mass was then washed with cold sterile distilled water, filtrated, immediately frozen with liquid nitrogen and reduced to a fine powder. Total RNA was extracted with the RNeasy plant kit (QIAGEN Inc, Venlo, The Netherlands) and purified with the Turbo DNA free kit (Thermo Fisher Scientific, Reinach, Switzerland). RNA concentration was measured with Nanodrop-1000 (Witec AG, Switzerland) and adjusted at the concentration of 9 ng/µl in RNA-free water. Tubes were kept frozen at -80°C until analysis.

Quality of total RNA samples was checked with a Fragment AnalyzerTM (Advanced Analytical Technologies) prior to the preparations of the RNA libraries with TruSeq Stranded mRNA Library Prep Kit (Illumina), according to manufacturer's instructions. Samples were sequenced in an Illumina HiSeq 2000 sequencing platform using the 100-nt single-end protocol with all the samples on same lane (25).

The analysis was performed with three biological replicates for each condition. RNA-seq data were processed using CLC Genomic Workbench Version 10.1.1 (Qiagen). Reads were aligned to the *A. fumigatus* genome Af293 and read counts normalized by the quantile approach method. All conditions were compared with each other and filtered according to a FDR cut-off (≤ 0.05).

Only genes with a significant increased expression (p value ≤0.05) in the caspofungin exposed strain compared to the untreated condition were considered. Raw sequence reads of the current RNAseq data can be found under Bioproject PRJNA486252.

Quantification of mycelial mass

Conidia of Ku80 and *pthiA*-hsp90 were harvested from fresh GMM agar plates, counted with a hemocytometer and adjusted for a quantity of 10^7 spores in flasks containing 25 mL of GMM liquid medium. Both strains were incubated at 37°C for 22h and an additional 2h in the absence or presence of caspofungin 2 µg/mL, for untreated and caspofungin-treated conditions, respectively. The mycelial mass was filtered and completely dried overnight at 60°C, before weighting. The experiment was performed in triplicates.

Measurement of caspofungin paradoxical effect

A 10 µl aqueous suspension with 10^4 conidia of the tested *A. fumigatus* strain (Ku80, AF293 or *pthiA*-hsp90) was inoculated on GMM agar plates supplemented with caspofungin at a concentration of 1, 2 or 4 µg/ml. The paradoxical effect of caspofungin was defined as a significant increase of fungal growth between caspofungin concentrations of 1 and 2 or 4 µg/mL, respectively. Different inhibitors of the MRC were tested at different concentrations for their ability to abolish the paradoxical effect: rotenone (a MRC complex I inhibitor), antimycin A (MRC complex III inhibitor), oligomycin (ATPase inhibitor), and sodium azide (MRC complex IV inhibitor). The effect of hypoxic conditions on paradoxical growth was also tested in GENbox Anaerobic (Biomérieux, France). Pictures were taken after 5 days of incubation at 37°C. Experiments were performed in triplicates.

Quantification of intracellular adenosine triphosphate (ATP)

The Ku80 and *pthiA*-hsp90 strains were cultured at a concentration of 10^6 conidia/ml in GMM broth at 37°C under constant agitation (225 rpm) for 24h. Caspofungin (2µg/mL) was added

at 22h for an additional 2h for the treated conditions. Cultures were filtered and washed with sterile water using a Büchner funnel. The mycelial mass was immediately frozen with liquid nitrogen, reduced to fine powder with mortar and pestle and kept at -20°C. Proteins were extracted with lysis buffer (NaCl 150mM, Tris-HCl pH7.5 10mM, EDTA 0.5mM, Triton 0.1%, DTT 1mM, PMSF 1mM and protease inhibitory cocktail 1X). Protein concentration was measured by the Bradford method (26) and adjusted to a concentration of 30 µg/mL for each sample. Lysates of 100 µL of each strain were incubated with 100µL of Celltiter-glo™ one solution (Promega, Fitchburg, Wisconsin, USA) in a 96-well plate (black clear flat bottom) for 10 min before measurement of luminescence with LUMIstarOmega microplate reader (BMG LABTECH, Ortenberg, Germany). A standard curve was made using ATP at a concentration range of 0-1µM. The experience was performed in three biological replicates and the mean of final results was expressed as fold change compared to Ku80 in basal condition.

Visualization of active mitochondria

A total of 10^4 conidia of Ku80 and *pthiA*-hsp90 were incubated on microscope coverslips in GMM broth at 37°c for 24h. Caspofungin (2 µg/ml) was added after 22h for an additional 1-hour incubation. Cultures were incubated with MitoTracker™ Deep Red FM or MitoTracker™ Red CM-H_2XRos (ThermoFisher, Waltham, Massachusetts, USA) 1µM for 45 min at 37°C and then observed with a fluorescent microscope (Axioplan 2, Zeiss, Oberkochen, Germany). Fluorescence quantification was determined by ImageJ software.

Oxygen measurement

Oxygen measurement was performed with the Dual Digital Model 20 oximeter (Rank brothers, Cambridge, England). Ku80 and *pthiA*-hsp90 strains were cultured at a concentration of 10^7 conidia/ml in GMM broth at 37°C for 7h under constant agitation (225 rpm) to allow start of germination and then transferred to the oximeter chambers that were saturated in oxygen, sealed with parafilm for 4h and maintained at 37°C. Caspofungin (2 µg/ml) was added with a syringe 1h before measurement or 30 min after the beginning of

measurement. Effect of rotenone (158 µg/mL) was also tested. Oxygen consumption was measured every 15 seconds for 3-4 hours.

Calcium measurement

10^6 conidia of Ku80-AEQ were incubated in calcium-free medium (GMM supplemented with 50 mM uridine and 25 mM uracil) in a white 96-well plate for 18h at 37°C, washed with PGM (20 mM PIPES, pH 6.7, 50 mM glucose, 1 mM $MgCl_2$) and incubated for 4h at 4°C in PGM supplemented by 1.06 µg/mL of water soluble Coelenterazine (Sigma-Aldrich, Saint-Louis, Missouri, USA) for protein reconstitution (27). Cells were then incubated at room temperature for 1h in the presence of inhibitors: Geldanamycin 4 µg/mL (Sigma-Aldrich, Saint-Louis, Missouri, USA) and Rotenone 158 µg/mL (Sigma-Aldrich, Saint-Louis, Missouri, USA). Caspofungin 2 µg/mL was added 6 minutes after start of luminescence measurement using LUMIstarOmega microplate reader (BMG LABTECH, Ortenberg, Germany). The experiment was repeated with addition of 1mM of the Ca^{2+} chelator BAPTA (Sigma-Aldrich, Saint-Louis, Missouri, USA) 1h before measurement to remove all source of extracellular Ca^{2+}.

Statistical analyses

For transcriptomic data (RNA sequencing), statistical analyses were performed in R (V3.1.1) using the edgeR Bioconductor Package implemented in the CLC software (28). This software implements the 'Exact Test' for two-group comparisons accounting for overdispersion caused by biological variability (29). Non-parametric tests were performed using the software GraphPad Prism 7.03. P-value were calculated by multiple comparisons using Kruskal-Wallis test and considered as significant if ≤0.05.

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FIGURE LEGEND

Figure 1. Transcriptomic analyses of MRC genes in *A. fumigatus* Ku80 (parental strain) and *pthiA*-hsp90 in basal conditions (untreated) and after 2h caspofungin (CAS) exposure.

A) Gene expression in fold-change compared to the reference condition (Ku80, untreated). Black: Ku80 untreated (Ku80), Dark grey: Ku80 with caspofungin (Ku80 CAS), grey: *pthiA*-hsp90 untreated (*phtiA*-hsp90), light grey: *pthiA*-hsp90 with caspofungin (*phtiA*-hsp90 CAS). The p-values are expressed as: * ≤0.01, **≤0.001, **** ≤0.00001, *****≤0.000001. Numbers I to V correspond to the MRC complex to which the genes were assigned according to nBlast. ND: not determined.

B) Dried mycelial mass (mg) of the different strains (Ku80 and *pthiA*-hsp90) under the experimental conditions of the transcriptomic analyses (24h untreated and 22h untreated with an additional 2h of caspofungin exposure). Error bars represent standard deviation of experiments in triplicates. Numbers represent p-values, ns: not significant.

Figure 2. Effect of *hsp90* repression (*pthiA*-hsp90 strain) and MRC complex I inhibition (rotenone) on the caspofungin paradoxical effect of *A. fumigatus.*

A) Pictures were taken after 5 days of growth at 37°C on glucose minimum medium (GMM) agar plates supplemented with caspofungin (CAS) at increasing gradient concentration. Rotenone (ROT) was added at a fixed concentration of 158 µg/mL.

B) Graphs represent the mean diameter of the colonies. Error bars represent standard deviation of experiments in triplicates. P-values are represented for comparisons of the diameters of the colonies exposed to caspofungin 1 µg/mL versus 2 and 4 µg/mL, respectively, in order to demonstrate the paradoxical effect (significant recovery of the growth at concentrations above 1 µg/mL). **** P value ≤0.0001, ns = not significant.

Figure 3. Visualization of active mitochondria in Ku80 and *pthiA*-hsp90 in the absence or presence of caspofungin.

A) Cultures performed on coverslips in GMM broth at 37°C for 24h in the absence or presence of caspofungin (2 µg/ml after 22 hours). Light microscopy (left) and fluorescence microscopy stained with MitoTracker™ Red CM-H_2XRos (right).

B) Graphs represents fluorescence quantification for each conditions, measured with imageJ software. Fluorescence is expressed in Relative Fluorescent Unit (RFU).

Figure 4. Measurement of ATP production in Ku80 and *pthiA*-hsp90 in the absence or presence of caspofungin.

Measurement of ATP concentration by Celltiter-glo™ in cell lysates after 24h growth in GMM broth at 37°C in the absence or presence of caspofungin (2 µg/ml added after 22 hours). Bars represent means with standard deviations of three biological replicates with results expressed as fold-change of luminescence compared to the untreated Ku80 strain. Numbers represent p-values, ns: not significant.

Figure 5. Oxygen measurement in Ku80 and *pthiA*-hsp90 in the absence or presence of caspofungin and rotenone.

Graphs represents percentage of oxygen (y-axis) in the chambers over time (x-axis).

A) Ku80 in the absence or presence of caspofungin (CAS, 2 µg/mL) added 1h before start of measurement.

B) *pthiA*-hsp90 in the absence or presence of caspofungin (CAS, 2 µg/mL) added 1h before start of measurement.

C) Ku80 and *pthiA*-hsp90 with addition of caspofungin (CAS, 2 µg/mL) after 30 min.

D) Ku80 in the absence or presence of rotenone (ROT, 158 µg/mL) added 1h before start of measurement.

Figure 6. Intracellular calcium (Ca^{2+}) measurement using the bioluminescent reporter aequorin expressed in Ku80.

A) The $AEQ^{\Delta akuB}$ strain was pre-incubated in the absence or in the presence of geldanamycin (GDA, 4 µg/mL) or rotenone (ROT, 58 µg/mL), added 1h before start of measurement at room temperature. Caspofungin (CAS, 2 µg/mL) was injected 6 min after start of the measurement.

B) Same experiment with addition of BAPTA (1mM) for 1h at room temperature before start of measurement.

Results represent mean curves of triplicates and are expressed in Relative Luminescence Unit (RLU) over time.

Figure 1

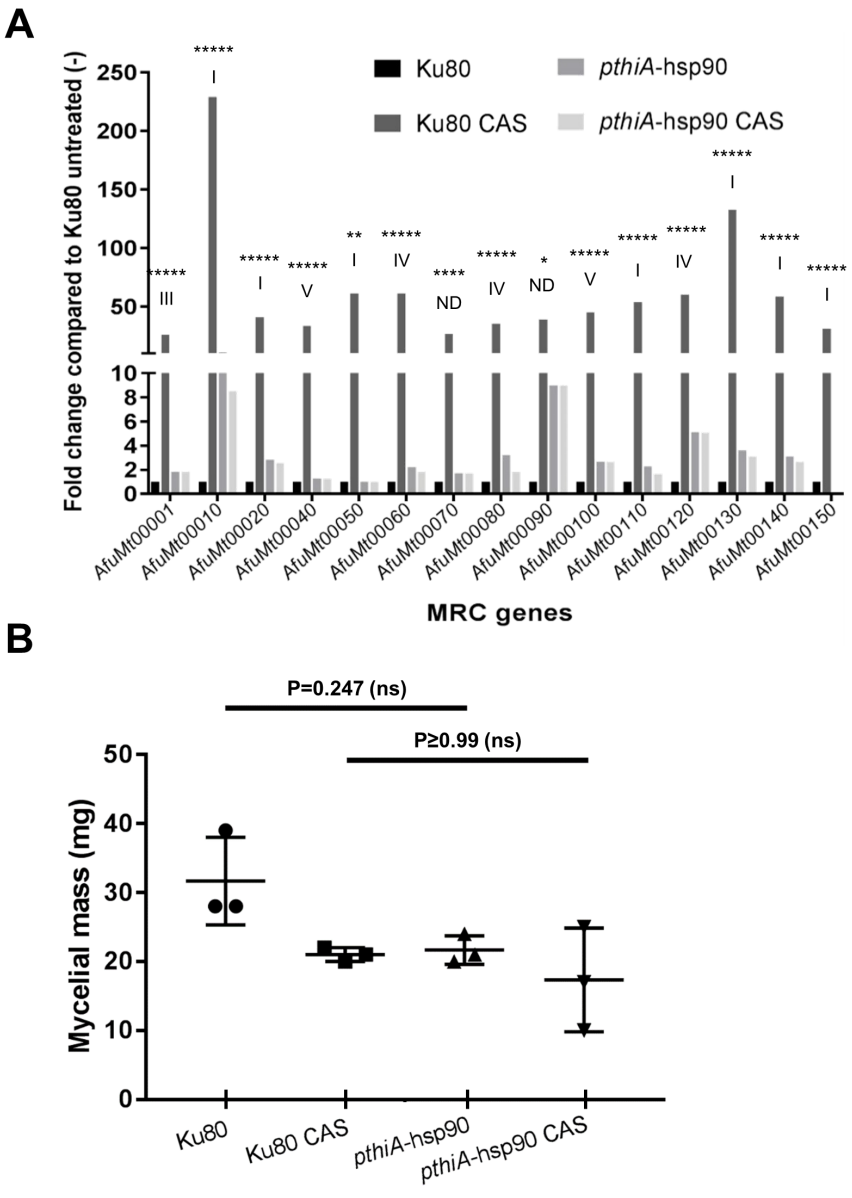

A
Ku80
pthiA-hsp90
Ku80 CAS
pthiA-hsp90 CAS
Fold change compared to Ku80 untreated (-)
MRC genes
AfuMt00001
AfuMt00010
AfuMt00020
AfuMt00040
AfuMt00050
AfuMt00060
AfuMt00070
AfuMt00080
AfuMt00090
AfuMt00100
AfuMt00110
AfuMt00120
AfuMt00130
AfuMt00140
AfuMt00150
B
P=0.247 (ns)
P≥0.99 (ns)
Mycelial mass (mg)
Ku80
Ku80 CAS
pthiA-hsp90
pthiA-hsp90 CAS

Figure 2

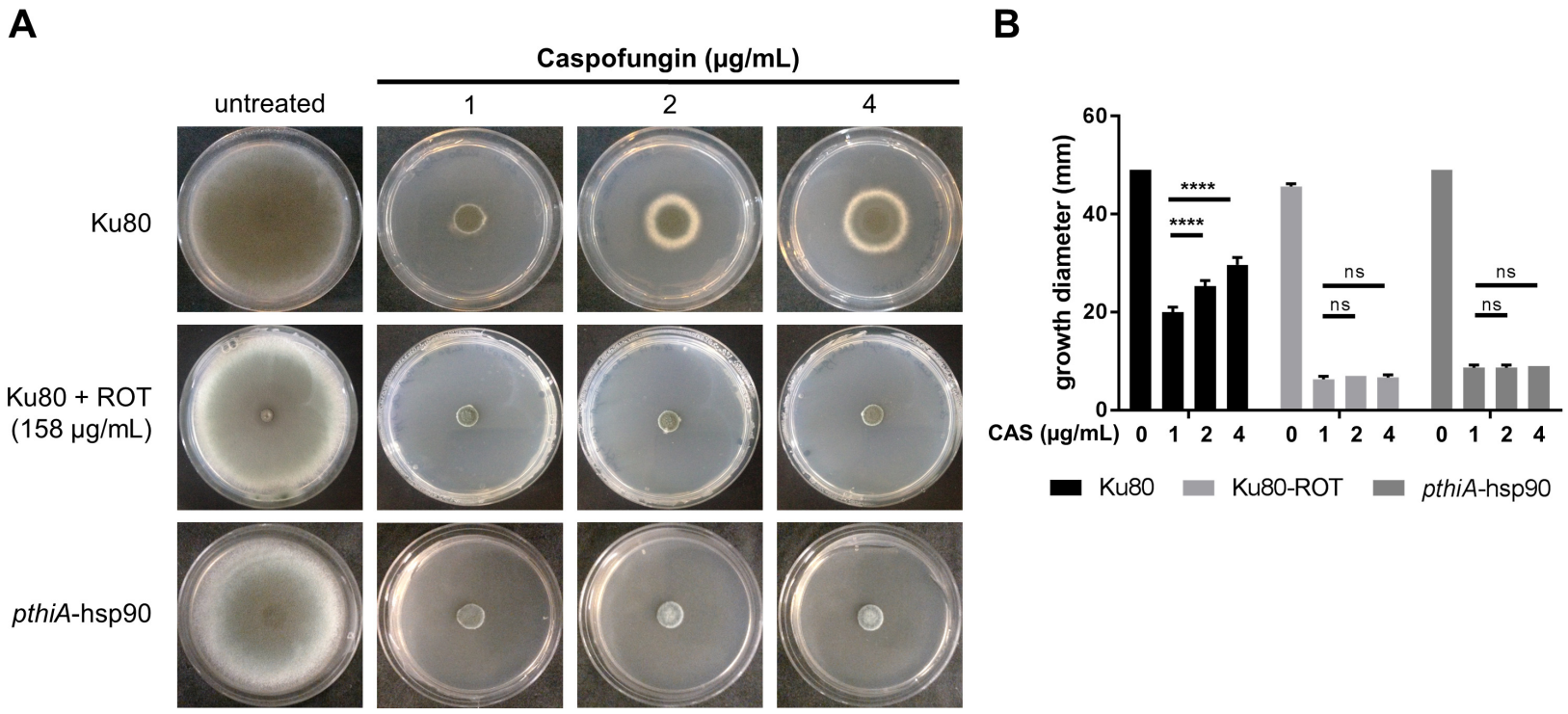

A
Caspofungin (µg/mL)
untreated
1
2
4
Ku80
Ku80 + ROT
(158 µg/mL)
pthiA-hsp90
B
growth diameter (mm)
60
40
20
0
****
****
ns
ns
ns
ns
CAS (µg/mL) 0 1 2 4 0 1 2 4 0 1 2 4
Ku80
Ku80-ROT
pthiA-hsp90

Figure 3

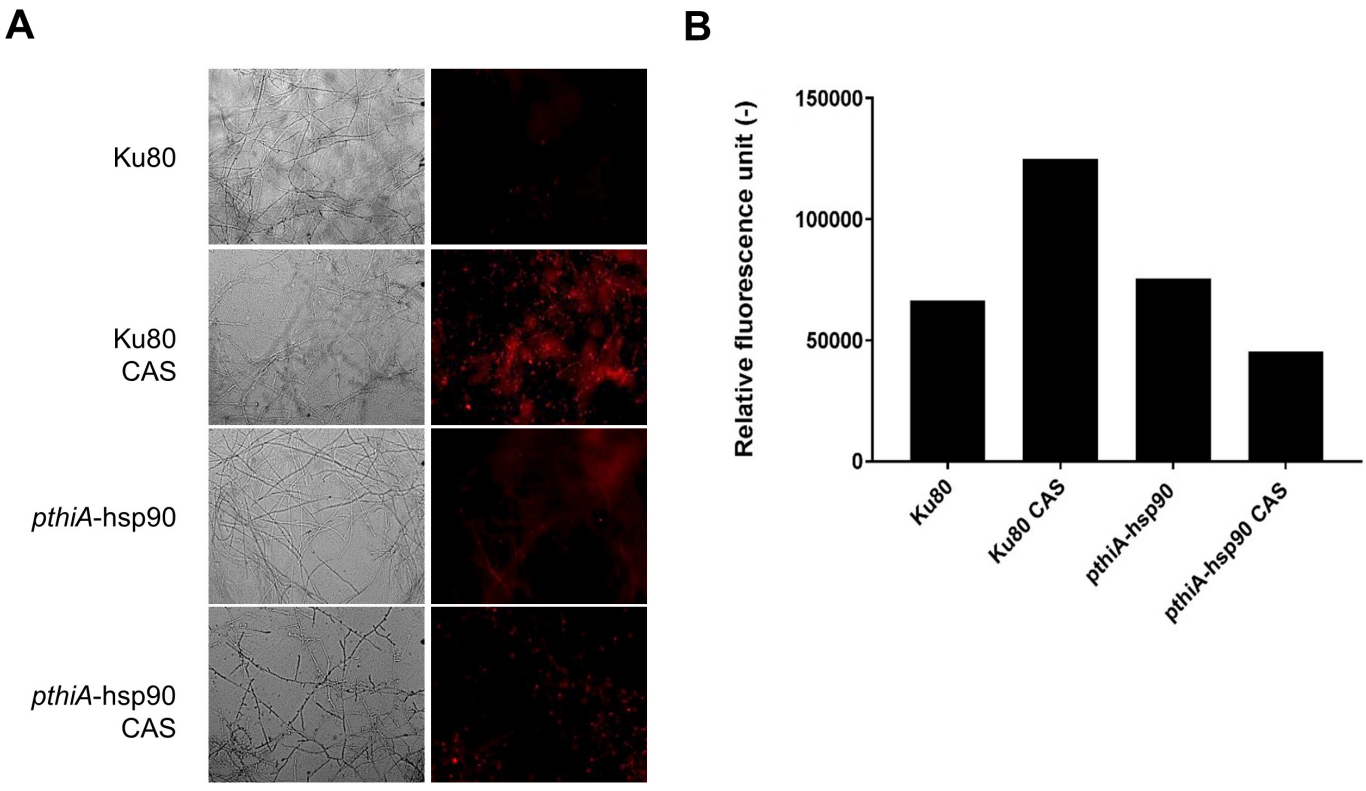

A
Ku80
Ku80
CAS
pthiA-hsp90
pthiA-hsp90
CAS
B
Relative fluorescence unit (-)
150000
100000
50000
0
Ku80
Ku80 CAS
pthiA-hsp90
pthiA-hsp90 CAS

Figure 4

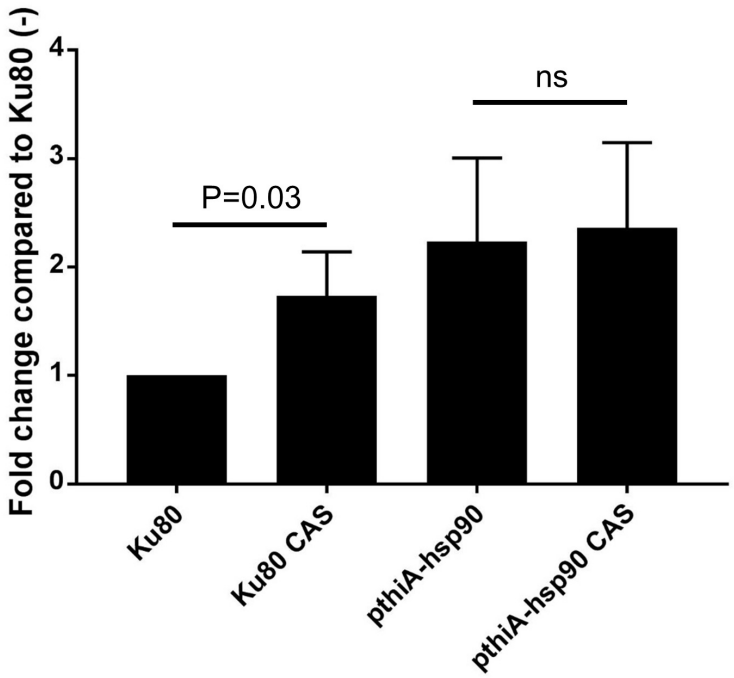

Fold change compared to Ku80 (-)
0
1
2
3
4
P=0.03
ns
Ku80
Ku80 CAS
pthiA-hsp90
pthiA-hsp90 CAS

Figure 5

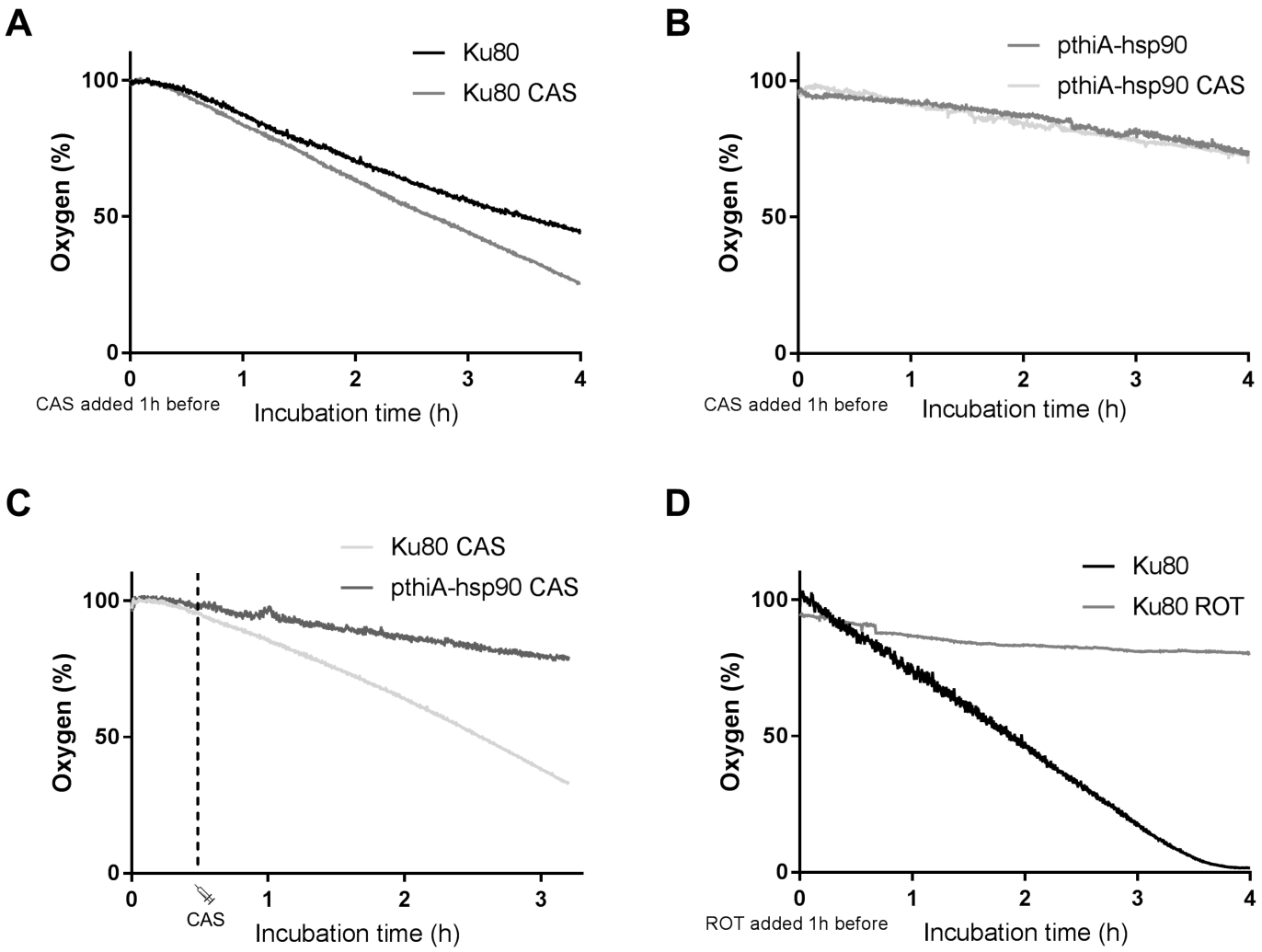
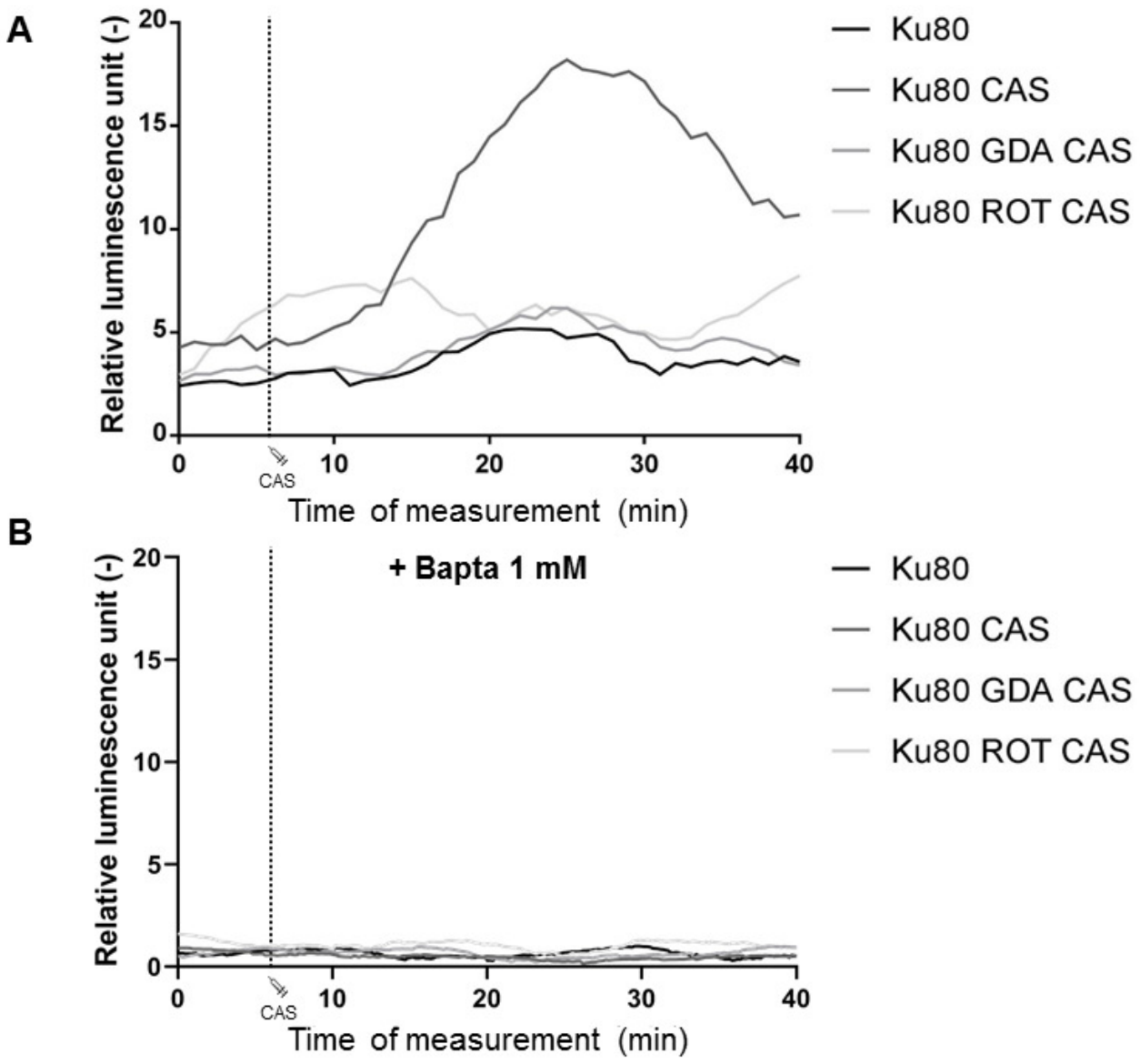

A
Ku80
Ku80 CAS
Oxygen (%)
100
50
0
0
1
2
3
4
CAS added 1h before
Incubation time (h)
B
pthiA-hsp90
pthiA-hsp90 CAS
Oxygen (%)
100
50
0
0
1
2
3
4
CAS added 1h before
Incubation time (h)
C
Ku80 CAS
pthiA-hsp90 CAS
Oxygen (%)
100
50
0
0
1
2
3
CAS
Incubation time (h)
D
Ku80
Ku80 ROT
Oxygen (%)
100
50
0
0
1
2
3
4
ROT added 1h before
Incubation time (h)

Figure 6


A
Relative luminescence unit (-)
20
15
10
5
0
0
10
20
30
40
CAS
Time of measurement (min)
Ku80
Ku80 CAS
Ku80 GDA CAS
Ku80 ROT CAS
B
+ Bapta 1 mM
Relative luminescence unit (-)
20
15
10
5
0
0
10
20
30
40
CAS
Time of measurement (min)
Ku80
Ku80 CAS
Ku80 GDA CAS
Ku80 ROT CAS