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Resistance Mechanism in Adapted Cells to Cancer Drugs

Master of Science Thesis [in the Biotechnology Programme]

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CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden, 2012

THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Resistance Mechanism in Adapted Cells to Cancer Drugs

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Abstract

Background: One obstacle to greater success with chemotherapy treatment is drug resistance. Patients receiving chemotherapy become adapted to previously effective drugs and no longer respond to the effects of drugs. This is due to an evolutionary process, whereby cancer cells accumulate mutations randomly and the ones with mutations causing resistance will prevail during drug treatment through simple Darwinian selection. It is a daunting challenge to predict how genetic adaptation will occur and which adaptive mutations are likely to arise and become fixed during selection. The challenges are less formidable if using model systems that lend themselves to analysis of the mutational and selective process. Therefore, to study evolutionary adaptation in response to controlled selective pressures, a unique opportunity has been provided by experimental evolution of yeast populations.

Results: A strain of *Saccharomyces cerevisiae* was evolved for 200 generations during exposure to four anti-cancer drugs. Three of these are mutagenic and the remaining one, rapamycin, inhibits the TOR signaling pathway. The drugs were applied both in single treatments and in all pair-wise combinations. Four replicate populations of each treatment were phenotyped by Bioscreen analyser C and promising results obtained from three growth variables; adaptation time, doubling time and growth efficiency. Also, the viability of founder strain and evolved populations under selective pressure was measured by drop test. The results of drop test show no significant difference between founder strain and the last generation of adapted populations in case of survival.

Conclusions: In general adaptation occurs faster in single treatments than pair-wise treatments. In pair-wise treatment the mode of adaptation depends on the combination of drugs. It can be rapid and strong or slow and weak. Variability between populations is low, suggesting few allowed evolutionary paths. Survival has not been changed whereas proliferation and efficiency have been affected.

Keywords: Anti-cancer drug resistance, Adaptation, *Saccharomyces cerevisiae*, Bioscreen.

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List of abbreviation:

ATM	Ataxia Telanagiectasia Mutated
Atr	ATM and RAD3-Related
Bmh	1,6-bismaleimidohexane
bp	base pair
CI	Cisplatin
Chk2	Checkpoint kinase 2
CSM	Complete Supplement Mixture
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
DO	Doxorubicin
4E-BP1	Eukaryotic translation initiation factor 4E-BP1
elF4E	Eukaryotic initiation factor elF4E like protein
FDA	Food and Drug Administration
FK506 (FKBP5)	FK506 binding protein5 (Peptidyl-prolyl cis-trans isomerase FKBP5)
FKBP12	Peptidyl-prolyl cis-trans isomerase FKBP12
G1	Gap1
GT	Generation Time
HIF-1α	Hypoxia inducible factor-1α
Ho	homing endonuclease
HU	Hydroxyurea
HygMX	Constructed genotype of Hygromycin resistance gene
KanMX	Constructed genotype of Kanamycin resistance gene
LSC	Logarithmic Strain Coefficients
Mat a/α	Mating type a/α
MDR	Multiple Drug Resistance protein

Mec1	Mitosis entry checkpoint protein
OD	Optical Density
O/N	Over Night
$p27^{Kip1}$	$p27^{Kip1}$ protein
PCR	Polymerase Chain Reaction
P-glycoprotein	Permeability glycoprotein
PP2A	Protein Phosphatase 2A
RA	Rapamycin
RAD52	Repair and Recombination protein 52
RNA	Ribonucleic Acid
RNR	Ribonucleotide Reductase
S6K1	S6 Kinase1
SC	Synthetic Complete
SD	Synthetically Defined
SGRP	*Saccharomyces* Genome Resequencing Project
TBE	Tris Borate Ethylenediaminetetraacetic acid
TOR	Target of Rapamycin
URA3	Uracil3
YFP	Yellow Fluorescent Protein
YNB	Yeast Nitrogen Base

1. Introduction

1.1. Background

The generation of random variation through mutation and concomitant natural selection of the fittest phenotypes resulting from this variation is the fundamental process in biology. The consequence of these processes are the increase in frequency of beneficial variation within a population, the increase in average fitness within the population, and the continuous diversification of populations living in different ecological contexts and for which different phenotypes are beneficial (1). In the cellular scale, adaptation is defined as changes in gene-products made by a cell in response to different environmental cues and it can be either physiological (normal) or pathological (abnormal) (2). In case of cancer, adaptation is problematic when cells become resistant to chemotherapy. When cancer cells become resistant to the effects of the chemotherapy, they stop responding to the drug and begin to divide. In this situation the drug should be changed. In the list below, there is a mixture of types of mutation on the DNA level, and the physiological changes resulting from changes in the corresponding gene products (proteins).

1- Altered cell cycle checkpoints.

2- Changes in specific metabolism of a drug.

3- Alteration of the specific target of a drug and loss of a cell surface receptor or transporter for a drug.

4- Activation of the DNA breaks repair mechanisms.

5- Increased efflux which is observed when cancer cells pump the drug out of the cell with the same or higher speed than uptaking the drug.

6- Gene amplification which leads to the production of a particular gene as much as hundreds of copies, consequently, the overproduction of a protein can make the drug ineffective due to increased drug targets.

7- Development of a mechanism that inactivates the drug.

8- Decreased uptake; sometimes the protein that transports the drug across the cell wall stops working, then cancer cells stop taking in the drug.

Using drugs in combination seem a functioning strategy against drug resistance development. It is thought that the probability of developing resistance to any one drug is reduced in this way (3).

In this study, four different drugs were examined; cisplatin, hydroxyurea, doxorubicin and rapamycin. To observe the difference between single treatment and combined treatment in causing adaptation, cisplatin, hydroxyurea, doxorubicin and rapamycin as single treatments and cisplatin+hydroxyurea, cisplatin+doxorubicin, cisplatin+rapamycin, hydroxyurea+doxorubicin, hydroxyurea+rapamycin and doxorubicin+rapamycin as combined

treatments were selected to study. Also, to investigate the effect of ploidy on this phenomenon, both haploid and diploid cells were exposed to doxorubicin.

For over 30 years, cisplatin has been used in treatment of cancers including testicular, ovarian and lung cancer. When it enters the cell, cisplatin becomes positively charged and then interacts with nucleophilic molecules such as; DNA, RNA and proteins. Cisplatin toxicity is mainly believed due to interaction with DNA, and then formation of inter- and intra-strand adducts which hinder both RNA transcription and DNA replication, consequently, this process leads to cell cycle arrest and apoptosis.

Apparently, multiple resistance mechanisms are induced by cisplatin and it is difficult to determine which of these plays more important role in resistance as many of these mechanisms are linked by the cellular stress response. The contributed mechanisms to cisplatin resistance include increased efflux, reduced uptake, increased detoxification, inhibition of apoptosis, increased ability to replicate past DNA adducts and increased DNA repair. It is demonstrated that combination of gemcitabine which is a cell cycle specific antagonist with cisplatin is more effective than either drug alone. By this combination, toxicity is enhanced in cisplatin resistant cells, revealing that gemcitabine reverses cisplatin resistance (4).

Hydroxyurea inhibits the enzymatic activity of ribonucleotide reductase (RNR); as a consequence of this, it inhibits DNA replication in a wide variety of cells, including *Saccharomyces cerevisiae.* It has been discovered that hydroxyurea inhibition of DNA synthesis occurs by starving the DNA polymerase at the replication forks for dNTPs. Adapted cells to hydroxyurea have evolved a mechanism for arresting replicative DNA elongation before exhaustion of dNTP pools. Conserved mechanisms for controlling RNR levels and activity, by the Mec1/Rad53 pathway in yeast and the homologous Atr/Chk2 pathway in mammals reveals the importance of boosting dNTP levels in response to DNA damage. Longer time for transcriptional and post transcriptional processes causing by the Mec1/Rad53 pathway to boost dNTP pools clarify the advantages of arresting DNA synthesis at replication forks to conserve the dNTP pools for DNA synthesis at repair sites. In summary, replication arrest compensates time for DNA repair to take place and preserves the activated precursor pools needed for DNA repair (5).

Doxorubicin (14-hydroxydaunorubicin) was isolated from the soil bacteria *Streptomyces peucetius* subspecies *caesius* around half a century ago. It can be found with three other anthracycline antibiotics ε-rhodomycinone, daunorubicin (daunomycin), and baumycins as well as other intermediates of baumycin biosynthesis (6). Doxorubicin exact mechanism of action is unknown but its anti cancer activity can be categorized in three ways. It intercalates into DNA and binds the strands of genetic material together; this insertion prevents cells making DNA, RNA and proteins. It also appears to interfere with the enzyme topoisomerase II, which is involved in DNA replication and this stops the cancer cells growing and multiplying. Moreover, it can form free radicals and damage the malignant cells with these reactive molecules. Anthracycline resistance occurs through increases in antioxidant defenses, overexpression of P-glycoprotein, lung resistance proteins and multidrug resistance proteins, proteasome subunits and alterations in apoptotic signaling (7).

Rapamycin, was isolated and identified as an antifungal agent from the soil bacteria *Streptomyces hygroscopicus* around 40 years ago. Rapamycin (sirolimus) and the macrolide antibiotic FK506 (tacrolimus, Prograf®) are structural analogues. Therefore, like FK506, it was found to suppress the immune system. Finally, as an immunosuppressive drug, rapamycin (Rapamune®) was approved by the Food and Drug Administration (FDA) in the USA in 1999 and the European Commission in 2000, respectively. From coming results, it has been discovered that rapamycin, in contrast to FK506, is not only an immunosuppressant, but also an active antitumor agent. As a cytostatic, it can slow or arrest cells in G1 phase and in some special cases it can induce apoptosis in culture. Rapamycin inhibits mTOR downstream signaling pathway which is highly conserved from budding yeast to mammalian cells. TOR acts to sense nutritional status in *S. cerevisiae* and regulates response to starvation via well studied pathways, and it controls translation initiation, protein turnover, transcription, and actin cytoskeleton organization. It is clear that mTOR signaling is critical for proliferation of many cancer cells in vitro and for tumor growth in vivo. Some certain characteristics of malignancy such as; anchorage independent growth and angiogenesis through control of HIF-1α may be regulated by mTOR.

As a consequence of monotherapy, cells can become resistant to rapamycin. Some defined reasons underlying the adaptation mechanism include; mutations in FKBP12 and mTOR, deregulation of eIF4E (mTOR phosphorylates and regulates the function of 4E-BP1, the suppressor of eIF4E. Resistance to rapamycin is associated with decreased levels of 4E-BP1.), mutations in S6K1 (one of the principal downstream effector of mTOR). Mutations of PP2A-Related Phosphatases, defective regulation of $p27^{Kip1}$, mutations of ATM, and mutations of 14-3-3 (In *S. cerevisiae*, Bmh1 and Bmh2 are two homologous of the mammalian 14-3-3 proteins which overexpression of *BMH1* or *BMH2* leads to resistance to rapamycin and in case of *BMH1* and/or *BMH2* disruption the yeast cells become sensitized to rapamycin.) (8).

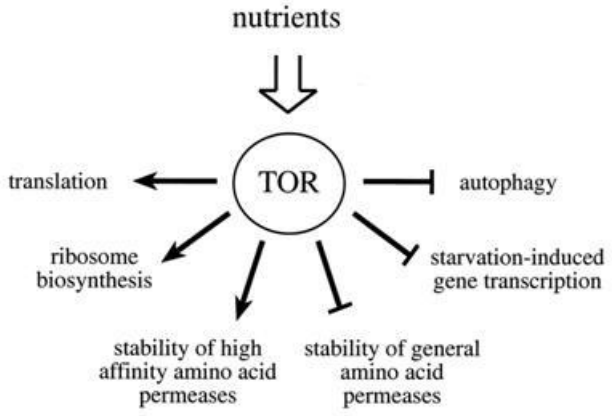

Figure 1; the TOR proteins have a key role to balance between protein synthesis and protein degradation. To enrich protein synthesis, TOR signaling should be active and presence of sufficient nutrients makes it possible for this signaling to become active. The TOR signal causes translation initiation, ribosome biosynthesis, and the stabilization of high affinity amino acid permeases. On the other hand this signaling destabilizes general amino acid permeases, represses the transcription of genes needed for amino acid biosynthesis and inhibits autophagy. This figure was reproduced with permission from (9).

The budding yeast *Saccharomyces cerevisiae* was used in this study. As an ideal organism in which to address many of the central questions facing biologist, some advantages featuring genetics, simplicity, microorganism and popularity can be mentioned. It is very amenable to genetic manipulation because of low genomic complexity of yeast and extremely high rates of homologous recombination. The short generation time greatly benefits multistep genetic manipulations, unlimited amounts of cells can be grown from an isogenic colony for biochemical studies, much is known about the biology of yeast as a consequence of its popularity and it becomes increasingly useful by technological innovations. Finally, the limited number of behaviors can be counted as the advantages of using yeast (10).

In this study, the experiments were carried out using the heterothallic strain S288c. Historically, this strain was obtained through genetic crosses from the EM93 progenitor and it is popular because naturally it is a rare heterothallic (ho) strain meaning that its mating type is stable and it cannot undergo mating type switching, whereas the large number of *S. cerevisiae* strains are homothallic diploids meaning that they can undergo mating type switching which is a gene conversion process and it is initiated by a site-specific endonuclease HO that it can be followed by mother-daughter mating (11), (12).

1.2. Objective

To slow down evolutionary adaptation and to improve the functionality of chemotherapy treatment, it is assumed that application of anti-cancer drugs in combinations can be more effective than single ones.

This study has been performed for two reasons;

1- Investigation of resistance mechanisms in treated cells with anti-cancer drugs in single patterns and in pair-wise combinatorial patterns.
2- Investigation of resistance mechanisms caused by DNA-damaging agents and non DNA-damaging agents.

2. Materials and methods

2.1. Strains and media: Throughout the study, the yeast strain S288c *Mat a* and S288c *Mat a/α* (*Mat a*, *Mat a/α*, *ho::HygMX*, *ura3::KanMX-6bpBarcode(GGATCC)*) was used (13). All experiments were performed using synthetic complete (SC) medium (2% glucose, CSM (complete supplement mixture) and YNB (yeast nitrogen base)) at 30 °C.

2.2. Microcultivation technique: This method was applied for various studies. Growth in microplates was automatically recorded using a Bioscreen analyser C (Thermic Labsystems Oy, Finland). The optical density was measured using a wide band (450–580 nm) filter to reduce the contribution to the reading from the medium. Incubation was kept at 30.0 °C, ±0.1 °C (10 min preheating time). The plates were subjected to shaking at the highest shaking intensity with 60 s of shaking every other minute. OD measurements were taken every 20 min during a 24 h period. This time-span allows for maximum throughput while still allowing most yeast cells (with a doubling time <4.5–5 h) to reach stationary phase. Strains were run in duplicates (separated by day of the run and plate position). Inoculation cultures were taken from cultures that had been inoculated from loop-fulls of cells from agar plates and incubated overnight (approximately 18 h) at 30.0 °C on a rotator in 5 ml SD medium in 15 ml plastic test tubes (Falcon). Each well contained 350 µl solutions (175 µl media which is 2x YNB and 175 µl different drugs), then 10 µl of preculture and/or any desired cells depending on the experiment is added (14).

2.2.1. Dose-response assay: Pre-cultivation and cultivation in a Synthetically Defined (SD) medium, with and w/o drugs, were performed as earlier described (14). For initial testing of drug dose-response correlations, a ladder of concentrations selected.

CI	**300.0**	**240.0**	**192.0**	**153.6**	**122.9**	**98.3**	**78.6**	**62.9**	**50.3**	**40.3**	**32.2**	**25.8**	**20.6**	**16.5**	**13.2**	**10.6**	**8.4**	**6.8**	**5.4**	**4.3**
HU	**30.0**	**24.0**	**19.2**	**15.4**	**12.3**	**9.8**	**7.9**	**6.3**	**5.0**	**4.0**	**3.2**	**2.6**	**2.1**	**1.6**	**1.3**	**1.1**	**0.8**	**0.7**	**0.5**	**0.4**
DO	**200.0**	**160.0**	**128.0**	**102.4**	**81.9**	**65.5**	**52.4**	**41.9**	**33.6**	**26.8**	**21.5**	**17.2**	**13.7**	**11.0**	**8.8**	**7.0**	**5.6**	**4.5**	**3.6**	**2.9**
RA	**1000.0**	**800.0**	**640.0**	**512.0**	**409.6**	**327.7**	**262.1**	**209.7**	**167.8**	**134.2**	**107.4**	**85.9**	**68.7**	**55.0**	**44.0**	**35.2**	**28.1**	**22.5**	**18.0**	**14.4**

Table 1; 20 concentrations of each drug including; Cisplatin= (µg/ml), Hydroxyurea= (mg/ml), Doxorubicin= (µg/ml) and Rapamycin= (ng/ml).

The relative growth of each treatment was calculated. To select the range of pair-wise treatments concentrations, the relative growth of generation time for each treatment was chosen in a way that when it was added to the component in its defined combinatorial pattern, the relative growth of combination varied between -1 and -3.

Relative Growth	CI+HU	CI+DO	CI+RA	HU+DO	HU+RA	DO+RA
-3	**220+30**	**220+67**	**220+770**	**30+67**	**30+770**	**59+770**
-2.78	**210+27**	**210+65.5**	**210+750**	**29.5+66**	**29.5+600**	**52.4+600**
-2.56	**200+25**	**200+67**	**200+700**	**24+40**	**24+700**	**29+700**
-2.34	**180+22**	**180+60**	**180+700**	**22+37**	**22+670**	**35+670**
-2.12	**170+20**	**170+33.6**	**170+660**	**21+39**	**21+640**	**33.6+640**
-1.9	**155+17**	**155+30**	**155+640**	**19+33.5**	**19+620**	**32+620**
-1.68	**130+13.5**	**130+30**	**130+600**	**15+30**	**15+555**	**31+600**
-1.46	**123+12.3**	**123+25**	**123+565**	**10+25**	**10+500**	**26.8+460**
-1.24	**110+8**	**110+22**	**110+405**	**8+22**	**8+405**	**22+409**
-1.02	**99+6.3**	**99+15**	**99+400**	**6.3+13**	**6.3+403**	**17+403**

Table 2; 10 concentrations for each pair-wise treatment. The concentration unit for each drug is; Cisplatin= (µg/ml), Hydroxyurea= (mg/ml), Doxorubicin= (µg/ml) and Rapamycin= (ng/ml).

2.2.2. Phenotyping assay: From 200 generations, 11 different generations were selected. Generations 5,15,30,45,60,80,100,120,140,160 and190 were phenotyped for hydroxyurea, doxorubicin (haploid cells), doxorubicin (diploid cells), rapamycin, hydroxyurea+rapamycin, doxorubicin+rapamycin treatments and generations 5,15,30,45,60,80,100,120,135,160 and190 were phenotyped for cisplatin, cisplatin+hydroxyurea, cisplatin+doxorubicin, cisplatin+rapamycin, hydroxyurea+doxorubicin treatments. Adapted cells of different treatments from generations mentioned above, were inoculated in 350 µl of synthetic defined (SD) medium and drugs. Concentrations of drugs used for this experiment; cisplatin=190 µg/ml, hydroxyurea=30.15 mg/ml, doxorubicin (haploid cells)=53.6 µg/ml, doxorubicin (diploid cells)=53.6 µg/ml, rapamycin=0.75 µg/ml, cisplatin+hydroxyurea(cisplatin=73.0 µg/ml, hydroxyurea=7.3 mg/ml), cisplatin+doxorubicin(cisplatin=73.0 µg/ml, doxorubicin=12.0 µg/ml), cisplatin+rapamycin (cisplatin=98.21 µg/ml, rapamycin=0.4 µg/ml), hydroxyurea+doxorubicin(hydroxyurea= 9.805 mg/ml, doxorubicin=17.2 µg/ml), hydroxyurea+rapamycin(hydroxyurea=4.5 mg/ml, rapamycin=0.39 µg/ml), doxorubicin+rapamycin(doxorubicin=63.755 µg/ml, rapamycin=0.74 µg/ml).Two precultures were run (haploid cells and diploid cells, separately). For experimental runs (duplicates) were cultivated at 30 °C for 72h with duplicates on separate plates. Rate of growth, efficiency of growth and adaptation time were calculated. In each run, eight wells of founder strain were included and used for normalization of strain behavior, forming strain coefficients [logarithmic (natural logarithm) strain coefficients (LSC)] (15).

2.3. Adaptation assay: This experiment was started with four replicates of each founder strain, populations were cultivated for 200 generations, and strains grew in different environments (cisplatin, hydroxyurea, doxorubicin, rapamycin, cisplatin+hydroxyurea, cisplatin+ doxorubicin, cisplatin+ rapamycin, hydroxyurea + doxorubicin, hydroxyurea+ rapamycin, doxorubicin+ rapamycin, cisplatin+ hydroxyurea+doxorubicin). Every second well in 96-well plate was used to avoid cross-contamination and each well was filled with 95 µl 2x YNB and 95 µl drug, then 10 µl cells were added. Cells were incubated at 30 °C until they reached to stationary phase and they transferred to another 96-well plate, again exposing to fresh media with drugs (drugs concentrations were exactly the same). Addition of cells to each well was followed with the same order.

Drug Concentrations in Single Treatments	
Cisplatin=122.9 µg/ml	Hydroxyurea=30.15 mg/ml
Doxorubicin=53.6 µg/ml	Rapamycin=0.75 µg/ml
Drug Concentrations in Pairwise Treatments	
Cisplatin+Hydroxyurea	Cisplatin=49.15 µg/ml Hydroxyurea=4.9 mg/ml
Cisplatin+Doxorubicin	Cisplatin=49.15 µg/ml Doxorubicin=8.6 µg/ml
Cisplatin+Rapamycin	Cisplatin=98.21 µg/ml Rapamycin=0.205 µg/ml
Hydroxyurea+Doxorubicin	Hydroxyurea=9.805 mg/ml Doxorubicin=17.2 µg/ml
Hydroxyurea+Rapamycin	Hydroxyurea=22.0 mg/ml Rapamycin=0.7 µg/ml
Doxorubicin+Rapamycin	Doxorubicin=63.755 µg/ml Rapamycin=0.74 µg/ml

Table 3; drugs concentrations for single treatments and pair-wise treatments used in adaptation experiments.

2.4. Viability assay (Drop test): For this experiment the founder strains both haploid cells and diploid cells and generation 200 of adapted cells from all treatments were tested. O/N culture of strains mentioned above were inoculated in fresh 1x YNB medium and they grew from OD=0.2 to OD=1.0. After spinning down for 3 minutes at 5000 rpm, they were washed with 1x YNB-glucose, then they inoculated 200 µl solution containing 100 µl of drugs both single treatments and pair-wise treatments (concentrations were the same as used in adaptation experiment) and 100 µl 2x YNB-glucose, for no stress environment, the founder strains exposed to 200 µl 1x YNB-glucose. Incubation was performed for 72h at 30 °C, then 10 fold dilutions repeated four times and 5 µl of each dilution was spotted on 1x YNB agar plate, two replicates were prepared. Again, they were incubated at 30 °C for 2 days. The number of colonies was counted and data related to the environments with stress were normalized with data from no stress condition. Finally, logarithmic scale with base 10 was used.

2.5. Diagnostic PCR (Verification of Contamination) assay: The last generation of each treatment (generation 200) was streaked on 1xYNB plate and incubated for 2 days at 30° C to form several single colonies. From each plate, 10 colonies were selected to perform the Yeast Colony PCR. According to Blackburn protocol this method has two steps:

2.5.1. Yeast Cell Lysis; 3 µl of 0.02 M NaOH was aliquot into PCR tubes. Using a sterile pipette tip, a small colony was picked and resuspended in NaOH to have a cloudy solution meaning that enough cells were added. To boil the samples on a PCR machine, the tubes were incubated at 99° C for 10 minutes.

2.5.2. PCR; the master mix solution containing: 2.5 µl 10x PCR buffer, 0.5 µl dNTPs (10 mM each), 0.5 µl forward primer (100 µM) with this sequence (5'-3'): GAAACGAAGATAAATCATGGGATCCCGTACGC, 0.5 µl reverse primer (100 µM) with this sequence (5'-3'): GAAACGTGAGTCTTTTCCTTACCCATGGT, 0.25 µl Dream Taq polymerase and 17.75 µl ddH2O was prepared. Then PCR was run for every 25 µl reaction tube with this programme; predenaturation step at 94° C for 5 minutes, denaturation step at 94° C for 30 seconds, annealing step at 60° C for 30 seconds, elongation step at 72° C for 1 minute and the last three steps repeated for 30 cycles. The final elongation was at 72° C for 10 minutes (16).

The last step of diagnostic PCR was performing the gel electrophoresis. Ten PCR products, negative control and GeneRuler 50 bp DNA Ladder were loaded in separate wells of gel containing 0.7 gr agarose dissolved in 100 ml 1xTBE buffer and 7 µl ethidium bromide. The gel was run at 100 V for 40 minutes.

3. Results and discussions

3.1. Dose-response assay results and discussions

There are three types of interactions between two drugs. It can be additive (no effect), synergistic, or antagonistic when their combined effect is equal, greater or less than that predicted according to their individual effects (17).

X-axis in all results of dose-response experiment corresponds to predicted effects, assuming a multiplicative model of drug effects in pair-wise treatments. The predicted effect was calculated for both generation time (GT) phase and efficiency phase.
GT Predicted Effect =LOG_2 GT [drug 1] + LOG_2 GT [drug 2]
Efficiency Predicted Effect= LOG_2 Efficiency [drug 1] + LOG_2 Efficiency [drug 2]

Y-axis in all results of dose-response experiment corresponds to observed effects of drugs in pair-wise treatments. The observed effect was obtained using a Bioscreen™ machine for three variables including; adaptation time, rate of growth and efficiency of growth. The predicted effect and observed effect of each pair-wise treatment were plotted against each other to find out the antagonism, synergism and no effect of two drugs in combination. It is assumed that the effect of two drugs in combination is antagonistic when the majority of data are located above the 1:1 line; it is synergistic when the majority of data are located below the 1:1 line and there is no effect when the majority of data are located around the 1:1 line.

3.1.1. Dose-response at growth doubling time (generation time)

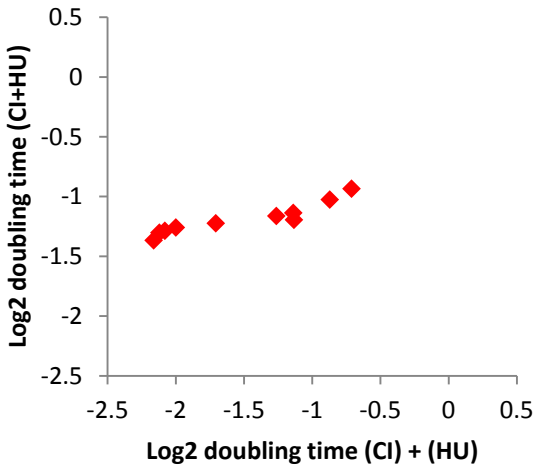

Figure 2; dose-response experiment of cisplatin+hydroxyurea treatment, generation time phase. Most of the dots are accumulated above the 1:1 line and the rest of them are very close to it. Higher drug concentrations show more synergistic effect with respect to doubling time compared to lower drug concentrations.

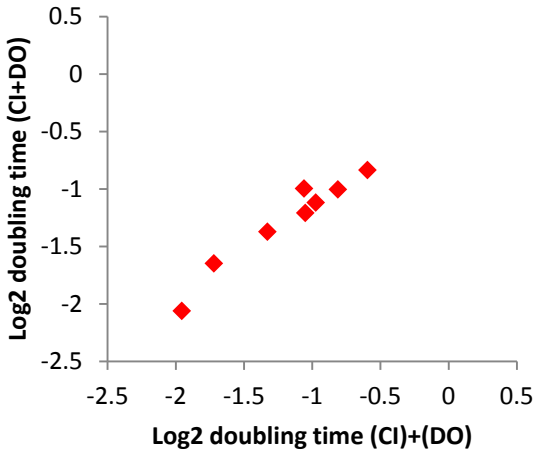

Figure 3; dose-response experiment of cisplatin+doxorubicin treatment, generation time phase. The dots are located very close to 1:1 line.

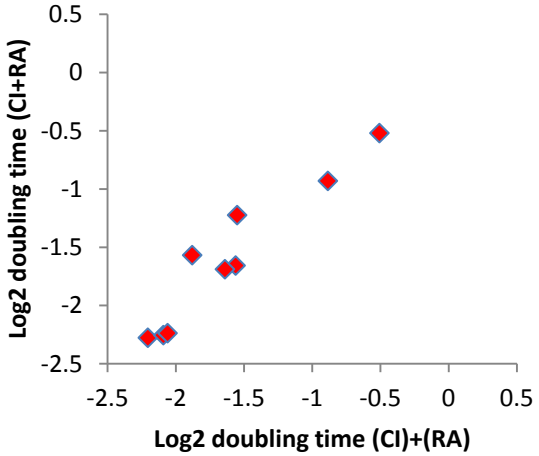

Figure 4; dose-response experiment of cisplatin+rapamycin treatment, generation time phase. The dots are located very close to 1:1 line.

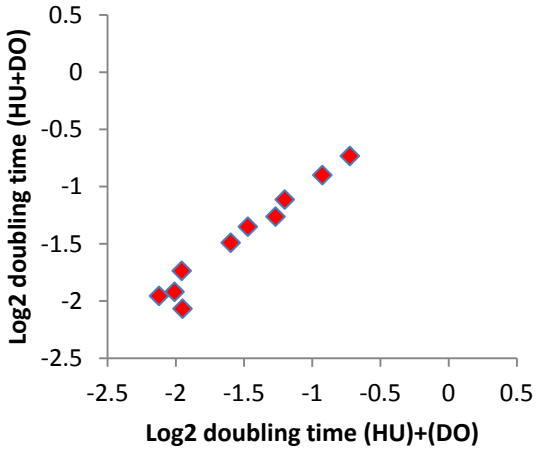

Figure 5; dose-response experiment of hydroxyurea+doxorubicin treatment, generation time phase. The dots are located very close to 1:1 line.

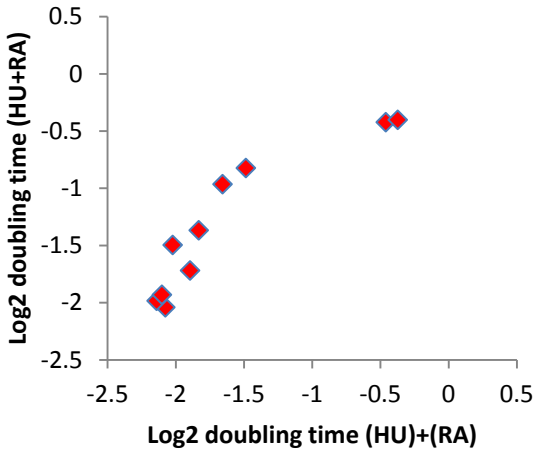

Figure 6; dose-response experiment of hydroxyurea+rapamycin treatment, generation time phase. Most of the dots are accumulated above the 1:1 line and the rest of them are almost on the line.

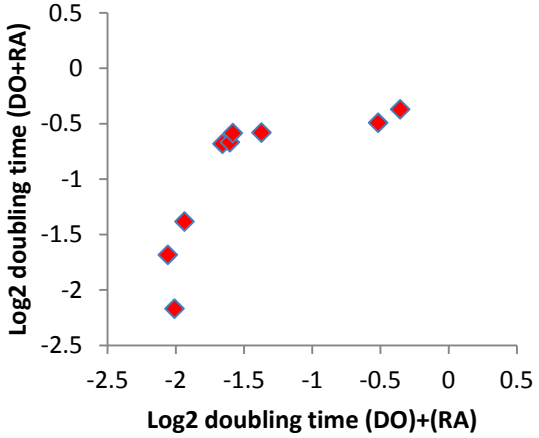

Figure 7; dose-response experiment of doxorubicin+rapamycin treatment, generation time phase. Most of the dots are accumulated above the 1:1 line and the rest of them are almost on the line.

3.1.2. Dose-response at growth efficiency (yield)

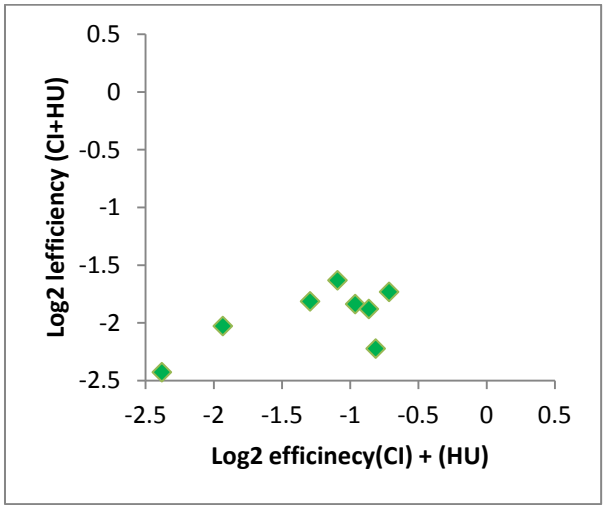

Figure 8; dose-response experiment of cisplatin+hydroxyurea treatment, efficiency phase. Most of the dots are accumulated below the 1:1 line and the rest of them are almost on the line.

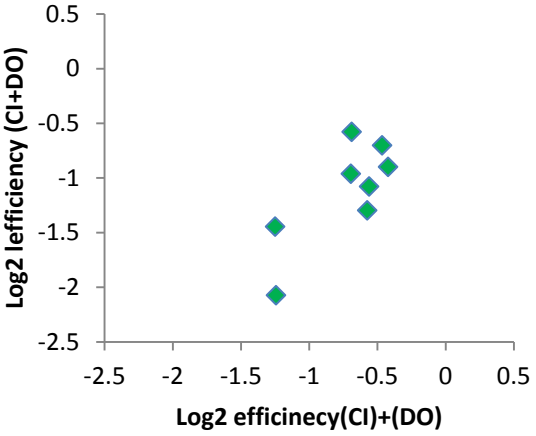

Figure 9; dose-response experiment of cisplatin+doxorubicin treatment, efficiency phase. Most of the dots are accumulated below the 1:1 line and the rest of them are very close to the line.

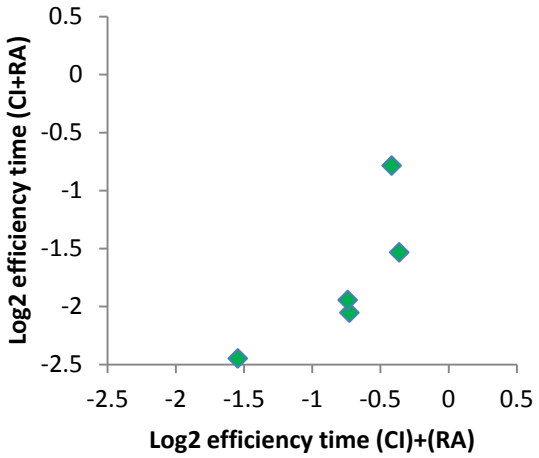

Figure 10; dose-response experiment of cisplatin+rapamycin treatment, efficiency phase. The dots are located below the 1:1 line.

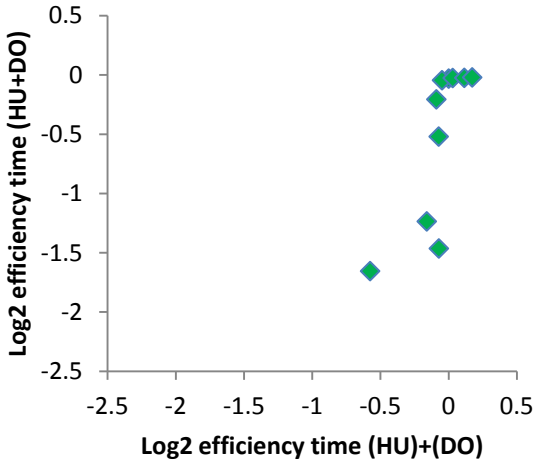

Figure 11; dose-response experiment of hydroxyurea+doxorubicin treatment, efficiency phase. Most of the dots are located below the 1:1 line and the rest of them very close to the line.

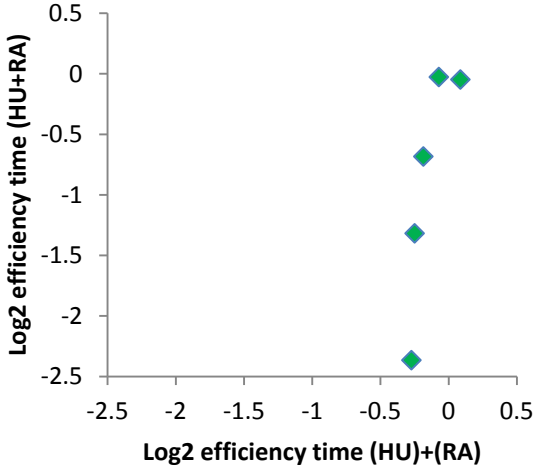

Figure 12; dose-response experiment of hydroxyurea+rapamycin treatment, efficiency phase. The dots are located below the 1:1 line.

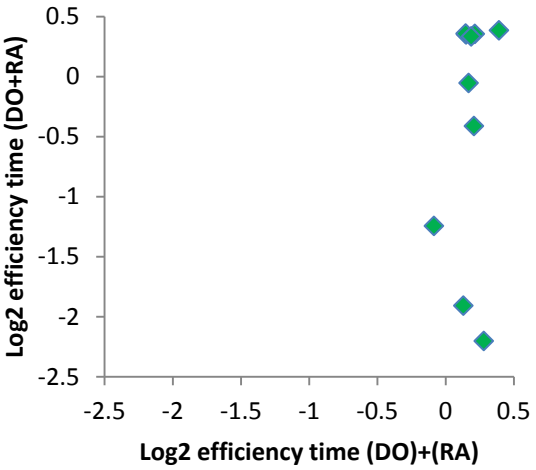

Figure 13; dose-response experiment of doxorubicin+rapamycin treatment, efficiency phase. Most of the dots are located below the 1:1 line and the rest of them very close to the line.

The results of figures above can be summarized in the table below.

Drugs	**Log_2 Doubling time**	**Log_2 Efficiency**
Cisplatin +Hydroxyurea	Slightly Antagonistic	Synergistic
Cisplatin+ Doxorubicin	No Effects	Synergistic
Cisplatin+ Rapamycin	No Effects	Synergistic
Hydroxyurea+ Doxorubicin	No Effects	Synergistic
Hydroxyurea+ Rapamycin	Slightly Antagonistic	Synergistic
Doxorubicin+ Rapamycin	Slightly Antagonistic	Synergistic

Table 4; drugs interactions in pair-wise treatments.

3.2. Phenotyping assay results and discussions

A sigmoid growth curve is followed by living cells defining by three fundamental growth variables; growth lag, growth rate, and growth efficiency. "Growth rate is extracted as the slope in the exponential phase converted into population doubling time (h), growth lag (h) is given by the intercept of the initial density and the slope and growth efficiency is calculated as the change in density for cultures having reached stationary phase" (18). The graph shows three windows of growth that can be observed from microcultivation (Figure 14).

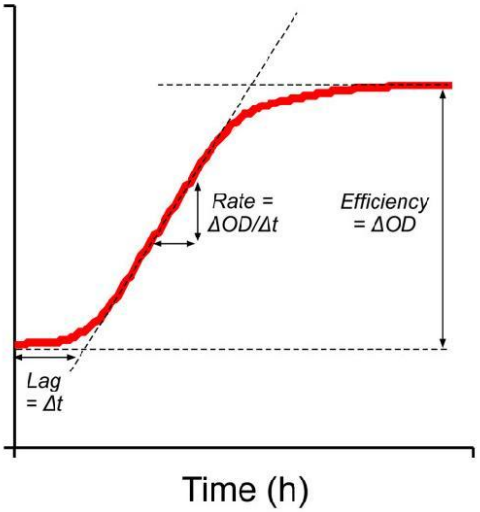

Figure 14; growth variables; growth lag, growth rate, and growth efficiency. Figure was reproduced with permission from (18).

To have clear vision of the mechanism of drug induced resistance and considered changes in fitness extraction of the composite growth measure was performed.

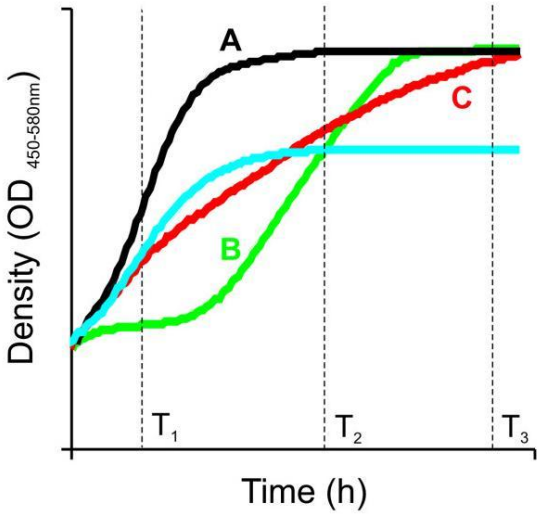

Figure 15; "Extraction of the composite growth measure (density reached) at various time-points, T1, T2 and T3, in absence of stress (A) and in presence of a compounds that impact on growth lag (B) growth rate (C) or growth efficiency (D)"(1). Figure was reproduced with permission from (18).

Having the possibility of exact observation of growth variables for adapted cells to each treatment, growth lag, growth doubling time, and growth efficiency figures were produced, separately.

3.2.1. Growth lag results

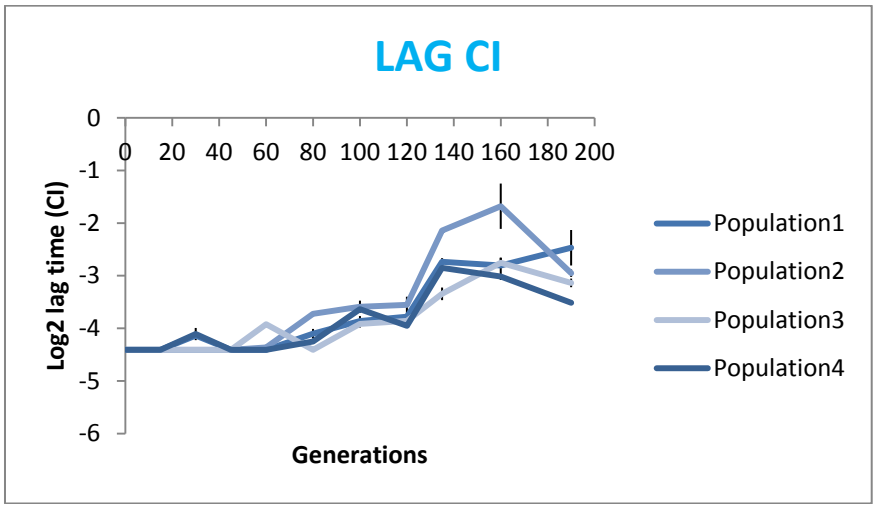

Figure 16; adapted cells to cisplatin treatment, lag phase. It seems in cisplatin treatment lag phase is really involved (long lag time for majority of generations); from generation (120) to generation (135) lag time has started to become shorter. Bars show the mean and standard error for two replicates.

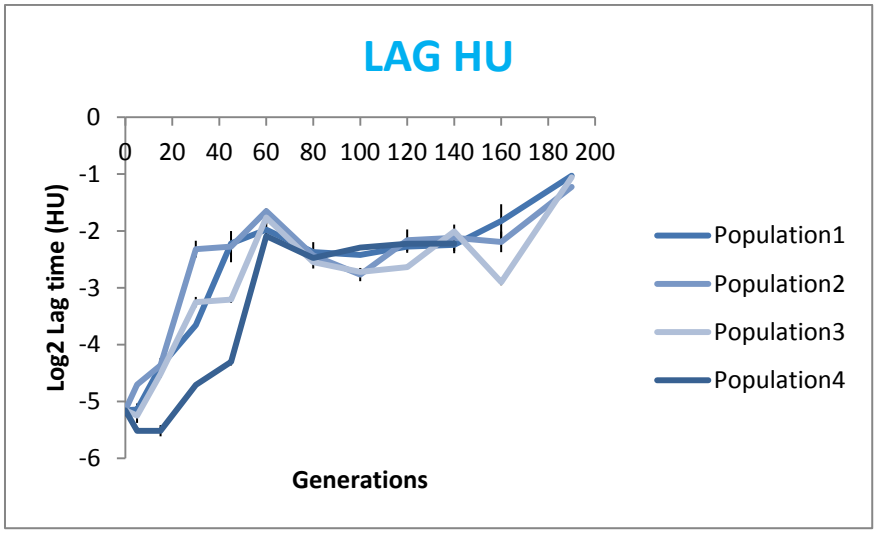

Figure 17; adapted cells to hydroxyurea treatment, lag phase. From generation (0) to generation (60) lag time has been shortened and no significant changes occurred till generation (160). Again, from generation (160) lag time has started to become shorter. Bars show the mean and standard error for two replicates.

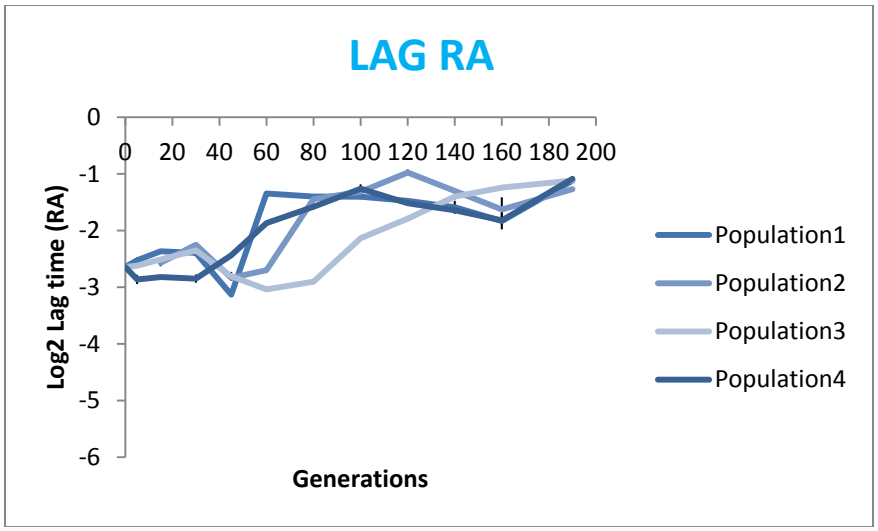

Figure 18; adapted cells to rapamycin treatment, lag phase. Four populations show different adaptive trends with respect to lag time. Bars show the mean and standard error for two replicates.

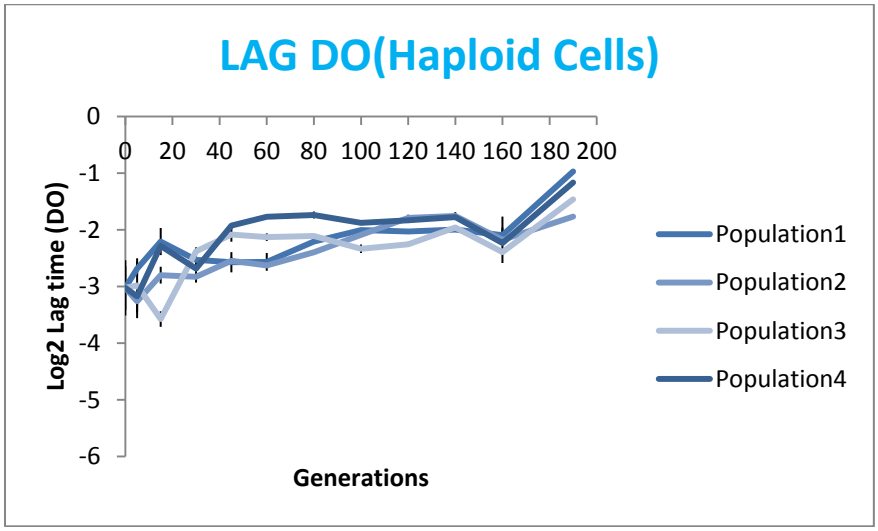

Figure 19; adapted cells (haploid cells) to doxorubicin treatment, lag phase. From generation (0) to generation (160) no significant changes occurred but from generation (160) adaptation time has started to be shortened. Bars show the mean and standard error for two replicates.

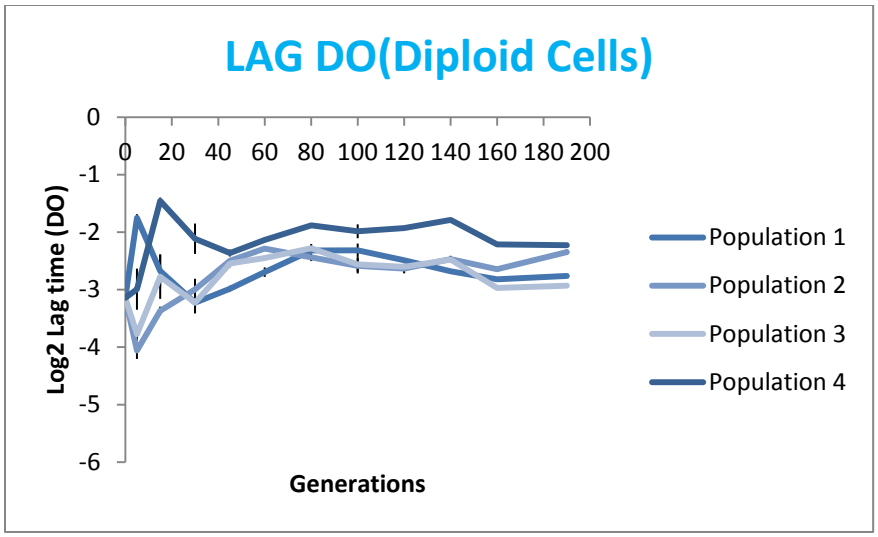

Figure 20; adapted cells (diploid cells) to doxorubicin treatment, lag phase. No remarkable change in lag time is observable. Bars show the mean and standard error for two replicates.

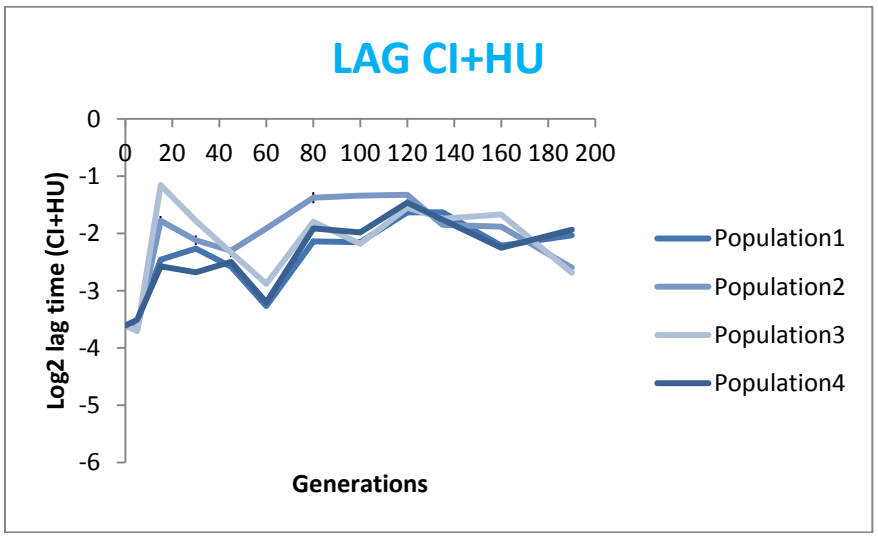

Figure 21; adapted cells to cisplatin+hydroxyurea treatment, lag phase. It seems four populations hesitate to follow a regular trend with respect to lag time. Several increases and decreases can be observed, alternatively. Bars show the mean and standard error for two replicates.

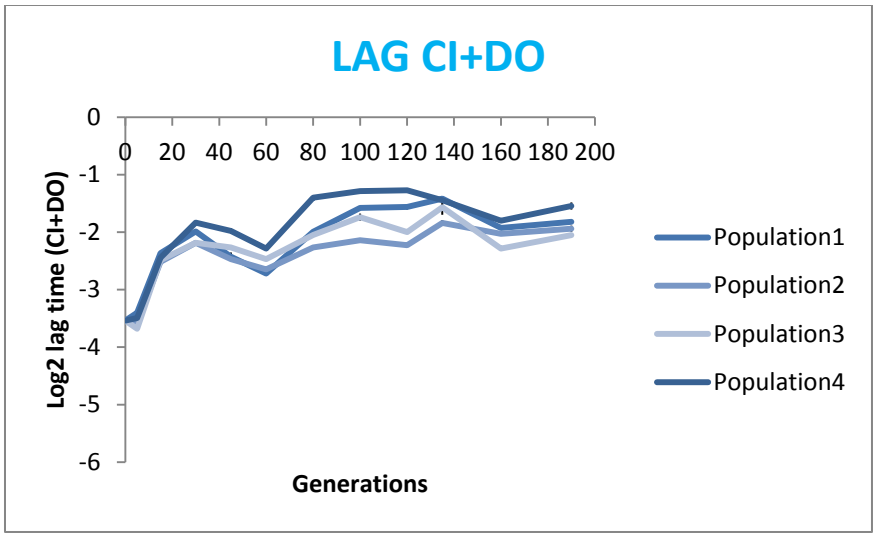

Figure 22; adapted cells to cisplatin+doxorubicin treatment, lag phase. From generation (0) to generation (24) lag time has been shortened and then prolonged but from generation (80), populations show steady state with respect to lag time. Bars show the mean and standard error for two replicates.

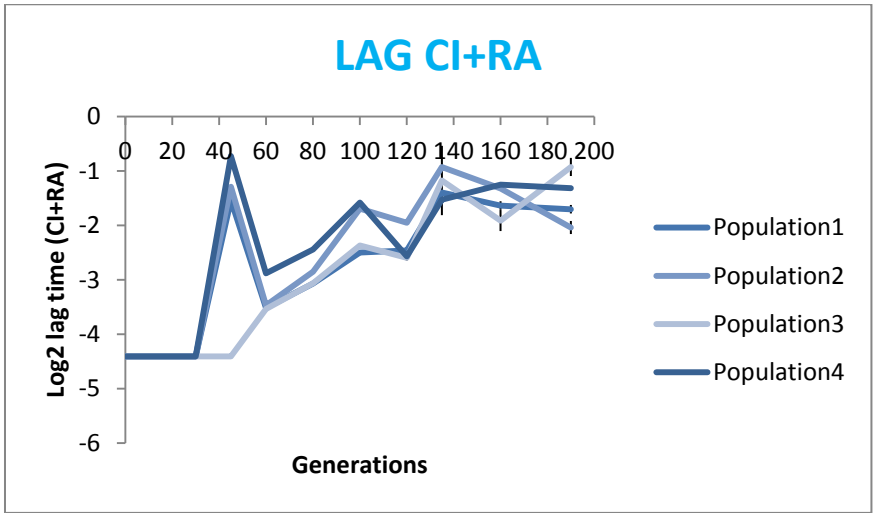

Figure 23; adapted cells to cisplatin+rapamycin treatment, lag phase. There is a rapid change in lag time from generation (15) to generation (45). Surprisingly, one more rapid change is observable with respect to lag time from generation (45) to generation (60) and with a slight change in between, from generation (135) no significant changes is observable. Bars show the mean and standard error for two replicates.

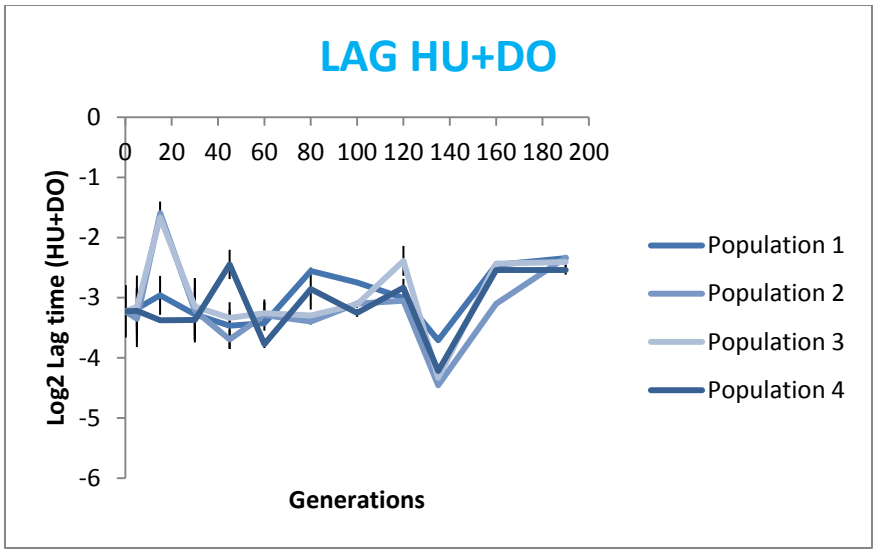

Figure 24; adapted cells to hydroxyurea+doxorubicin treatment, lag phase. It seems four populations hesitate to follow a regular trend with respect to lag time. Bars show the mean and standard error for two replicates.

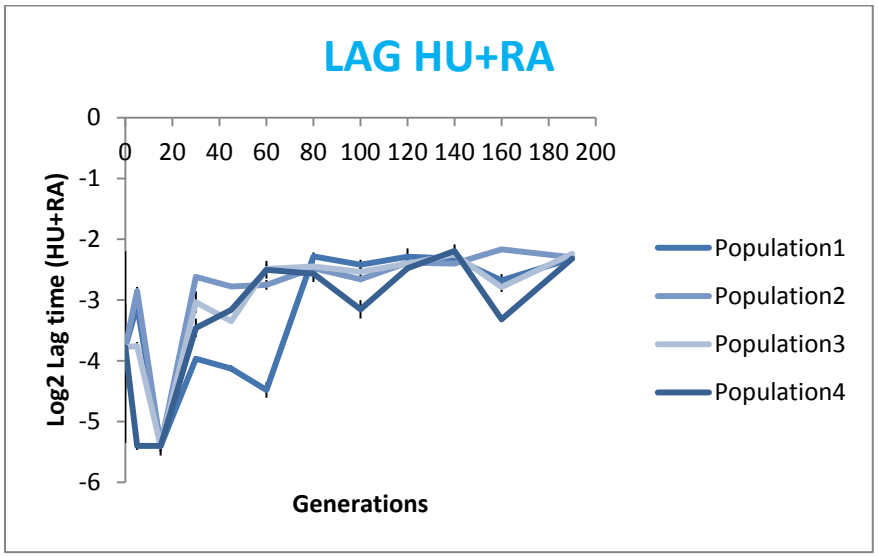

Figure 25; adapted cells to hydroxyurea+rapamycin treatment, lag phase. With some irregularities in lag time from very initial generations, from generation (15) to generation (24) lag time has become shorter and then two populations show steady state. The other two populations show also steady state, but with some irregularities in between. Bars show the mean and standard error for two replicates.

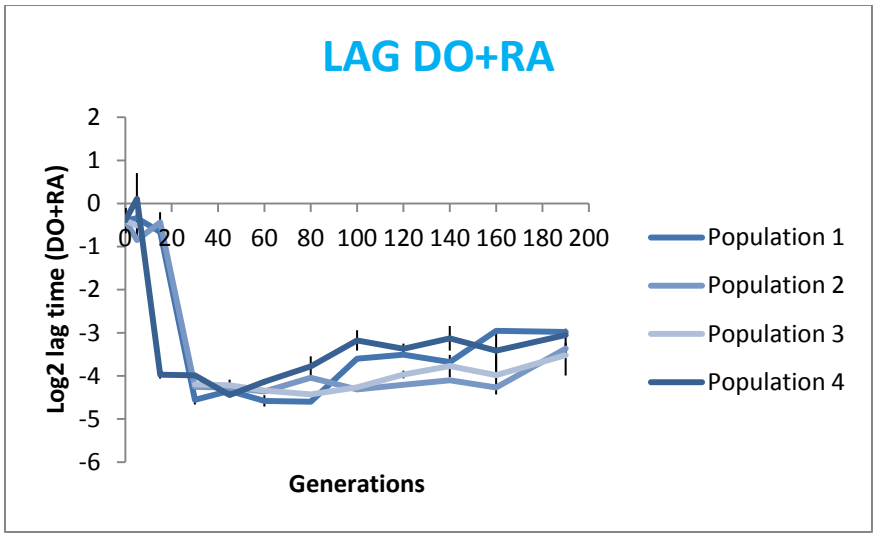

Figure 26; adapted cells to doxorubicin+rapamycin treatment, lag phase. With ignorance to unexpected increase in lag time from generation (0) to generation (24), all populations show steady state since then. Bars show the mean and standard error for two replicates.

3.2.2. Growth doubling time (generation time) results

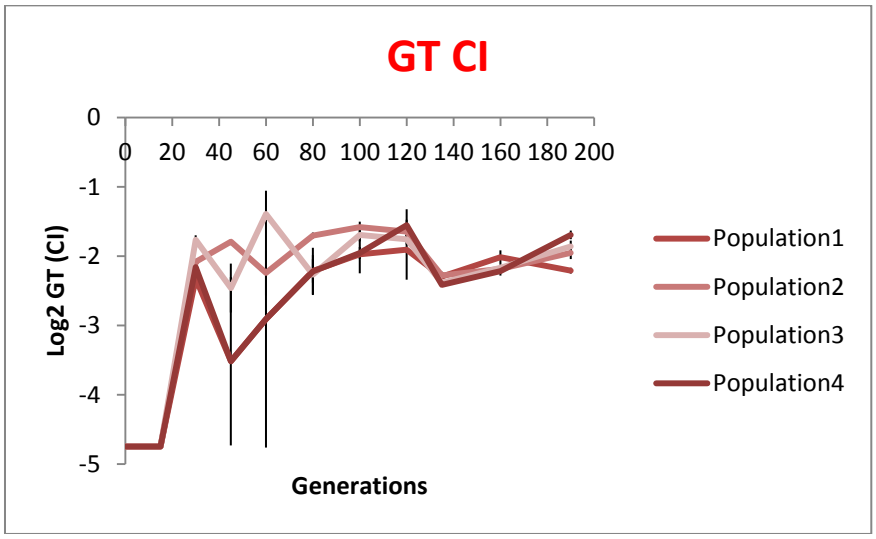

Figure 27; adapted cells to cisplatin treatment, generation time phase. There is a rapid change in generation time from generation (15) to generation (24) and with some slight changes in between, from generation (135) all of the populations show steady state. Bars show the mean and standard error for two replicates.

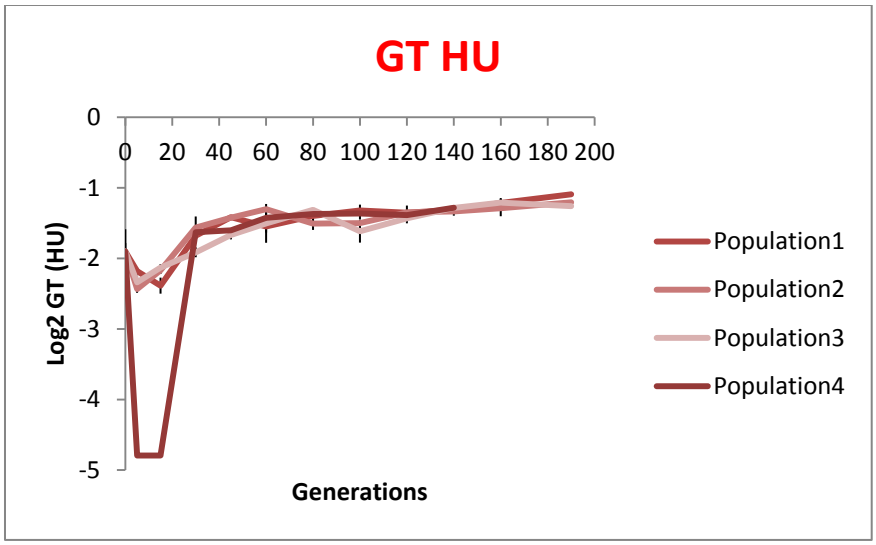

Figure 28; adapted cells to hydroxyurea treatment, generation time phase. It seems no significant changes has been occurred for three populations with respect to generation time but population 4 shows a rapid adaptive mutations at very initial generations. Bars show the mean and standard error for two replicates.

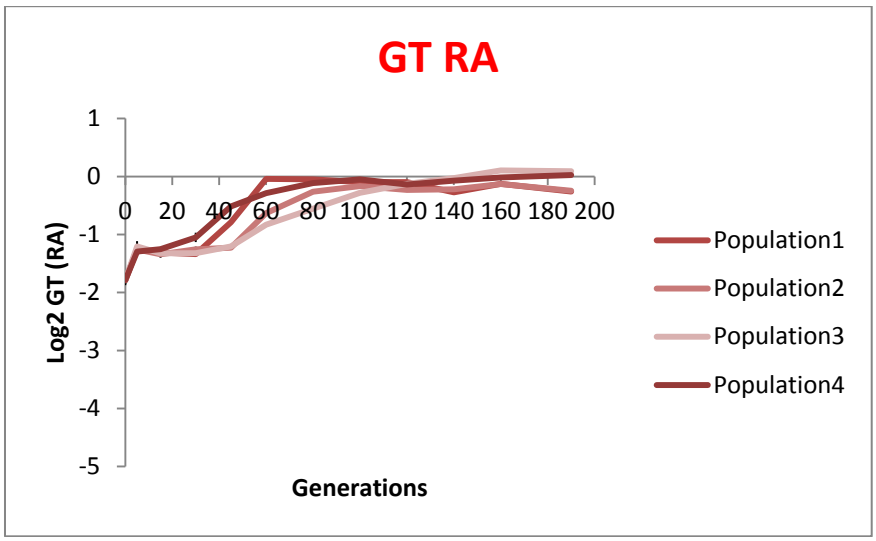

Figure 29; adapted cells to rapamycin treatment, generation time phase. The generation time curves of three populations look almost sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). Population 1 shows almost completed adaptation from generation (60), populations 2 and 4 show almost completed adaptation from generation (100) but population 3 has reached to steady state very late. Bars show the mean and standard error for two replicates.

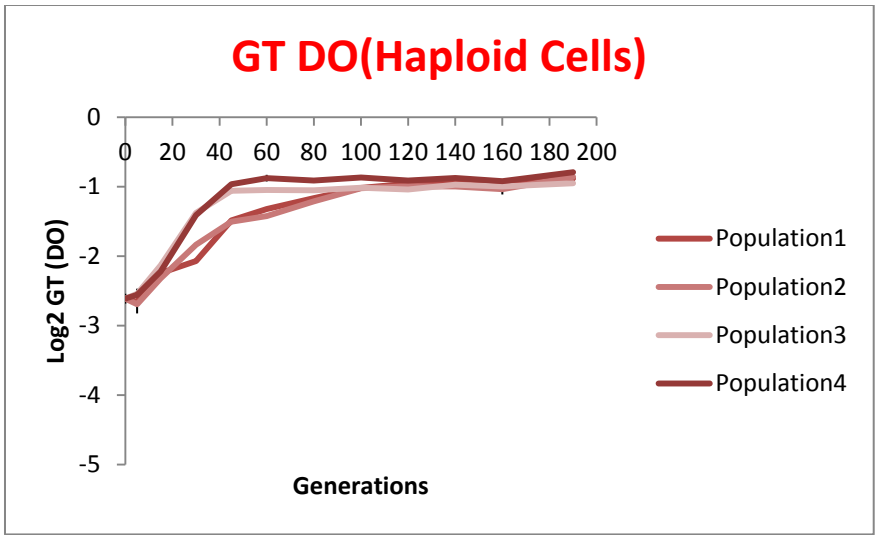

Figure 30; adapted cells (haploid cells) to doxorubicin treatment, generation time phase. The four populations' generation time curves look sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). Two populations show completed adaptation from generation (45) and the other two populations show steady state from generation (100). Bars show the mean and standard error for two replicates.

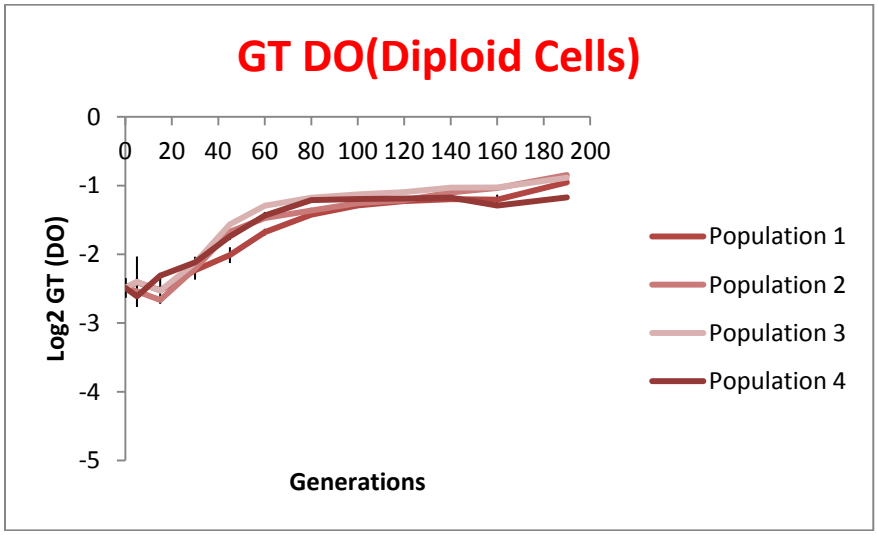

Figure 31; adapted cells (diploid cells) to doxorubicin treatment, generation time phase. The four populations' generation time curves look sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). All of the populations show steady state from generation (80) and start changing very slightly from generation (160). Bars show the mean and standard error for two replicates.

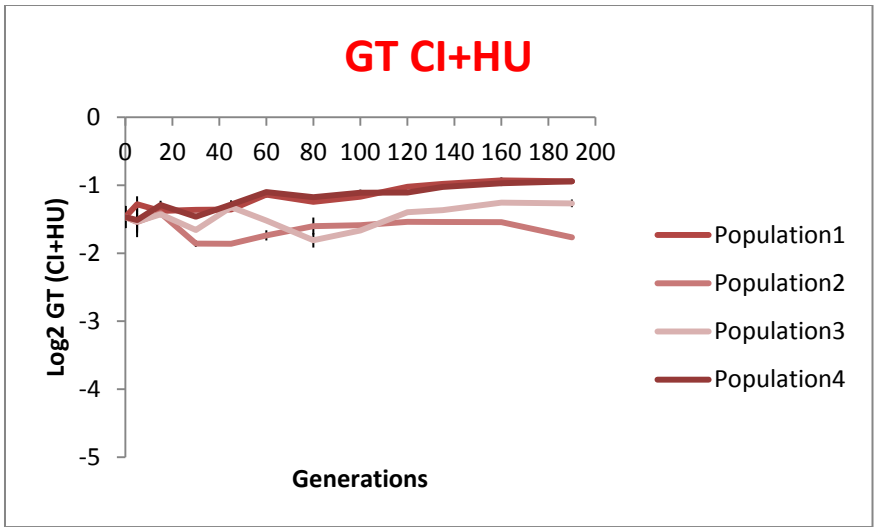

Figure 32; adapted cells to cisplatin+hydroxyurea treatment, generation time phase. It seems no significant change has been occurred. Bars show the mean and standard error for two replicates.

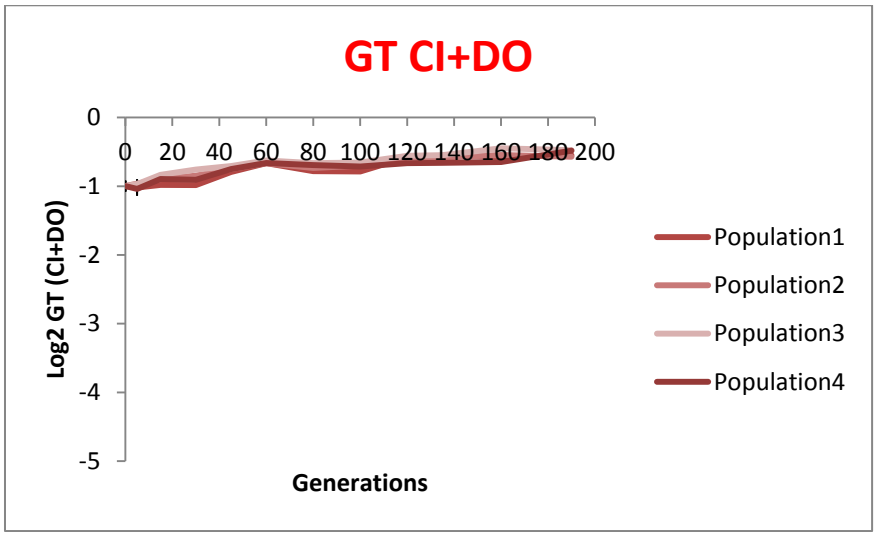

Figure 33; adapted cells to cisplatin+doxorubicin treatment, generation time phase. All of the populations show mild changes with respect to generation time from the beginning to generation (60), and then with very slight slope which can be ignored they show steady state. Bars show the mean and standard error for two replicates.

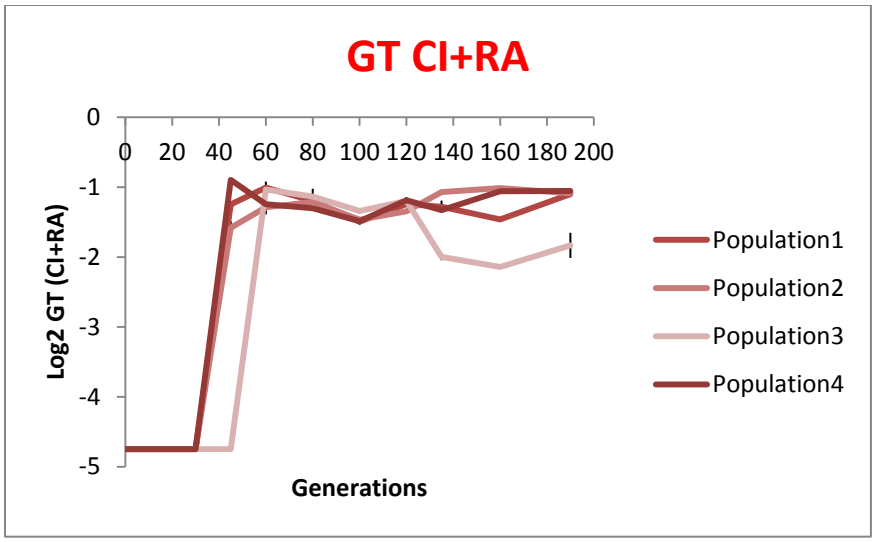

Figure 34; adapted cells to cisplatin+rapamycin treatment, generation time phase. It seems a very rapid change has been occurred for all of the populations from generation (30) in populations 1, 2 and 4. In population 3, this sharp change is observable from generation (45) to generation (60). The radical adaptation occurred early and populations show steady state since then. Bars show the mean and standard error for two replicates.

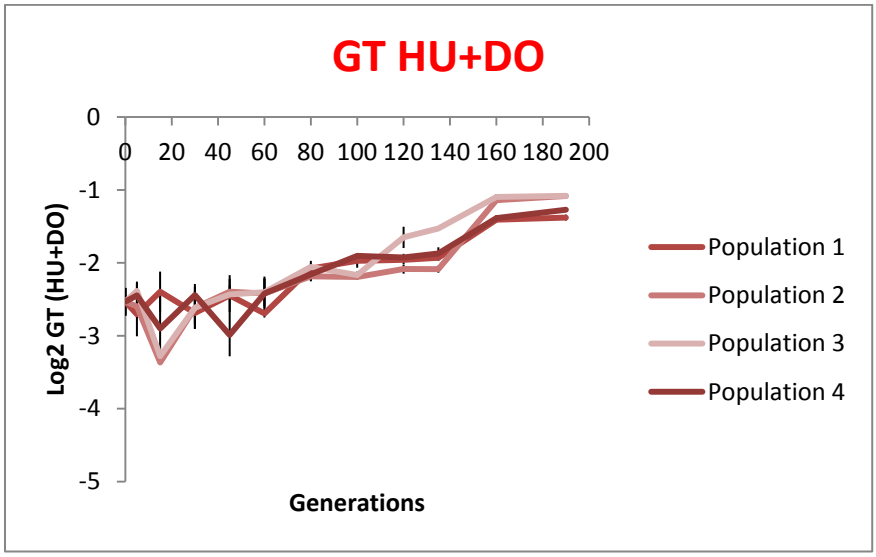

Figure 35; adapted cells to hydroxyurea+doxorubicin treatment, generation time phase. With ignorance to several irregularities at initial generations, it seems populations were involved with adaptive mutations slightly. They show complete steady state from generation (160). Bars show the mean and standard error for two replicates.

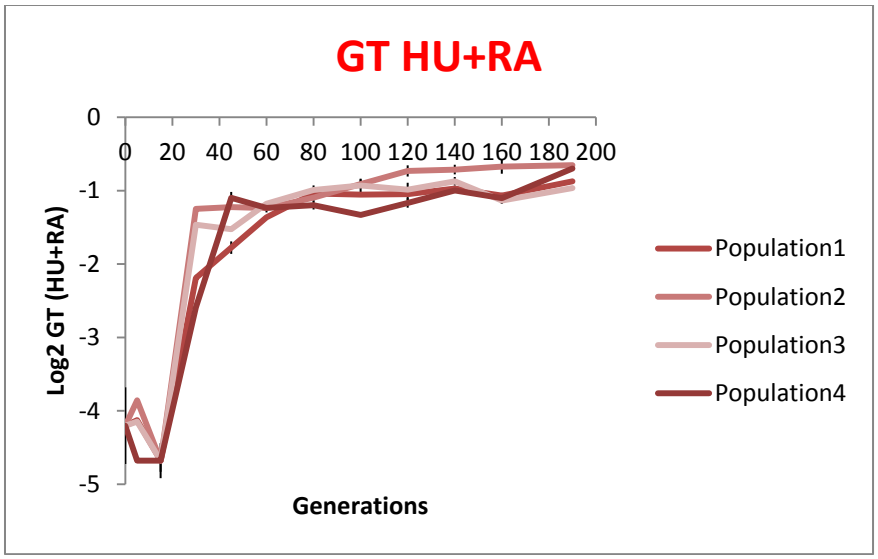

Figure 36; adapted cells to hydroxyurea+rapamycin treatment, generation time phase. It seems radical adaptive mutations occurred from generation (15) to generation (24) and with very slight changes in between, all of the populations show completed adaptation from generation (60). Bars show the mean and standard error for two replicates.

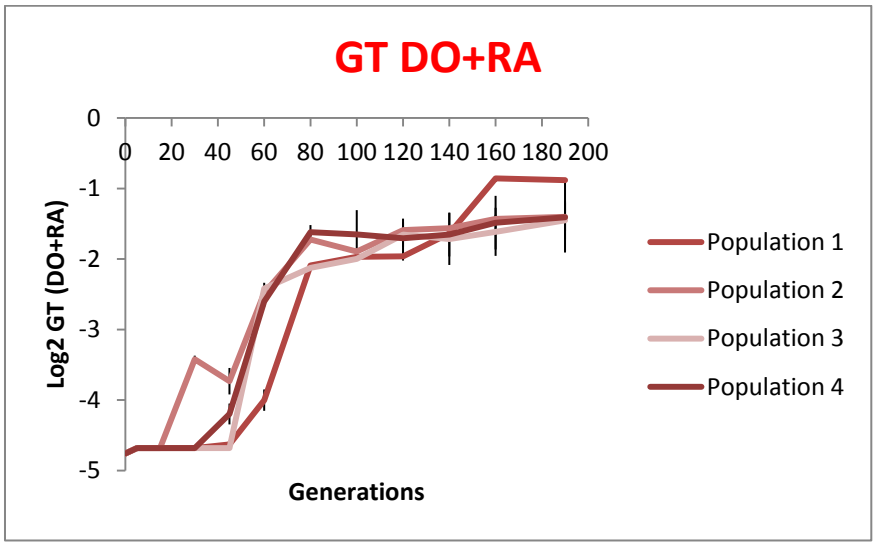

Figure 37; adapted cells to doxorubicin+rapamycin treatment, generation time phase. The radical adaptive mutations started from generation (45) in populations 2, 3 and 4. This sharp change started from generation (60) in population 1. Populations 2 and 4 reached to steady state at generation (80), population 1 reached to steady state at generation (160) and population 3 shows completed adaptation at generation (120). Bars show the mean and standard error for two replicates.

3.2.3. Growth efficiency (yield) results

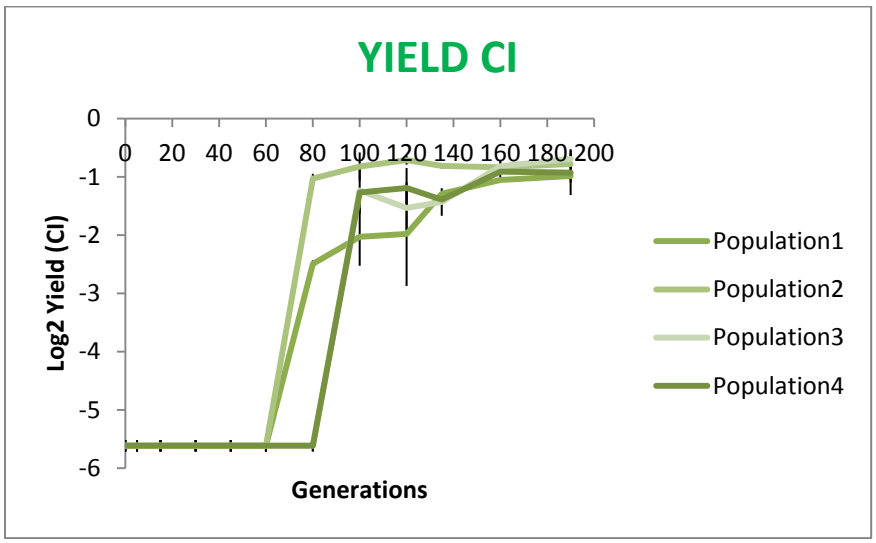

Figure 38; adapted cells to cisplatin treatment, yield phase. It seems adaptive mutations occurred sharply from generation (60) to generation (80) in populations 1 and 2, this rapid change occurred from generation (80) to generation (100) in populations 3 and 4. Population 2 shows completed adaptation from generation (80), with slight changes in between the rest of the populations show completed adaptation from generation (160). Bars show the mean and standard error for two replicates.

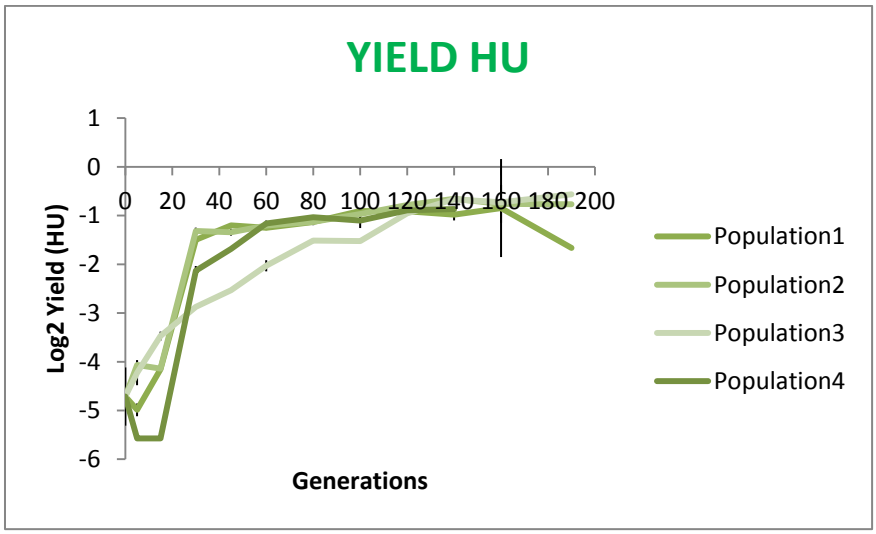

Figure 39; adapted cells to hydroxyurea treatment, yield phase. The population 3´s yield curve looks almost sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). Populations 1, 2 and 4 show very rapid adaptive mutations from generation (15) to generation (30). Populations 1 and 2 show almost steady state from generation (30), population 4 shows this condition from generation (60) and for population 3 this completed adaptation is observable from generation (140). Bars show the mean and standard error for two replicates.

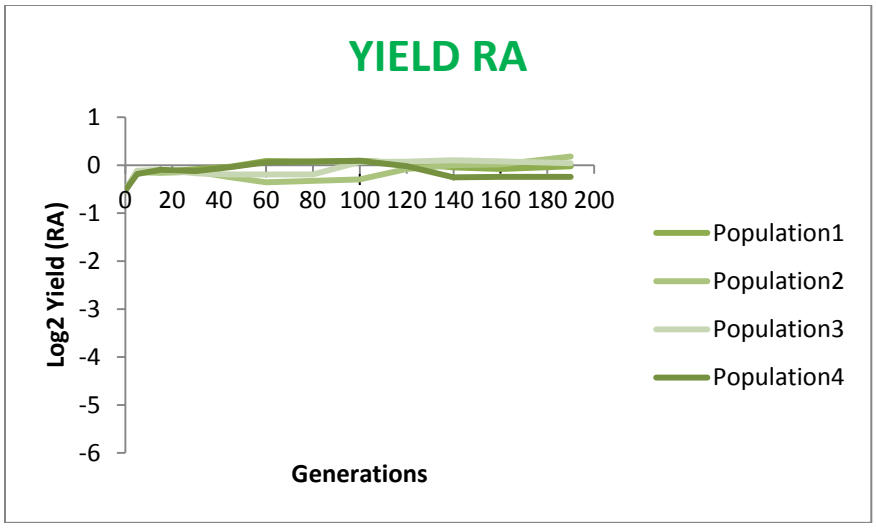

Figure 40; adapted cells to rapamycin treatment, yield phase. Overall, the extent of adaptive mutations do not look remarkable, they can be ignored. Bars show the mean and standard error for two replicates.

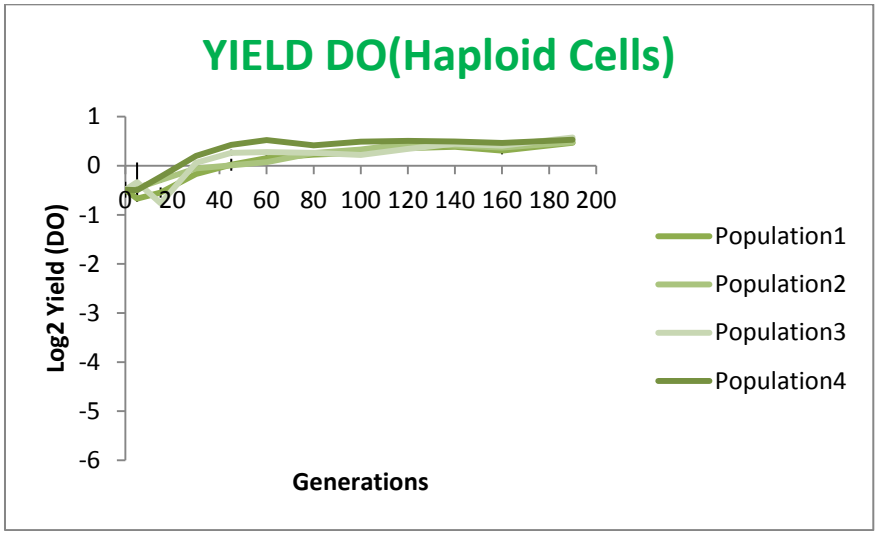

Figure 41; adapted cells (haploid cells) to doxorubicin treatment, yield phase. The four populations' yield curves look sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). Populations 1 and 2 show steady state from generation (120) and the extent of adaptive mutations look slight for intermediate generations. Population 3 shows almost steady state from generation (40) and population 4 shows completed adaptation from generation (60). Bars show the mean and standard error for two replicates.

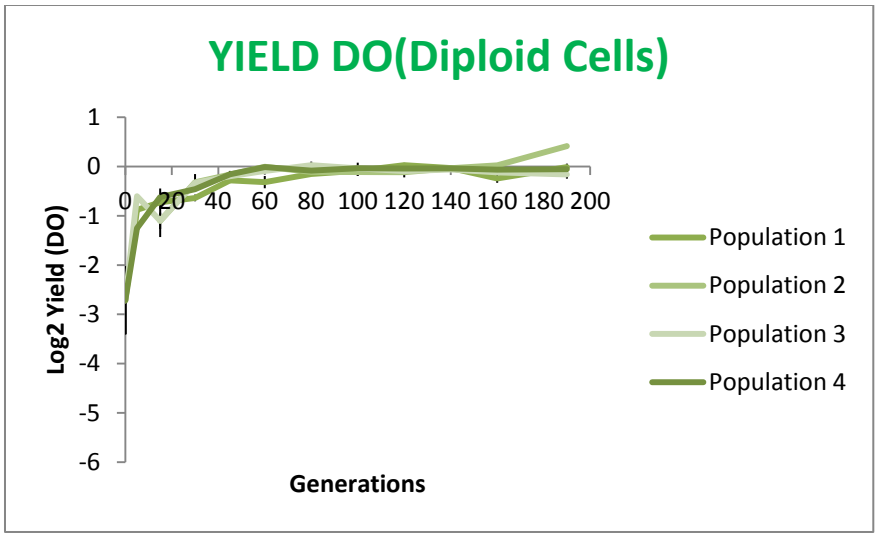

Figure 42; adapted cells (diploid cells) to doxorubicin treatment, yield phase. The radical changes occurred from generation (0) to generation (5) for populations 1 and 3; these extensive adaptations occurred from generation (0) to generation (15) for populations 2 and 4. It seems all of the populations had gained completed adaptation at very initial generations. Bars show the mean and standard error for two replicates.

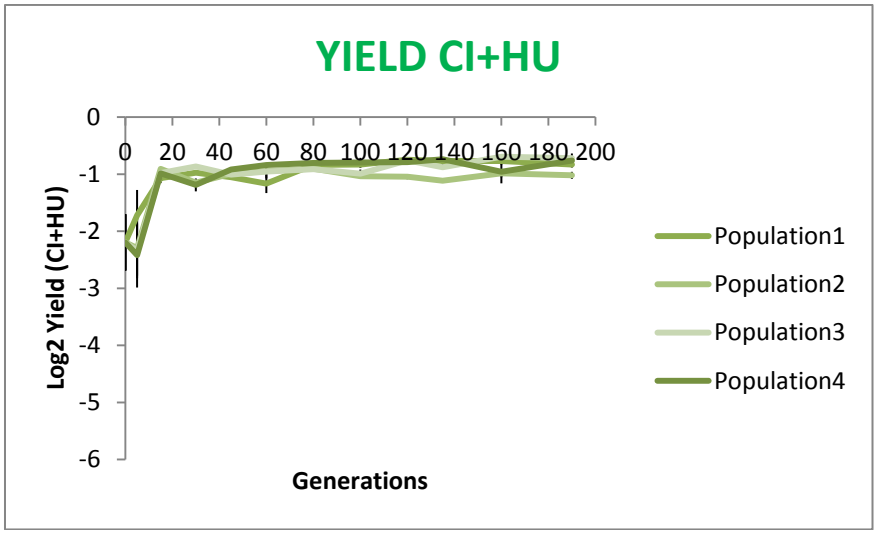

Figure 43; adapted cells to cisplatin+hydroxyurea treatment, yield phase. Rapid adaptive mutations occurred at very initial generations, and then all of the populations gained completed adaptation from generation (15). Bars show the mean and standard error for two replicates.

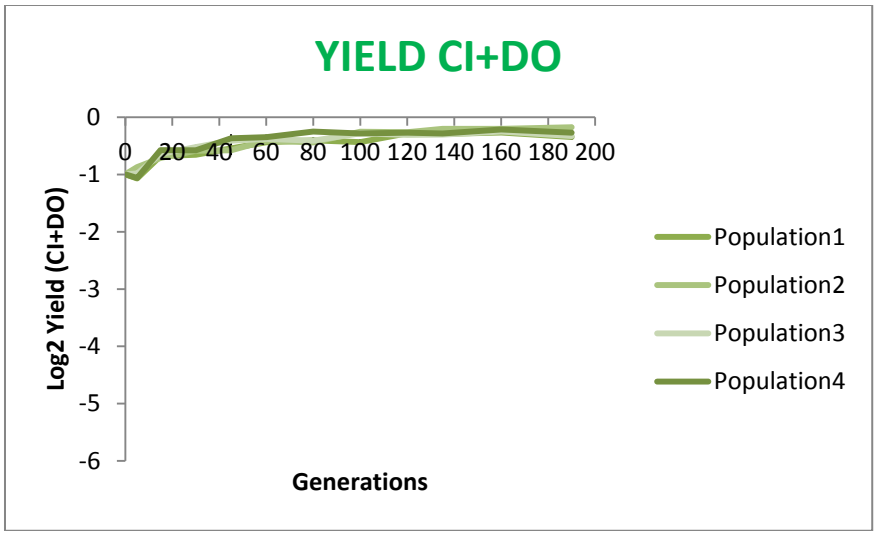

Figure 44; adapted cells to cisplatin+doxorubicin treatment, yield phase. The four populations' yield curves look slightly sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). Bars show the mean and standard error for two replicates.

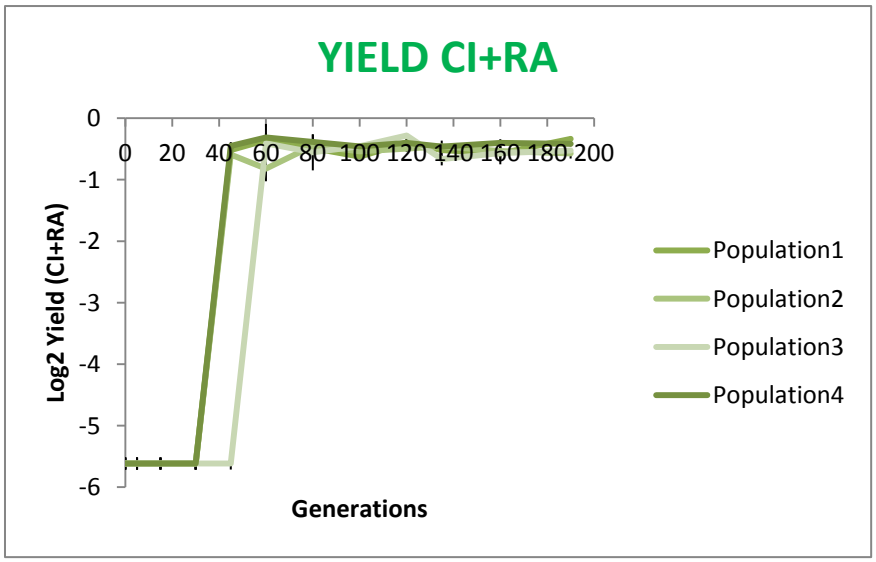

Figure 45; adapted cells to cisplatin+rapamycin treatment, yield phase. In populations 1, 2 and 4 the extensive adaptive mutations occurred from generation (30) to generation (45) and in population 3 happened from generation (45) to generation (60). After this huge shift, it seems all of the populations are completely adapted (continuous steady state). Bars show the mean and standard error for two replicates.

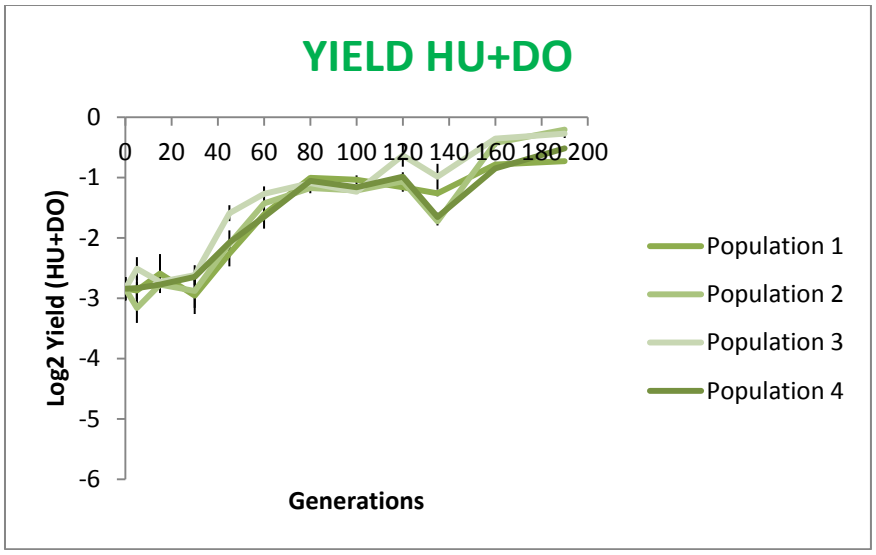

Figure 46; adapted cells to hydroxyurea+doxorubicin treatment, yield phase. With some irregularities at initial generations, a huge shift is observable for each population from generation (30) to generation (80). Again, another chaos is observable starting from generation (100). Bars show the mean and standard error for two replicates.

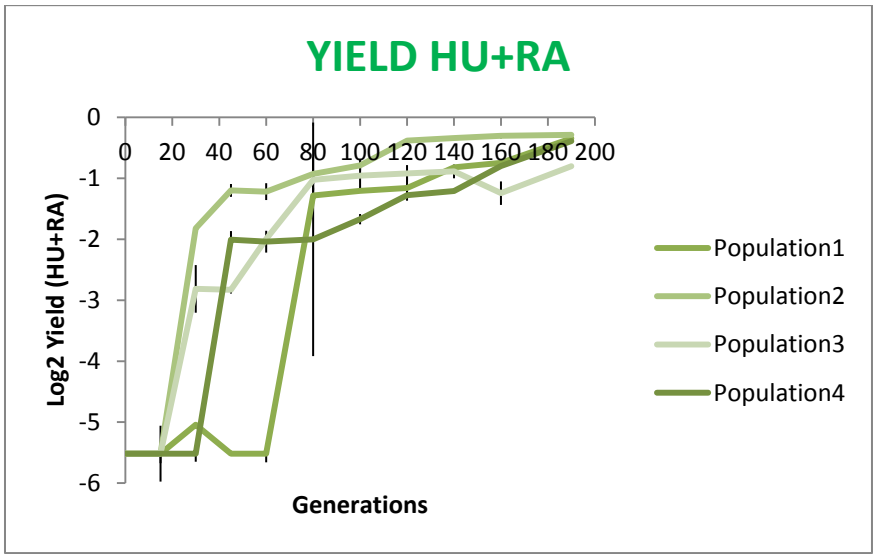

Figure 47; adapted cells to hydroxyurea+rapamycin treatment, yield phase. In different period of time rapid adaptive mutations occurred for all of the populations then they show almost completed adaptation. Bars show the mean and standard error for two replicates.

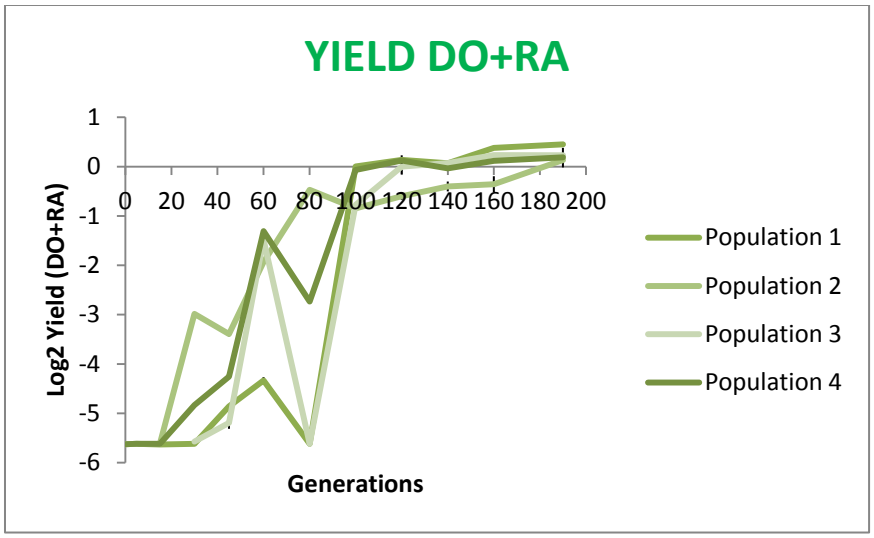

Figure 48; adapted cells to doxorubicin+rapamycin treatment, yield phase. In all of the populations with huge irregularities for half of the generations, the steady state regarding to completed adaptation is observable. Bars show the mean and standard error for two replicates.

3.2.4. Phenotyping assay discussions

Cisplatin treatment- Lag is really involved and adaptation time for majority of generations is long, cisplatin adapted populations show extensive adaptive mutations at initial populations with respect to generation time, also, a huge shift is observable in different period of time for all of the populations´ efficiency.

Hydroxyurea treatment- Almost rapid adaptive mutations occurred at initial generations for all of the populations with respect to lag time It seems no significant changes has been occurred for three populations with respect to generation time but population 4 shows a rapid adaptive mutations at very initial generations. In growth efficiency, the population 3´s yield curve looks almost sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). Populations 1, 2 and 4 show very rapid adaptive mutations from generation (15) to generation (30). Populations 1 and 2 show almost steady state from generation (30), population 4 show this condition from generation (60) and for population 3 this completed adaptation is observable from generation (140).

Rapamycin treatment- Four populations show different adaptive trends with respect to lag time. The generation time curves of three populations look almost sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). Population 1 shows almost completed adaptation from generation (60), populations 2 and 4 show almost completed adaptation from generation (100) but population 3 has reached to steady state very late. Overall, the extent of adaptive mutations occurred in growth efficiency do not look remarkable, they can be ignored.

Doxorubicin treatment (haploid cells) - No significant changes occurred with respect to growth lag but from generation (160) adaptation time has started to be shortened. The four populations' generation time curves look sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). Two populations show completed adaptation from generation (45) and the other

two populations show steady state from generation (100). The four populations' yield curves look sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). Populations 1 and 2 show steady state from generation (120) and the extent of adaptive mutations look slight for intermediate generations. Population 3 shows almost steady state from generation (40) and population 4 shows completed adaptation from generation (60).

Doxorubicin treatment (diploid cells) - No remarkable change in lag time is observable. The four populations' generation time curves look sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly). All of the populations show steady state from generation (80) and start changing very slightly from generation (160). In growth efficiency, the radical changes occurred from generation (0) to generation (5) for populations 1 and 3; these extensive adaptations occurred from generation (0) to generation (15) for populations 2 and 4. It seems all of the populations had gained completed adaptation at very initial generations.

Cisplatin+hydroxyurea treatment- It seems four populations hesitate to follow a regular trend with respect to lag time. Several increases and decreases can be observed, alternatively. In growth generation time, it seems no significant changes had been occurred. Compared to growth generation time in cisplatin treatment, the effect of cisplatin is reversed in combination with hydroxyurea. With respect to growth yield, rapid adaptive mutations occurred at very initial generations, and then all of the populations gained completed adaptation from generation (15).

Cisplatin+doxorubicin treatment- From generation (0) to generation (24) lag time has been shortened and then prolonged but from generation (80), populations show steady state with respect to lag time. All of the populations show mild changes with respect to generation time from the beginning to generation (60), and then with very slight slope which can be ignored they show steady state. The four populations' yield curves look slightly sigmoid meaning that the number of adaptive mutations has become fewer and fewer generation by generation (linear coefficient decreased constantly).

Cisplatin+ rapamycin treatment- There is a rapid change in lag time from generation (15) to generation (45). Surprisingly, one more rapid change is observable with respect to lag time from generation (45) to generation (60) and with a slight change in between, from generation (135) no significant changes is observable. In growth generation time, it seems a very rapid change has been occurred for all of the populations from generation (30) in populations 1, 2 and 4. In population 3, this sharp change is observable from generation (45) to generation (60). The radical adaptation occurred early and populations show steady state since then. In growth yield, in populations 1, 2 and 4 the extensive adaptive mutations occurred from generation (30) to generation (45) and in population 3 happened from generation (45) to generation (60). After this huge shift, it seems all of the populations are completely adapted (continuous steady state).

Hydroxyurea+doxorubicin treatment- It seems four populations hesitate to follow a regular trend with respect to lag time. In growth generation time with ignorance to several irregularities at initial generations, it seems populations were involved with adaptive mutations slightly. They show complete steady state from generation (160). In growth yield with some irregularities at initial generations, a huge shift is observable for each population

from generation (30) to generation (80). Again, another chaos is observable starting from generation (100).

Hydroxyurea+rapamycin treatment- With some irregularities in lag time from very initial generations, from generation (15) to generation (24) lag time has become shorter and then two populations show steady state. The other two populations show also steady state, but with some irregularities in between. In growth generation time, it seems radical adaptive mutations occurred from generation (15) to generation (24) and with very slight changes in between, all of the populations show completed adaptation from generation (60). Growth generation time in this treatment compared to growth generation time in hydroxyurea treatment and rapamycin treatment, looks the average. It seems both drugs mitigated the effects of each other. In growth yield, in different period of time rapid adaptive mutations occurred for all of the populations then they show almost completed adaptation.

Doxorubicin+rapamycin treatment- With ignorance to unexpected increase in lag time from generation (0) to generation (24) which is the prob of question, all populations show steady state since then. In growth generation time, the radical adaptive mutations started from generation (45) in populations 2, 3 and 4. This sharp change started from generation (60) in population 1. Populations 2 and 4 reached to steady state at generation (80), population 1 reached to steady state at generation (160) and population 3 shows completed adaptation at generation (120). In growth yield, in all of the populations with huge irregularities for half of the generations, the steady state regarding to completed adaptation is observable.

Overall, adapted populations to cisplatin and hydroxyurea+rapamycin treatments show extensive mutations earlier than the other treatments with respect to generation time (fast resistance). It should be mentioned that in the cases of hydroxyurea, cisplatin+hydroxyurea and hydroxyurea+doxorubicin treatments, no rapid adaptive mutations observed in growth generation time. They should be rephenotyped with higher concentrations of drugs to compare the results. In growth yield, adapted populations to doxorubicin (diploid cells) and cisplatin+hydroxyurea treatments show extensive mutations earlier than the other treatments. In rapamycin treatment, no rapid adaptive mutation is observable with respect to growth yield. It should be rephenotyped with higher concentration of rapamycin to compare the results.

3.3. Viability assay (Drop test) results and discussions

Fitness is a central idea in evolutionary theory. Although a staggering number of definitions of fitness have been offered by biologists, they agree on the essence of the idea. To be defined, it can be either with respect to a genotype or to a phenotype. In either case, it describes the ability of organisms or, more rarely, populations or species to both survival and reproduction. The consequence of this survival and reproduction is contribution of genes to the next generation (19).

In this research, reproduction was studied by Bioscreen™, whereas survival (viability) was studied by counting the colony forming units.

In figure 49, each row of colonies, belongs to the founder strain (generation (0)) which had been exposed to a specific treatment with defined concentrations of drugs in 1xYNB- glucose liquid medium prior to cultivating on 1xYNB plate. 1x YNB- glucose was used in order to have a harsher environment for cells and observing how many cells can survive finally. The control rows belong to the founder strain (generation (0)) which had been exposed to 1xYNB-glucose liquid medium with no stress prior to cultivation on 1xYNB plate.

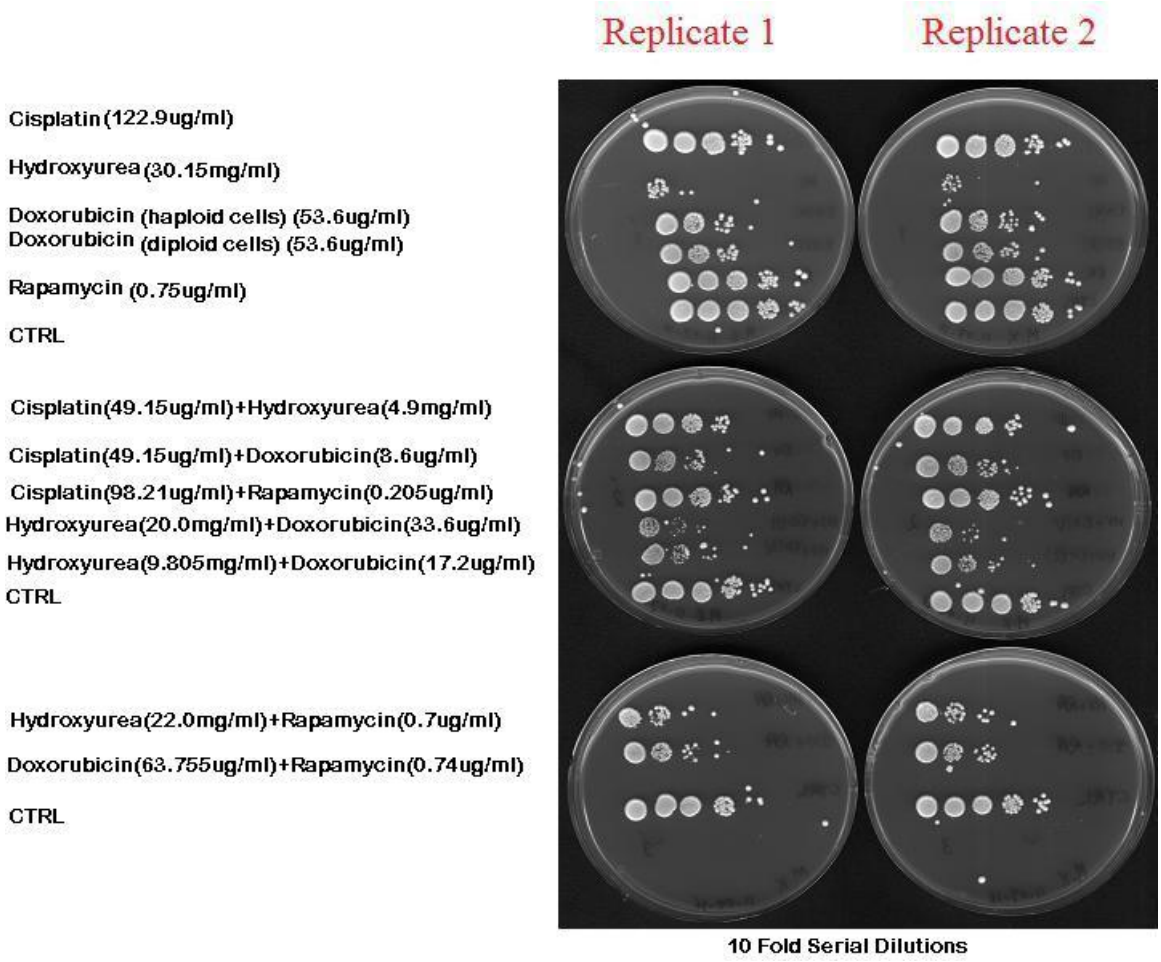

Figure 49; drop test of founder strain (generation (0)), the number of colonies belong to hydroxyurea treatment is much less than the other treatments and no significant difference between haploid cells and diploid cells both exposed to doxorubicin, exist.

In figure 50, each row of colonies, belongs to the generation (200) except control (founder strain) which had been exposed to a specific treatment with defined concentrations of drugs in 1xYNB- glucose liquid medium prior to cultivating on 1xYNB plate. The control rows belong to the founder strain (generation (0)) which had been exposed to 1xYNB-glucose liquid medium with no stress prior to cultivation on 1xYNB plate.

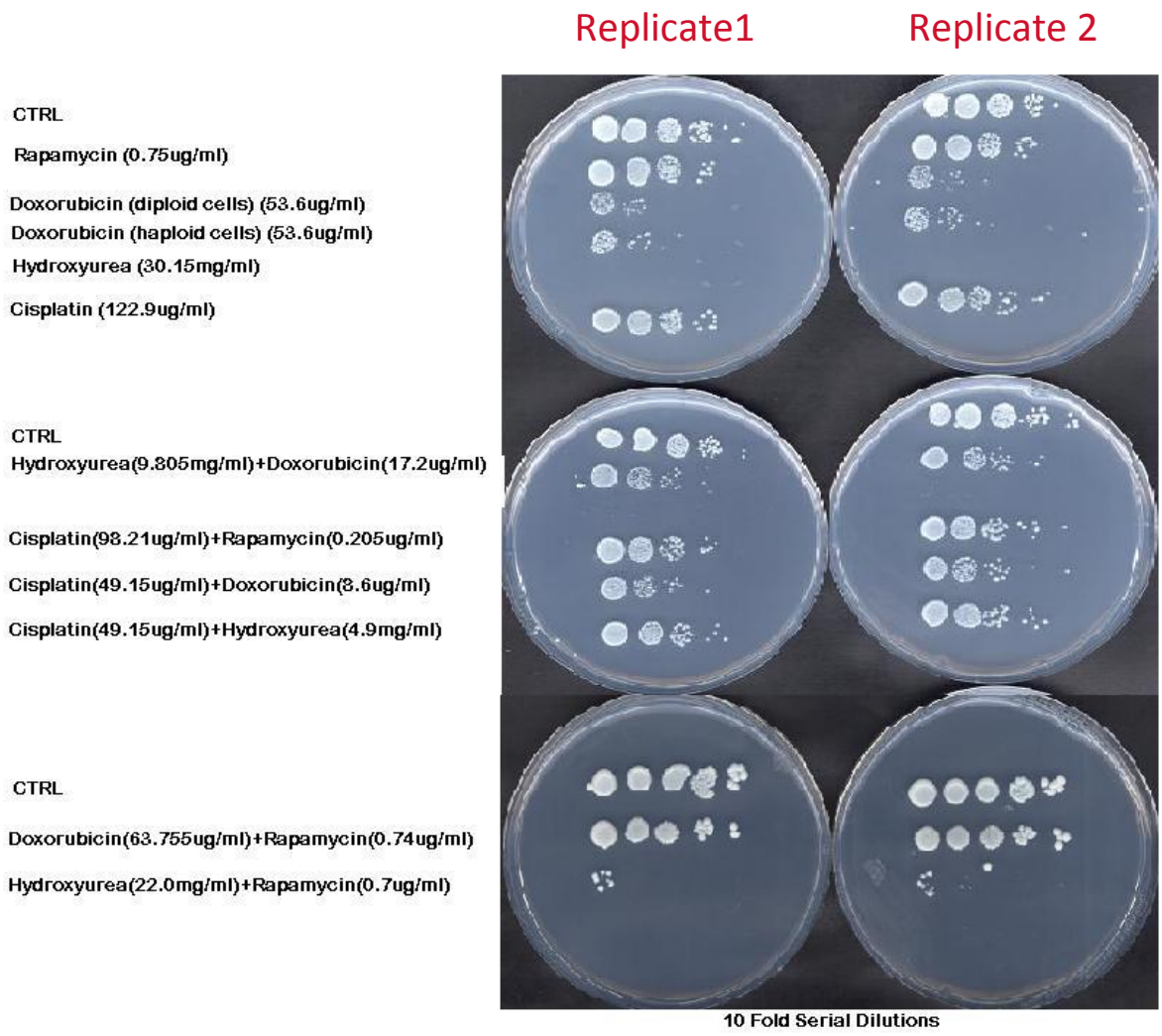

Figure 50; drop test of generation (200), no colonies belong to hydroxyurea treatment is observable and no significant difference between haploid cells and diploid cells both exposed to doxorubicin, exist.

The number of colonies belonging to each treatment obtained from drop test for generation (0) and generation (200) were multiplied to dilution factors to have the real number of the cells, and then divided to the number of the cells from no stress environment (control). Finally, the normalized values corresponding to the number of colonies varying from 10 to 30 were selected and converted into logarithmic scale based on 10.

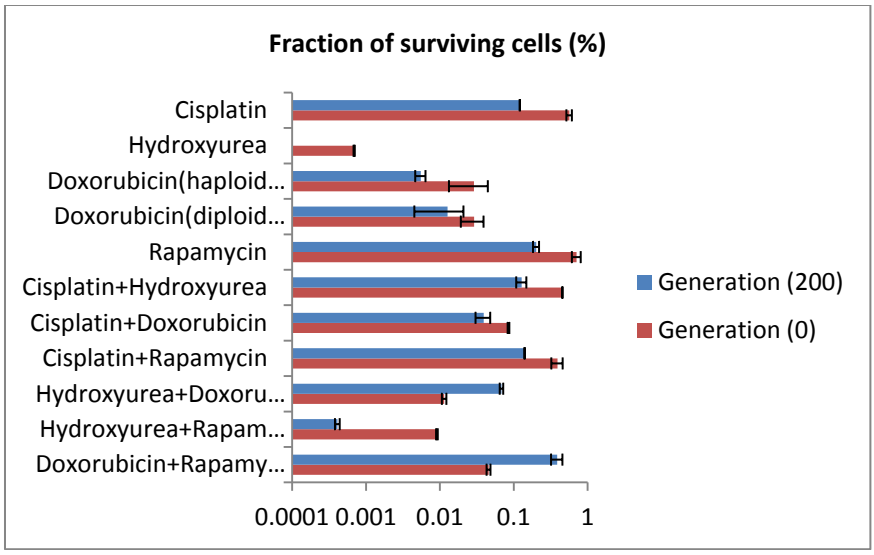

Figure 51; viability of founder strain and generation 200 of adapted cells in exposure to different treatments. For generation (200) treated with hydroxyurea, no value obtaind proving this drug is a real killer for cells and in aspect of viability, no adaptation occurred in this treatment. The viability of haploid cells and diploid cells both treated with doxorubicin is really the same. In general, there is not any significant difference between founder strain and the last adapted generation.

Comparing the viability of founder strain (generation (0)) with generation 200 reveals that adaptation with respect to survival did not occur at least for 200 generations. Adapted cells to hydroxyurea could not survive at all. This experiment was repeated twice for adapted cells to hydroxyurea, the results were the same in both cases.

3.4. Diagnostic PCR (Verification of Contamination) results and discussions

Because adaptation period for 200 generations was almost long (around 4 months) and the nature of the experiment was in a way that cross contamination was expected, it was essential to verify the contamination via diagnostic PCR, prior to phenotyping and genotyping.

S288c *Mat a* (haploid) and S288c *Mat a/α* (diploid) used in this study, belong to a set of 55 *S. cerevisiae* and *S. paradoxus* genetically tractable strains which previously sequenced in the *Saccharomyces* Genome Resequencing Project (SGRP). Three versions of each strain (haploid *Mat a* and *Mat α* and diploid *Mat a/α* all as *ura*3 :: *KanMX- Barcode*) are available through the National Culture Yeast Collection.

In the project mentioned above, for competition experiments, the barcode insertion was performed for specific strain identification and quantification. A 6-bp barcode was inserted upstream of the *KanMX* cassette. In addition to barcode insertion, *ura3* deletion was also performed because it is the most widely used selectable marker in plasmids (13).

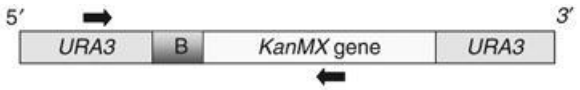

Figure 52; using the primers indicated by the horizontal arrows, PCR reaction was performed for amplification of the barcode insertion. Figure was reproduced with permission from (13)

To design the forward primer for this experiment, a 6-bp sequence specific to S288c strain barcode was included.

Fw. (5'-3'): GAAACGAAGATAAATCATG GGATCC CGTACGC
6-bp specific to barcode

The expected size of the PCR product was 509bp; therefore, observation of sharp band with this size was a proof for S288c strain. All of the adapted populations were diagnosed. If no right band was observed for some colonies of each adapted population, they would be rediagnosed. Finally, all of the colonies were confirmed to be S288c strain.

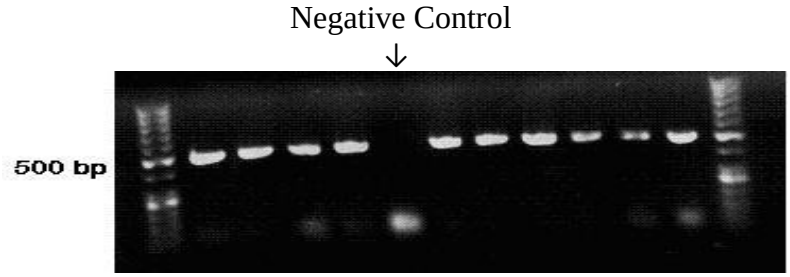

Figure 53; ten single colonies of adapted populations to hydroxyurea were diagnosed by colony PCR. All of the colonies were confirmed to be S288c strain.

4. Conclusions

Typically, fitness as a major trait rises rapidly at the start of the experiments and then plateaus as the population becomes close to a new optimal genotype and phenotype. Under harsher conditions (application of drugs in combinatorial patterns), it seems populations gain fitness by adaptive mutations slower with respect to time. It can be a meaningful reason to apply multiple drug treatments instead of single drug treatments to slow down the evolution. In combined treatments, the mode of adaptation process depends on the combinations of drugs. Variability between populations is low, suggesting few allowed evolutionary paths. Finally, it can be concluded that adaptation did not occur in population survivals but it affected the proliferation and efficiency.

5. Further approach

5.1. Founder and evolved strains genome sequencing; Experimental evolution of microbial populations provides a unique opportunity to study evolutionary adaptation in response to controlled selective pressures. However, until recently identification the precise genetic changes underlying adaptation at a genome-wide scale has been difficult. Undoubtedly, by applying new DNA sequencing technologies the genome of founderl and evolved populations of microorganisms should be rapidly determined.

5.2. Pleiotropy (cross phenotyping); Pleiotropy describes the genetic effect of a single gene on multiple phenotypic traits. The underlying mechanism is that the gene codes for a protein that has a signaling function on various targets. Cross phenotyping the adapted cells to other drugs (drugs not used in adaptation experiment) is helpful to study Multiple Drug Resistance (MDR). Cancer cells are able to become resistant to multiple different drugs by many of the same mechanisms such as; increased efflux of drug (by P-glycoprotein, multidrug resistance-associated protein, lung resistance-related protein, breast cancer resistance protein and reproductive cancer resistance protein), enzymatic deactivation (glutathione conjugation), decreased permeability, alternate metabolic pathway and altered binding sites.

5.3. Detection of DNA lesions by observation of RAD52 foci; RAD52 protein encoded by *RAD52* gene, shares similarity between human and *Saccharomyces cerevisiae.* Since, the plasmid containing *RAD52-YFP* genes with *URA3* marker and adapted populations already exist in our lab, DNA double-strand break repair can be studied with fluorescent microscopy.

5.4. Simulation of the evolution of chemotherapy resistance development; Despite the wealth of knowledge, not all of the mechanisms underlying resistance and the causes of theses mechanisms are correlated, starting the simulation will bring us conquers in this area.

6. Acknowledgment

This research project would not have been possible without the support of many people. I wish to express my deepest gratitude to my supervisor, Dr. Jonas Warringer who was abundantly helpful and offered invaluable assistance, support and guidance. He kept me encouraged and enabled me to develop an understanding of the subject.

I owe my sincere gratitude to Professor Per Sunnerhagen, who gave me the opportunity to work in his group in Department of Chemistry and Molecular Biology at University of Gothenburg.

I would like to thank my examiner Professor Jeanette Nilsson, for supporting me during the thesis.

Special thanks also to all my cosupervisor and friends, especially group members; Enikö Zörgö, Homan Alipour, Johanna Johansson Sjölander and Daniel Krumlinde for sharing the literature and invaluable assistance.

I wish to express my love and gratitude to my beloved family; for their understanding, endless love and spiritual support through the duration of my studies.

7. Appendix

7.1. Dose-response at growth lag

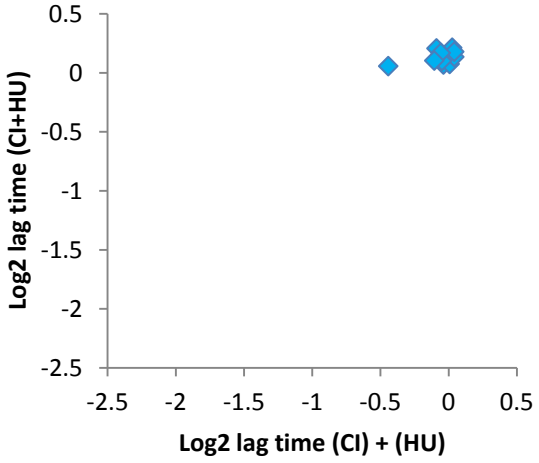

Figure 1; Dose-Response experiment of cisplatin+hydroxyurea treatment, lag phase.

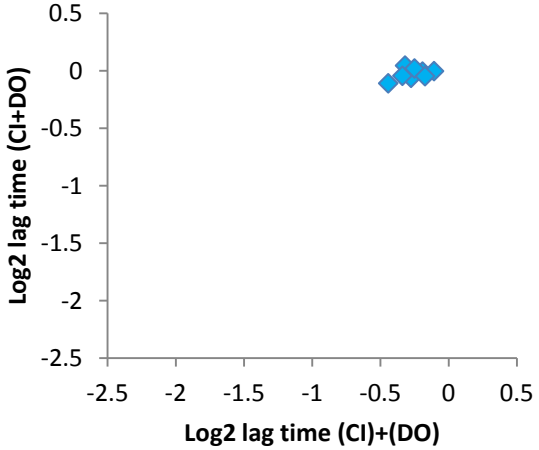

Figure 2; Dose-Response experiment of cisplatin+doxorubicin treatment, lag phase.

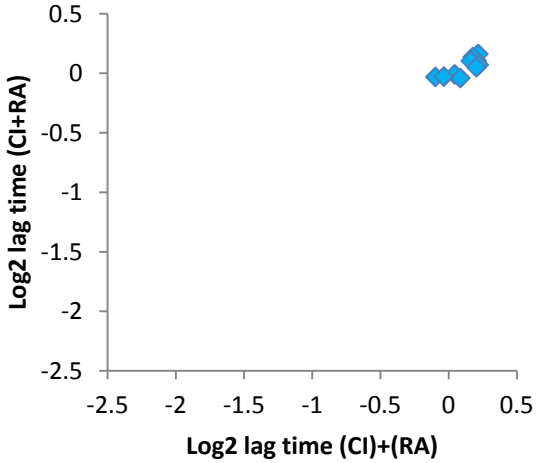

Figure 3; Dose-Response experiment of cisplatin+rapamycin treatment, lag phase.

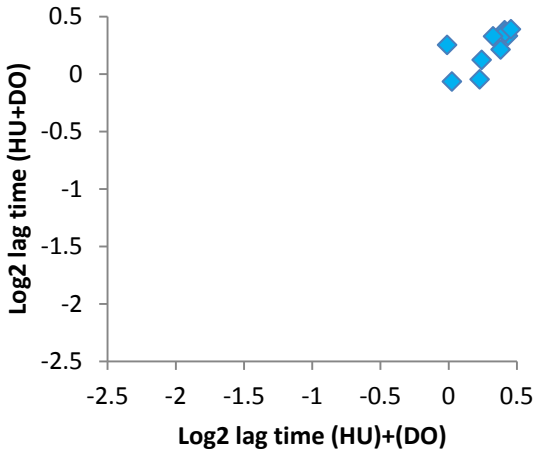

Figure 4; Dose-Response experiment of hydroxyurea+doxorubicin treatment, lag phase.

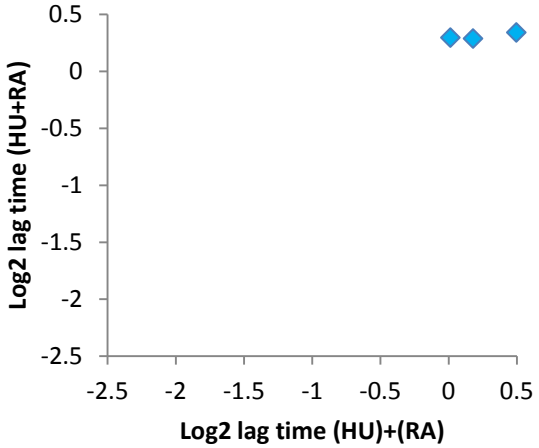

Figure 5; Dose-Response experiment of hydroxyurea+rapamycin treatment, lag phase.

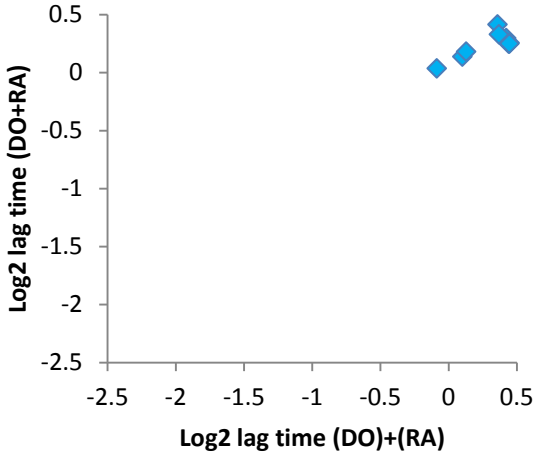

Figure 6; Dose-Response experiment of doxorubicin+rapamycin treatment, lag phase.

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