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Summary

Proguanil in combination with its synergistic partner atovaquone has been used for malaria treatment and prophylaxis for decades. However its mode of action is not fully understood. Here we used yeast to investigate its activity. Proguanil inhibits yeast growth, causes cell death and acts in synergy with atovaquone. It was previously proposed that the drug would target the system that maintains the mitochondrial membrane potential when the respiratory chain is inhibited. However our data did not seem to validate that hypothesis. We proposed that proguanil would not have a specific target but accumulate in the mitochondrial to concentrations that impair multiple mitochondrial functions leading to cell death. Selection and study of proguanil resistant mutants pointed towards an unexpected resistance mechanism: the decrease of CoQ level, which possibly alters the mitochondrial membrane properties and lowers proguanil intramitochondrial level.

Keywords: antimalarial drug, mitochondrial function, drug mode of action, drug resistance, yeast model

Introduction

The biguanide proguanil, in combination with its synergistic partner atovaquone, has been used for malaria treatment and prophylaxis for decades. Atovaquone is a well-known inhibitor of the mitochondrial respiratory chain cytochrome bc_1 and binds in the Q_o-site of the complex [1]. The drug is highly potent against the malaria agents *Plasmodium*. However, its inhibitory action is compromised by resistant mutations located in its binding site, such as Y268C and Y278S found in atovaquone resistant *P. falciparum* parasites (see [2] and refs within).

A potent fast-acting activity of proguanil is attributed to its metabolite cycloguanil, which acts as an inhibitor of the dihydrofolate reductase (DHFR). DHFR mutations causing resistance to cycloguanil were reported [3]. Proguanil itself (and a cyclization-blocked analogue) shows slow-acting anti-parasitic activity [4]. The efficiency of the combined atovaquone-proguanil therapy is not due to cycloguanil but rather to proguanil [4] [5]. Although the synergistic effect of the two compounds is well documented [5] [6] [7], the mode of action of proguanil is still poorly understood. The drug has no effect on the respiration of the parasite [5]. It was hypothesized that, in the parasite, proguanil target the alternative system of mitochondrial membrane potential generation, comprising the F1 domain of the ATP synthase with the

adenine nucleotide translocator and the phosphate carrier, which is essential for the cell in absence of an active respiratory chain [7]. This could explain why proguanil potentiates the activity of atovaquone. The combination of the two drugs would impair the generation of membrane potential by both the respiratory chain and the alternative system. It was reported that proguanil inhibited ATP hydrolysis by the ATP synthase prepared from animal source. However this effect did not seem specific to proguanil as it was also observed with cycloguanil and other biguanides [8].

Here we used the yeast model to study the mode of action of proguanil. Yeast, as *P.falciparum*, does not possess P450 cytochromes that could metabolise proguanil in cycloguanil, as it occurs in human liver. Therefore a growth inhibition observed upon addition of proguanil in the culture medium would be caused by that compound itself and not its metabolite.

As observed with the parasite, we showed that proguanil acted in synergy with atovaquone to inhibit yeast growth and that the inhibition of the bc_1 complex by atovaquone was required for the synergy. We then compared the wt strain and its rho° derivative that exclusively relies on the alternative system for the generation of membrane potential. We observed the same sensitivity to proguanil, thus suggesting that the drug would not act via the inhibition of that system. To obtain clues on the drug mode of action, proguanil resistant mutants were selected and studied.

Materials and Methods

Growth media and yeast strains

The following growth media were used: YPD (1% yeast extract, 2% peptone, 3% glucose), YPEth (1% yeast extract, 2% peptone, 2% ethanol), YPG (1% yeast extract, 2% peptone, 2% glycerol),YPGal (1% yeast extract, 2% peptone, 0.2% glucose, 2% galactose). Cells were cultured at 28°C with agitation (150 rpm) for a good aeration.

The strain AD1-9 and its derived mutants were used for the growth assays. AD1-9, lacking several membrane transporters (*α, ura3, his1, Δyor1, Δsnq2, Δpdr5, Δpdr10, Δpdr11, Δycf1, Δpdr3, Δpdr15, Δpdr1*) was kindly provided by M. Ghislain, UCL, Belgium. Δcoq5 mutant was constructed by PCR-based deletion.

Growth assays and drug sensibility tests

Drug sensitivity was assessed by monitoring the inhibition of yeast cell proliferation. Yeast were grown in YPEth or YPGal with increasing concentrations of drugs. Cultures were inoculated at an OD_{600nm} of 0.2 and incubated at 28°C with vigorous shaking for three days. $OD_{600\ nm}$ were then measured. Data were plotted for each strain as the percentage of growth relative to control, *i.e.* untreated. The experiments were repeated at least twice and the data averaged. IC_{50} (drug concentration required to obtained 50% growth inhibition) were estimated from inhibitor titration plots.

Drug combination

In vitro drug-drug interactions were investigated using the fixed-ratio isobologram method established by Fivelman et al. [9]. This approach is based on determining the IC_{50} values of each individual drug alone and the IC_{50} values of a mixture of both drugs at fixed concentration

ratios. Drugs were applied alone or in combination at fixed concentration ratios (A:B ratios [vol/vol] of 1:4, 2:3, 3:2, and 4:1). The FIC_{50} of each drug in a given combination was estimated as follows: FIC_{50} (A) = IC_{50} of A in combination / IC_{50} of A alone; FIC_{50} (B) = IC_{50} of B in combination / IC_{50} of B alone. All FIC_{50s} were confirmed by independent experiments. The isobolograms were drawn by plotting pairs of FIC_{50} values of drug A and drug B for each combination. The straight line indicated an indifferent interaction (no interaction or additive action), a concave curve towards the origin of the axes represented synergy and a convex curve towards the opposite indicated antagonism.

Measurement of O_2 consumption of mitochondria and of intact cells

Oxygen consumption activities were measured in a Clarke-type oxygen electrode. Mitochondria, prepared as described in [10], were added at around 50 µg of protein in 1 mL of 0.7 M sorbitol, 50 mM potassium phosphate pH 7.5 and 0.2 mM EDTA. The reaction was initiated by the addition of of 0.8 mM NADH.

Sequencing analysis

Total genomic DNA was isolated from the WT strain AD1-9 and from the derived mutants. The genomic libraries, the sequencing and bioinformatics analysis were performed by the Next Generation Sequencing Core Facility of I2BC (www.i2bc.paris-saclay.fr). The presence of the identified mutations was confirmed by PCR and sequencing of the mutated genes, using independent DNA preparations.

Results and discussion

Effect of proguanil and atovaquone combination on yeast respiratory growth

Before investigating the mode of action of proguanil in yeast, we addressed the question of the relevance of the yeast model. To that end, we checked whether yeast showed the same behaviour as the parasite in regards to the sensitivity to atovaquone-proguanil combination.
For the growth assays, we used the respiratory growth medium YPEth and the strain AD1-9 (and its derivatives) that lacks several membrane transporters, which renders the cells more sensitive to compounds as previously shown for atovaquone and other anti-malaria drug [11].

First, we checked whether proguanil and atovaquone acted synergistically to inhibit yeast growth, as observed in *P. falciparum*. We also tested the combination of proguanil with three others drugs: the antimalarial plasmodione, a potent yeast respiratory growth inhibitor acting via oxidative stress [12], and two bc_1 complex inhibitors, the antifungal azoxystrobin and the anti-parasitic ELQ-271 [13] [14]. Atovaquone and azoxystrobin bind at the Q_o-site of the complex while ELQ-271 targets its Q_i-site.

As shown in fig.1A, proguanil acts synergistically with atovaquone, azoxystrobin and ELQ-271 while it antagonizes plasmodione in growth inhibition assays. The antagonistic effect of plasmodione was previously observed with the malaria parasite, but not yet explained [15]. The synergistic effect of the bc_1 complex inhibitor ELQ-300, structurally similar to ELQ-271 was reported in *P.falciparum* [4].

Secondly, we asked whether atovaquone potentiates proguanil by inhibiting the bc_1 complex or whether another drug-drug interaction, independent of bc_1 complex inhibition, should be

proposed. To that end, we tested the effect of the drug combination on the atovaquone resistant mutant Y279C, derived from the control strain AD1-9. The cytochrome *b* mutation Y279C (corresponding toY268C in *P. falciparum*) confers a high level of atovaquone resistance in yeast as in the parasite [16]. In yeast growth assays, the IC_{50} of the control strain was around 0.1 µM atovaquone whereas it was >10 µM for Y279C. Fig.1B top panel shows the proguanil and combined proguanil-atovaquone inhibition curves of the control. A dramatically increased inhibition was observed with the drug combination. By contrast, as shown in the bottom panel, the proguanil and proganil-atovaquone inhibition curves were identical for Y279C. Thus atovaquone did not potentiate proguanil activity in the mutant. This confirmed that, as previously reported for the malaria parasite [17], the atovaquone-proguanil synergistic effect observed in the control (Fig.1B top panel) required the inhibition of the bc_1 complex by atovaquone. Another drug-drug interaction independent of atovaquone inhibitory action on the respiratory chain complex seems excluded.

Then, in order to pursue the comparison with data obtained with the malaria parasite, we tested the effect of proguanil and atovaquone-proguanil combination on the O_2 consumption of mitochondria isolated from yeast control strain (data not shown). Proguanil alone had not effect and did not potentiate the inhibitory effect of atovaquone on the respiratory chain actvity, which was also in agreement with results obtained with the parasites [5].

Thus based on these data, yeast seems an appropriate model to decipher the mode of action of proguanil.

Consequence of proguanil treatment on cell viability

We tested whether proguanil had a cytostatic or a cytotoxic effect on yeast. Cells were grown in YPEth to an OD_{600nm} of 2 and then treated with different concentrations of proguanil. After 24h treatment, the OD_{600nm} were recorded. The cells were then harvested, resuspended in water, counted, serially diluted and plated on YPD medium. After four days of incubation at 28°C, the colonies were counted and their number was reported to the number of plated cells (fig.2). Proguanil treatment at concentrations inhibiting cell proliferation severely lowered cell viability By contrast, treatment with bc_1 complex inhibitors had no effect on cell viability (not shown).

A role for the respiratory chain and for the alternative system for mitochondrial membrane potential generation

We then addressed the question of the role of the respiratory chain and the alternative mechanism for the generation of mitochondrial membrane potential in proguanil activity.
To that end, we compared the sensitivity to proguanil of the strain AD1-9 and its derived rho° mutant without mtDNA. Rho° cells lack the respiratory chain complexes III and IV and the F_O-part of complex V (or ATP synthase), as part of their components are mitochondrially encoded. The cells were grown in YPGal with a vigorous agitation for a good aeration of the cultures. In these conditions, the WT cells (rho^+) use mainly the respiratory function while the rho° cells exclusively produce their energy by glycolysis.

If the inhibitory activity of proguanil requires an active respiratory chain, the WT cells would be sensitive to the drug while the rho° cells would be unaffected. This is observed for plasmodione and primaquine [12] [18], and of course for atovaquone. By contrast, if the drug interferes with the alternative mechanism for mitochondrial membrane potential generation, the rho° cells should be more sensitive than WT as rho° cells rely exclusively on that mechanism.

In WT cells, the mitochondrial membrane potential is generated by the activity of the respiratory chain. In rho° cells, the mitochondrial membrane potential (indispensable even when ATP derived from glycolysis) is maintained by an alternative mechanism, by the electrogenic exchange of ATP^{4-} / ATP^{3-} via the adenine nucleotide translocator in concert with the ATP hydrolysis activity of the F1 domain of complex V and the phosphate carrier. Inhibition of the adenine nucleotide translocator by mutation or by bongkrekic acid, or inhibition of the F_1 domain of complex V is lethal for the rho° cells [19] [20].

As shown in fig.3, rho° cells were slightly less sensitive to proguanil, suggesting that the respiratory chain plays only a minor role, if any, in proguanil sensitivity. It could also be inferred that the activities of the adenine nucleotide translocator, the F_1-domain of complex V and the phosphate carrier were not impaired by proguanil.

It thus seems that the drug would not act (mainly) via the inhibition of the alternative system for membrane potential generation, and that other possible mechanisms need to be investigated. Its synergy with atovaquone (and other bc_1 complex inhibitors) strongly argues for mitochondrial targets. Its effect on cell viability points towards essential mitochondrial functions.

Cell strategy to decrease proguanil inhibitory effect

Uncovering the cell strategies to decrease a drug inhibitory effect could give clues about its mode of action. We thus selected resistant mutants.

Several subclones from AD1-9 were grown on YPD, and then incubated on YPG containing 150 μM of proguanil, a concentration that fully inhibited cell growth. Only a few resistant colonies appeared after two months of incubation at a frequency of around 10^{-10}, which was lower than the frequency we observed in other screens. Four mutants were studied, issued from different subclones, and analysed. They presented a weak resistance to proguanil with an IC_{50} (determined in YPEth medium) three-fold higher than the IC_{50} of the parental AD1-9 strain and unaffected growth competence.

Whole genomic sequencing was performed and revealed the same mutation: a T to G nucleotide substitution at position 41 downstream *PUF3* coding sequence, in the terminator region [21]. The mRNA-binding protein Puf3 was reported to have several mitochondrial targets. In particular it was shown to regulate Coq5 abundance and Co-enzyme Q (CoQ) biosynthesis [22]. Therefore we tested whether the deletion of *COQ5* could affect the sensitivity to proguanil. *COQ5* encodes the 2-hexaprenyl-6-methoxy-1,4-benzoquinone methyltransferase, forming a large complex with other CoQ biosynthetic enzymes.

We compared Δcoq5 with mutant rho°, both deriving form AD1-9 and both defective in respiratory function, and presenting same growth behaviour (fig.4A). The rho°, as shown in fig.3, presented a proguanil sensitivity very similar to WT. Interestingly, the absence of Coq5 and thus of CoQ synthesis, resulted in a decreased sensitivity to proguanil.

We then tested the effect of adding the CoQ analogue decylubiquinone in the growth medium, using the WT strain (AD1-9) (fig.4B). Addition of decylubiquinone increased proguanil inhibitory effect.

CoQ plays a key and well-known role in electron transfer and has been shown to act as an anti-oxidant that protects the membrane lipids against peroxidation (see for instance, [23] and references within). Yeast synthesize only saturated and monounsaturated fatty acids that are not prone to peroxidation but CoQ-deficient yeast cells were shown to be hypersensitive to the auto-oxidation of added polyunsaturated fatty acids [24].

CoQ was also reported to stabilize the phospholipid membrane. In presence of CoQ-10 the membranes are less permeable to hydrophilic solutes [25]. In absence of CoQ, one could thus expect that the membrane might be more permeable. The effect of decylubiquinone addition and of Δcoq5 mutation that abolishes CoQ biosynthesis on proguanil sensitivity might be related to that property. In the mutant lacking CoQ, a more permeable mitochondrial membrane might facilitate the diffusion of proguanil out of the mitochondria while adding decylubiquinol might increase proguanil retention, which could explain the lower or enhanced sensibility of the yeast growth to the drug. We could also hypothesize that proguanil would be transported into the mitochondria by a transporter whose activity is modulated by CoQ level.

Possible mode of action

Proguanil and other biguanides are positively charged molecules. It was suggested that biguanides could accumulate in the mitochondrial matrix at concentrations 1,000-fold higher than extracellular concentrations in response to cell and mitochondrial membrane potentials and depending on the presence of transporters [8].

Little is known about the transport of proguanil into the mitochondria. In mammalian cells, the drug seems unable to access the mitochondrial matrix contrarily to the related biguanide phenformin whose uptake would involve the organic cation/carnitine transporter (OCTN1) [8] [26] [27]. By contrast, in yeast as in the malaria parasite, proguanil could most likely access, and thus possibly accumulate into, the mitochondria as the synergy with atovaquone that specifically targets the bc_1 complex points toward a mitochondrial location of the drug activity.

We propose that proguanil would not have a specific target but accumulate at concentrations that impair multiple mitochondrial functions and/or block transporters, which would be lethal for the cells. The only resistance strategy would be to lower the mitochondrial level of proguanil.

The search for and study of proguanil resistant mutants seem to support this proposal. The low frequency of resistant mutants found would be in favour of a multi-target mode of action for proguanil. The sole mechanism of resistance (decrease in CoQ level) likely works by lowering the drug concentration. One could have expected that the resistance screens would identify mitochondrial transporter(s) possibly required for the transport of proguanil into the mitochondria. Our selection assays may not have fished all the possible resistance mutants or proguanil might have multiple transporters. The possible proguanil transporter(s) might also be essential and their activity could not be impaired.

If proguanil toxicity is due to its mitochondrial accumulation, resulting in multiple function defect, what could be the mechanism of the synergy with atovaquone (and other bc_1 complex inhibitors)? It should be noted that the synergy with atovaquone is not specific to proguanil but observed, in the parasite, with other biguanides [6].

A decrease of the mitochondrial membrane potential caused by the inhibition of the respiratory chain by atovaquone could not explain the synergy. Indeed, a rho° mutant (lacking a respiratory chain) is not more sensitive to proguanil than its parent rho^{+} (fig.3). On the contrary, a slightly decreased sensitivity was observed. Likewise, when the WT strain was grown in fermentation conditions (in glucose rich medium without aeration), its sensibility to proguanil was two- to three-fold decreased compared to its sensitivity in respiration conditions (not shown). An accumulation of proguanil depending on the mitochondrial membrane potential (as reported for other biguanides [8]) could explain that lower sensitivity.

More work would thus be needed to unravel the mechanism of synergy.

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Figure legends

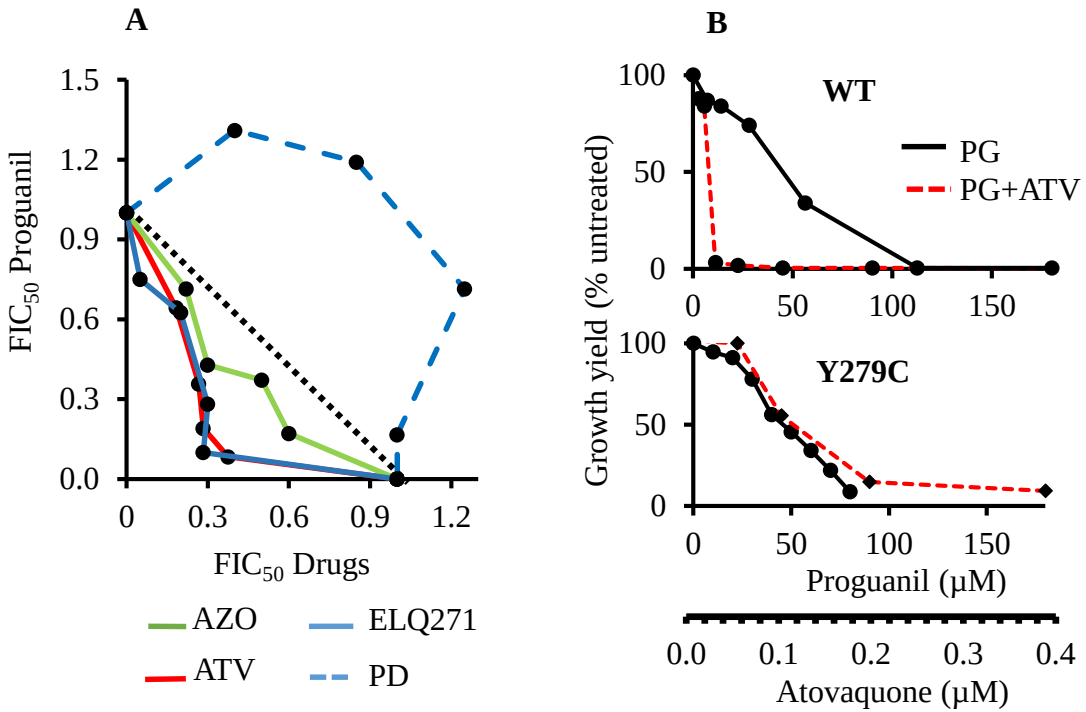
Fig. 1 Effect of proguanil combination with respiratory growth inhibitors. (A) Isobologram showing the effect of proguanil combination with atovaquone (ATV), azoxystrubin (AZO), ELQ271 and plasmodione (PD) on yeast growth. The dotted line shows a theoretical indifferent interaction. (B) Respiratory growth sensitivity of the atovaquone resistant strain Y279C and WT to proguanil alone (PG, straight line) and proguanil-atovaquone combination (PG+ATV, dotted line). Cells were grown in YPEth with increasing concentration of drugs. OD_{600nm} were measured after three days culture at 28°C. The results are presented as % of growth yield without treatment.

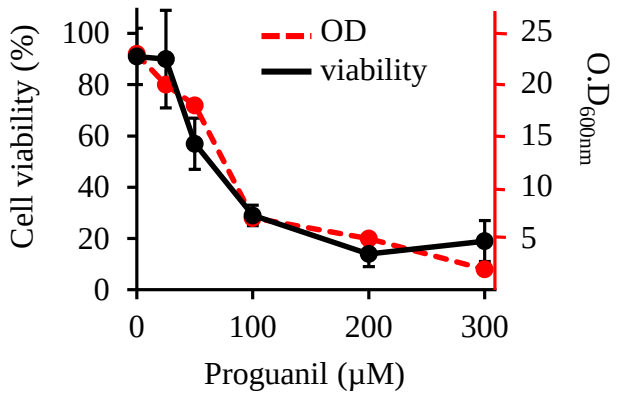
Fig. 2 Effect of proguanil treatment in cell growth and viability. WT cells were grown for 24h in YPEth to an OD_{600nm} of around 2, and then incubated for another 24h in YPEth without or with drugs. The OD_{600nm} of cultures were then recorded. The cells were then harvested, plated on YPD and incubated four days. The number of colonies observed was reported on the number of plated cells to determine the percentage of viability. Each measurement was repeated at least three times and averaged. Error bars represent standard deviation.

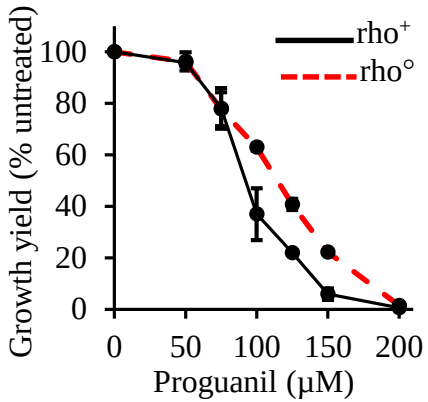
Fig. 3 Effect of respiratory chain on cell sensitivity to proguanil. WT (rho^+) and rho° strains were grown in YPGal with various concentrations of proguanil. After two days growth at 28°C, OD_{600nm} were measured. For each strain, the data are presented as percentage of the OD_{600nm} of the untreated culture. The experiments were repeated at least twice and the data averaged. Error bars represent standard deviation

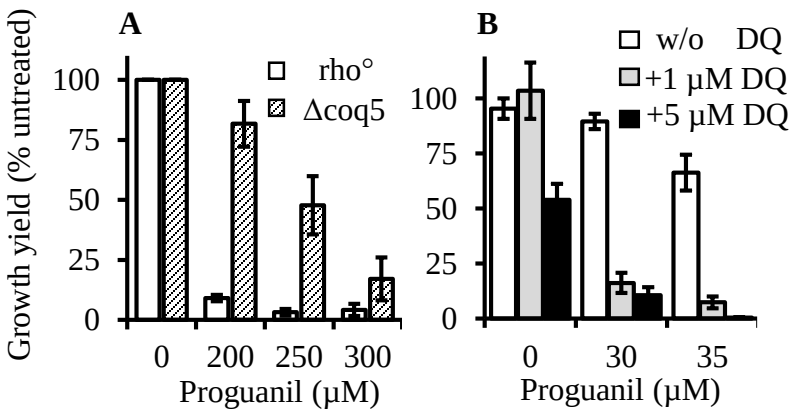
Fig. 4 Effect of CoQ biosynthesis mutant Δcoq5 and of decylbiquinone addition on growth sensitivity to proguanil. (A) Growth sensitivity of Δcoq5 and rho° mutant to proguanil; (B) Effect of decylbiquinone (DQ) addition on the growth sensitivity to proguanil. Cells were grown in YPGal (A) or YPEth (B) with various concentrations of proguanil. OD_{600nm} were

measured after three days. For each strain, the data are presented as percentage of the OD_{600nm} of the untreated culture. The experiments were repeated at least twice and the data averaged. Error bars represent standard deviation


A
1.5
1.2
0.9
0.6
0.3
0.0
FIC50 Proguanil
0
0.3
0.6
0.9
1.2
FIC50 Drugs
AZO
ELQ271
ATV
PD
B
WT
PG
PG+ATV
100
50
0
Growth yield (% untreated)
0
50
100
150
Y279C
100
50
0
0
50
100
150
Proguanil (µM)
0.0
0.1
0.2
0.3
0.4
Atovaquone (µM)


Cell viability (%)
100
80
60
40
20
0
OD
viability
25
20
15
10
5
O.D600nm
0
100
200
300
Proguanil (µM)


Growth yield (% untreated)
100
80
60
40
20
0
0
50
100
150
200
Proguanil (µM)
rho+
rho°


A
B
Growth yield (% untreated)
100
75
50
25
0
rho°
Δcoq5
0
200
250
300
Proguanil (μM)
w/o DQ
+1 μM DQ
+5 μM DQ
0
30
35
Proguanil (μM)