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The overexpression of the efflux pump Tpo1 leads to the bleomycin resistance in *Saccharomyces cerevisiae.*

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Ce mémoire intitulé:

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Présentée par:

Siham Berra

A été évalué par un jury composé des personnes suivantes:

Martine Raymond
présidente rapportrice

Dindial Ramotar
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Richard Bertrand
membre du jury

Résumé

La bléomycine est un antibiotique cytotoxique, son potentiel génotoxique est plus important quand elle est utilisée en combinaison avec des agents antinéoplasiques sur le cancer testiculaire, que sur les autres types qui développent souvent une résistance envers la drogue. Notre but consiste alors de mettre en évidence ce mécanisme de résistance en utilisant l'organisme modèle *Saccharomyces cerevisiae*.

Nous avons démontré au sein de notre laboratoire, que les levures délétées au niveau de leur coactivateur transcriptionnel Imp2, présentent une hypersensibilité à la bléomycine, en raison de son accumulation toxique dans la cellule. Ceci suggère que Imp2 pourrait réguler l'expression d'une ou de plusieurs pompes à efflux, capables d'expulser la bléomycine à l'extérieur de la cellule.

Pour tester notre hypothèse, nous avons recherché des suppresseurs multicopies capables de restaurer la résistance à la bléomycine chez le mutant *imp2Δ,* et c'est ainsi que nous avons identifié l'activateur transcriptionnel Yap1. Ce dernier se lie à une région spécifique localisée au niveau du promoteur et permet d'activer l'expression d'un sous-ensemble de gènes, codant pour des pompes à efflux, impliquées dans la résistance aux drogues.

Selon la littérature, au moins 27 pompes à efflux ont été identifiées chez la levure *Saccharomyces cerevisiae*, certaines d'entre elles disposent du site de liaison pour Yap1, tels que Qdr3, Tpo2 et Tpo1. Afin de déterminer si une de ces pompes expulse la bléomycine, nous avons créé des mutations simples et doubles en combinaison avec *IMP2*, aussi nous avons verifié si les mutants étaient sensibles à la drogue et enfin, nous avons testé si la surexpression de Yap1 pouvait restaurer le phénotype sauvage chez ces mutants, via l'activation de pompes à efflux.

Mots clés: bléomycine, cancer testiculaire, Imp2, Yap1, Tpo1, résistance à la drogue.

Abstract

Bleomycin is a cytotoxic antibiotic that, when used in combination with antineoplastic agents, has more genotoxic potential on testicular cancer than other types of cancer, which often develop resistance to the drugs. Our goal is to identify the resistance mechanism, using the organism *Saccharomyces cerevisiae* as a model.

In our laboratory, we have demonstrated that deleted yeast strains on their transcriptional coactivator Imp2 have presented hypersensitivity to bleomycin due to the toxic accumulation inside the cell. This led us to believe that Imp2 might regulate the expression of one or more efflux pumps capable of expelling bleomycin outside the cell.

To test our hypothesis, we sought multi-copy of suppressors capable of restoring bleomycin resistance in the mutant *imp2Δ*. As a result we identified the transcriptional activator Yap1, which binds to a specific region within the promoter and activates the expression of subset of genes, encoding efflux pumps that are involved in drug resistance.

Based on the literature, at least 27 efflux pumps have been identified in *Saccharomyces cerevisiae.* Some of these efflux pumps have binging sites for Yap1; such as Qdr3, Tpo2 and Tpo1. To determine whether or not one of these pumps expelled bleomycin, we proceded by single and double mutations in combination with *IMP2.* We also verified if these single and double mutants were sensitive to the drug, and then we have examined whether the overexpression of Yap1 could restore the wild phenotype in these mutants through the activation of efflux pumps.

Key words: Bleomycin, testicular cancer, Imp2, Yap1, Tpo1, drug resistance.

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3’ PG	3’phosphoglycolate
4-NQO	4-nitroquinoline 1-oxide
5'-G-Py-3'	5’ Guanine-Pyrimidine 3’
5'-G-Py-Pu-3 '	5’ Guanine-Pyrimidine-Purine-3’
5'-G-Py-Py-3’	5’ Guanine-Pyrimidine-Pyrimidine-3’
5’ P	5’ phosphate
ABC	ATP binding cassette
ADH	Alcohol dehydrogenase promoter
AGP2	High affinity polyamine permease
AP	Apurinic/apyrimidinic site
Apn1	Apurinic/apyrimidinic endonuclease
Atr1	Amino triazole resistance
BER	Base excision repair
BLM	Bleomycin
bZIP	Basic leucine zipper
$CaCl_2$	Calcium cloride
cAMP	Cyclic adenosine monophosphate
c-CRD	C-terminal cysteine-rich region
cDNA	Complementary DNA
Ci	Curie unit of measuring the radioactivity (1 Ci = 37 GBq)
Co^2+	Cobalt
Crm1 protein	β-karyopherin-like nuclear exporter
CTT1	Cytosolic catalase 1
Cys	Cystein
DAM	Decarboxylated S-adenosyl methionine
DHA1	Drug: H+ antiporter-1
dH_2O	Distilled water
DNA	Deoxyribose nucleic acid
DNase	Deoxyribonuclease

dNTP	Desoxy adénine tri-phosphate
dRpase	DNA deoxyribophosphodiesterases
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ENA1	Sodium extrusion 1
Fe II	Reduced iron
FLR 1	Fluconazole resistance 1
Flr1p	FLuconazole resistance1 protein
GAL2	Galactose 2 permease
GFP	Green fluorescent protein
GLR1	Glutathione reductase
Glu	Glutamic acid
Gpx3p	Glutathione peroxidise like protein 3
GRX1	Glutharedoxin 1
HAL3	Halotolerance
H_2O_2	Hydrogen peroxide
His	Histidine
IAA	Isoamyl alcohol
IMP2	Innositol monophosphate 2
K+	Potassium
KAc	Potassium acetate
Kan	Kanamycin
kV	Kilovolts
LB	Luria-Bertani media
Leu	Leucine
LRR	C-terminus leucine- rich repeat
MALS	Maltase permease
MALT	Maltose permease
MCPA	2-methyl-4-chlorophenoxyacetic acid
MDR	Multidrug resistance
MFS	Multifacilitator superfamily

MGBG	Methylglyoxal bis-guanylhydrazone
µFD	Microfaraday
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MMS	Methyl methanesulfonate
MOPS	Morpholino propanesulfonic acid
mRNA	Messenger ribonucleic acid
Msn2	Multicopy suppressor of SNF1 mutation 2
Msn4	Multicopy suppressor of SNF1 mutation 4
NaAc	Acetic acid
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NaI	Sodium iodide
NaOH	Sodium hydroxide
Na/MES	Morpholinoethanesulfonic acid sodium salt
n-CRD	N-terminal cysteine-rich region
NEM	N-ethylmaleimide
NES	Leucine-rich nuclear export signal
NLS	Nuclear localization Signal
NSAID	Non-steroidal anti-inflammatory drugs
O_2	Oxygen
OD	Optical density
ODC	Ornithine decarboxylase
Pdr 1-3	Pleiotropic drug resistance1-3
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Qdr2-3	Quinidine resistance 2-3
Rad	DNA repair protein
RNA	Ribonucleic acid
Rnase	Ribonuclease
Rpm	Rotation per minute
ROS	Reactive oxygen species
S. cerevisiae	*Saccharomyces cerevisiae*

SNQ2	Sensitivity to 4-NitroQuinoline-N-oxide
SAM	S-Adenosyl methionine
SAMD	Adenosylmethionine decarboxylase enzyme
SAMDC	S-adenosyl methionine decarboxylase enzyme
SDS	Sodium Dodecyl Sulfate
Ser	Serine
Skn7	Suppressor of Kre Null 7
SOD1-2	Superoxide dismutase1-2
SPD	Spermidine
SPM	Spermine
SS	Single stranded
SSAT	SPD acethyltransferase enzyme (SSAT)
SSAT	SPM acethyltransferase enzyme (SSAT)
TE/LiAc	Tris EDTA / Lithium acetate
TETRAN	Tetracycline transporter-like protein
Thr	Threonine
Tpo1-4	Polyamine transporter 1-4
Tris-HCl	2-Amino-2-hydroxymethyl-1, 3-propanediol hydrochloride
TRR1-2	Cytoplasmic thioredoxin reductase1-2
TSA1	Thiol peroxidase 1
URA	Uracil
UV	Ultraviolet
War1	Weak acid resistance 1
Yap1	Yeast activator protein
Ycf1p	Yeast cadmium factor 1 protein
YOR1	Yeast oligomycin resistance 1
YPD	Yeast extract peptone dextrose (YEPD)
YRE	Yeast response element
Yrr1	Yeast reveromycin-A resistant 1

To my family, for their enduring support,
Encouragements and love.

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Chapter 1: Introduction

1 Introduction

1.1 Bleomycin

Bleomycins are a family of glycopeptide antibiotics that were originally isolated as a fermentation product from *Streptomyces verticillis* cultures [1, 2] by Umezawa et al. in 1966 [1, 3]. Because of their cytotoxicity effect resulted from their DNA cleavage potency, they are considered as powerful anti-cancer drugs [1, 4, 5].

The clinically used compound is bleomycin sulfate (bleonoxane), which is a combination of 3 analogues of bleomycin – A2 (roughly 60%), B2 (approximately 30%) and minor amount of A5 [6]. Bleomycin has been used clinically since the early 1970's [7] to treat efficiently a number of malignancies, namely lymphomas, squamous cell carcinoma of the cervix, head, neck, and Hodgkin's disease [5, 8-11]. In combination therapy with cisplatin and etoposide, bleomycin is also used to treat testicular cancer, in spite of its side effect in inducing lung fibrosis in 10% of patients [12]. It is curative for 80 % patients [13, 14]; however, the 20 % remaining develop resistance towards the drug, limiting its therapeutic efficacity [13, 15].

Though the mechanism(s) describing the development of drug resistance towards bleomycin have not yet been found, a number of mechanisms whereby cells can become resistant to cytotoxic drugs include: 1) reduced permeability or uptake; 2) enhanced efflux; 3) inactivation of the drug by elevated levels of bleomycin hydrolase; 4) enhanced repair of bleomycin induced DNA lesions [16-20].

Depending on the level of the resistance they provide, most of these mechanisms that lead to resistance play a significant role in declining the efficacy of cancer chemotherapy.

1.1.1 Bleomycin structure

All bleomycin family members have a common structure but differ only in their amino acid side-chain at their C-terminal [21]. An example of bleomycin analogue A5 is illustrated in figure 1. Bleomycin A5 comprises three functional domains: C-terminal domain, N-terminal domain and the carbohydrate moety.

The C-terminal DNA-binding domain comprises a bithiazole moiety and a positively charged polyamine-like region [22-27]. It has been suggested that these two portions interact together with the DNA and might be involved in the sequence selective DNA cleavage.This was supported by previous studies showing that the substitution of bithiazole moity by two coumpounds; thriazole [28], and monothiazole [29] respectively resulted in altering the sequence from 5'-GC-3' to 5'-GT-3' [28] and loosing the ability of selective DNA cleavage [29].

The N-terminal metal-binding domain consists on β-hydroxyhistidine and pyrimidoblamic acid moieties [30]. It binds to the molecular oxygen as well as both iron and cobalt which are respectively redox-active and non-redox-active metal ions [24, 26, 31-34]. It interacts preferentially with iron, as it enhances the production of DNA lesions [35, 36].

The role of the carbohydrate moiety remains unknown, although some recent studies have shown that when bleomycin was lacking this domain, its DNA cleavage

potency became low [25, 37-39], and the ration of DSB to SSB was then reduced given that the deglycobleomycin generates essencially SSB, which were 300 fold less cytotoxic than the DSB [38].

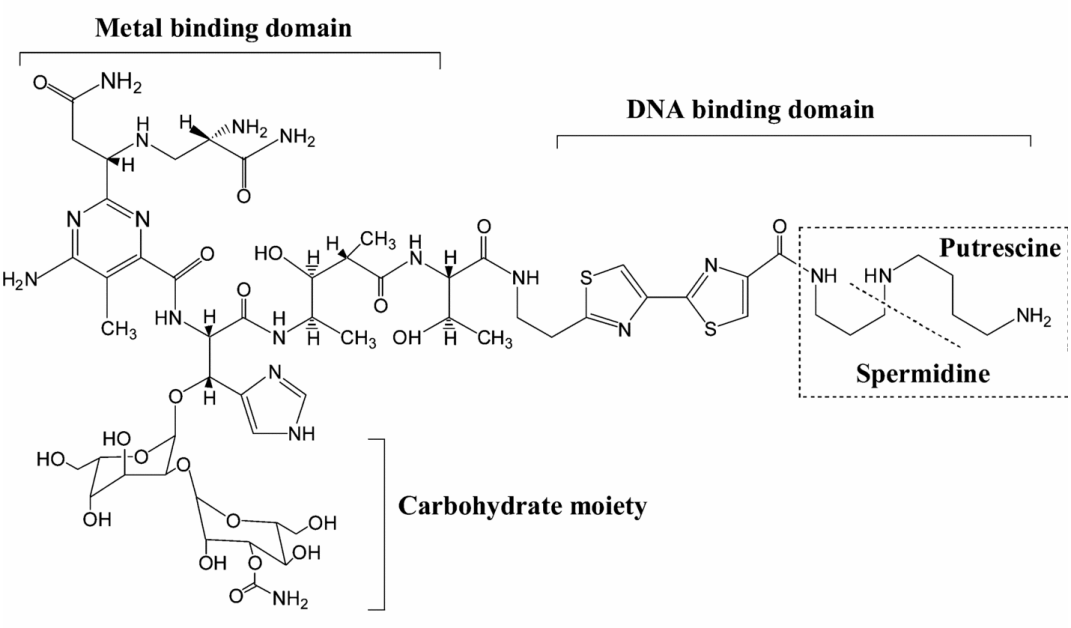


Figure 1. Bleomycin A5 structure
Bleomycin comprises 3 major domains: N-terminal metal binding domain, C-terminal metal binding domain and the carbohydrate moiety [40].

1.1.2 Mechanism of action of bleomycin

Bleomycin requires molecular oxygen and iron metal as a co-factor [41] to generate a narrow set of DNA lesions [42]. The reduced form of iron (Fe II) binds to the iron metal binding domain of bleomycin and becomes oxidized in the presence of oxygen. The complex (Blm–Fe(II)–O^2) is then converted to an activated form [43, 44] that is able to intercalate with the DNA as an oxidant causing an abstraction of the hydrogen atom from the 4' carbon of deoxyribose, and finally resulting in the

production of an intermediate radical (Fig. 2).

Depending on the absence or presence of oxygen, this intermediate will be either oxidized, generating oxidized apurinic/apyrimidinic (AP) sites [42, 45, 46], or reacts with O_2 to form a 4'peroxy radical that is reduced to a 4'hydroperoxide. The resulting product goes through chemical transformations, eventually inducing a single stranded DNA cleavage with 3'phosphoglycolate/5'-phosphate (3.-PG/5.-P) ends [42, 45, 47, 48], (Fig. 3), at a pyrimidine base (thymine or cytosine) to a guanine (5'-G-Py-3') [49]. Depending on the site of the residue 3', which is derived from the primary cleavage [50], a secondary cleavage occurs generating either 5'-staggered ends for the sequence 5'-G-Py-Pu-3 ', or blunt ends for 5'-G-Py-Py-3' (Fig. 4).

Such DNA lesions are considered highly genotoxic, and it is still not clear how cells could develop drug resistance to bleomycin.

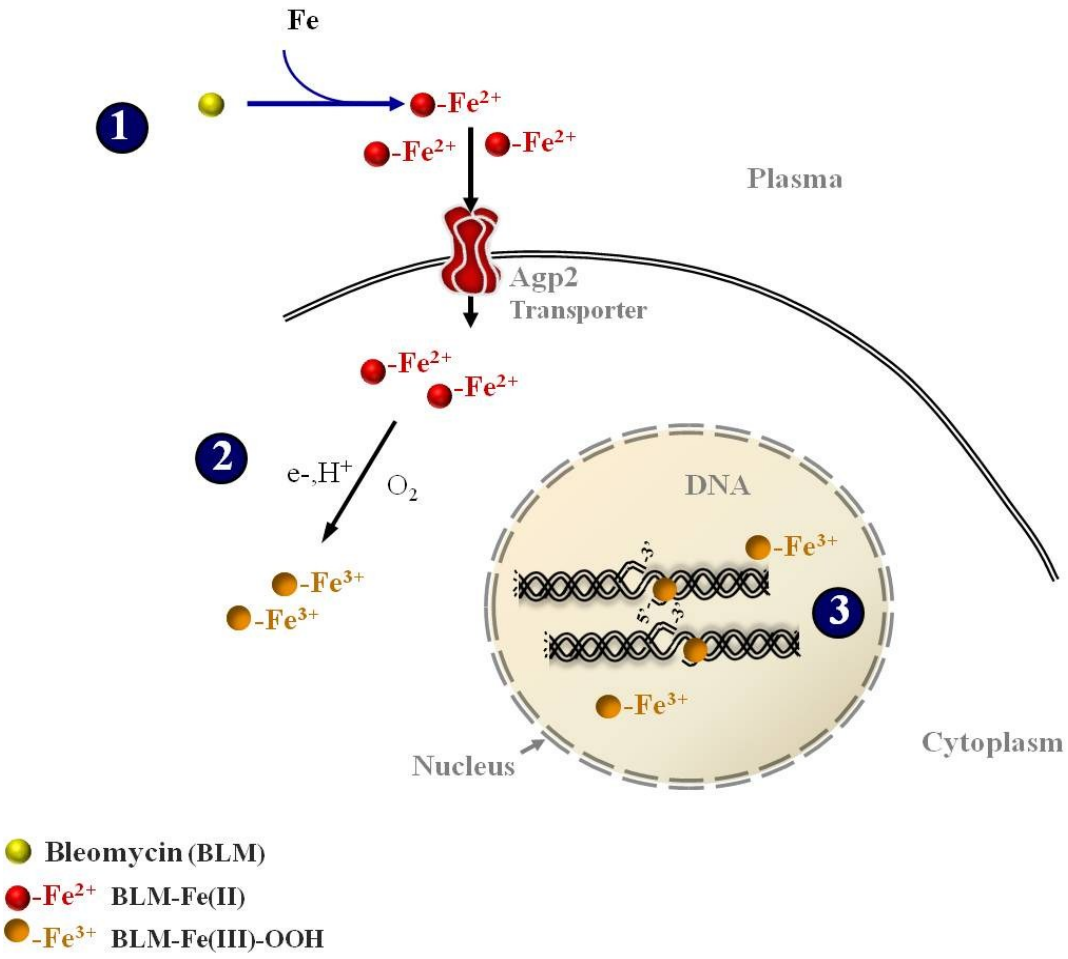


Figure 2. Mechanism of generation activated bleomycin
Bleomycin binds to the iron metal by forming a bleomycin-Fe (II) complex (1), this complex becomes activated once Fe (II) is intracellularly oxidized (2), generating free radicals and causing DNA lesions (3).

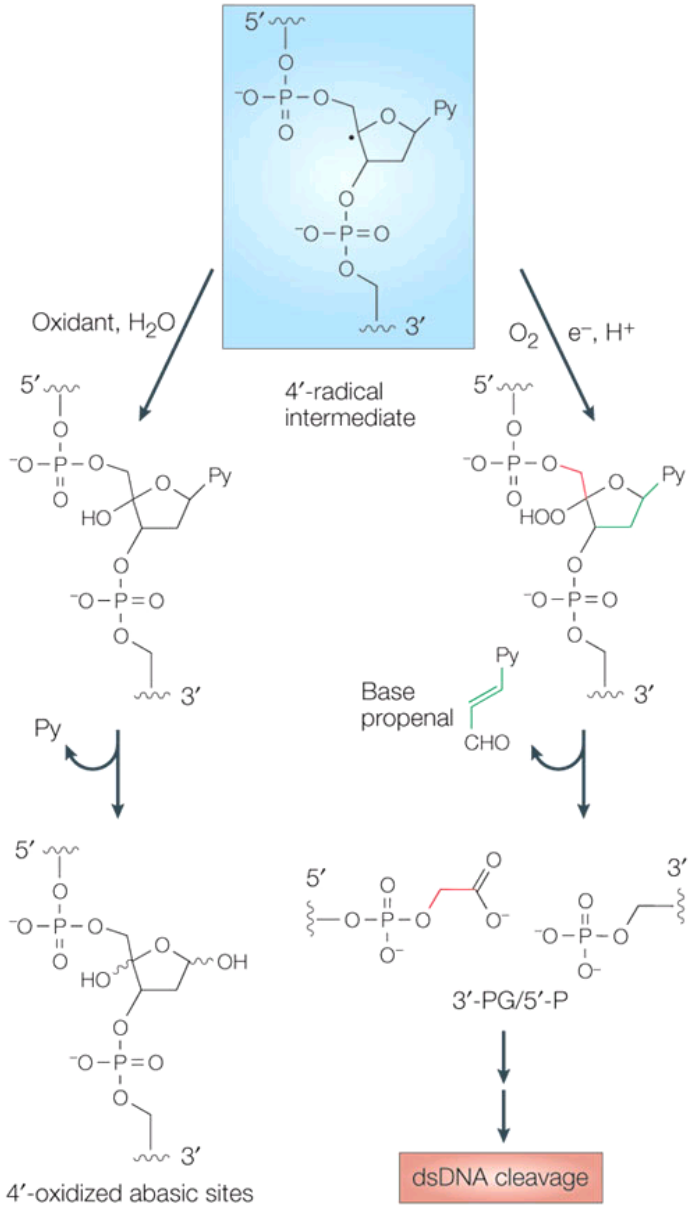


Figure 3. Mechanism of generation DNA lesions
Once bleomycin is activated, it intercalates with DNA, abstracts a hydrogen atom from the carbon 4' of desoxyribose and generates a radical intermediate. Depending on the availability of oxygen; the radical intermediate will generate either oxidative AP sites or single and double-strand break. *Nature review* [6].

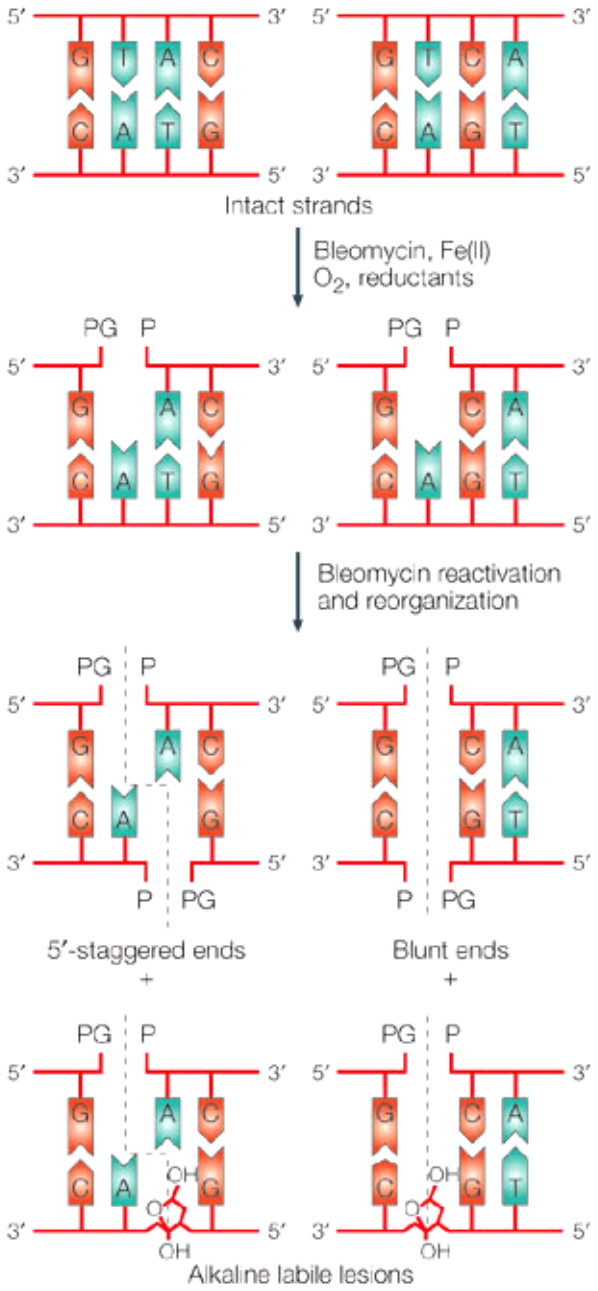


Figure 4. Bleomycin generates double strand breaks
The activated bleomycin initiates a single-stranded DNA cleavage, followed by a second cleavage that depends on the residue 3' created during the first cleavage. *Nature review*[6].

1.2 Mechanisms of resistance

Up to now, there is not a well accepted theory that is able to explain with certainty why some types of cancer respond better to the bleomycin treatment than those that are resistant. Cancer cells are selectively killed by anticancer drugs, because most of them are defective in their checkpoint responses [51-54], and proliferate rapidely; they would not have sufficient time to repair the DNA damage, and therefore keep dividing despite of unrepaired DNA. Furthermore, some cancer cells are repair-defective [55-62] in the first place, and this leads to their genomic instability [62-64].

Although, normal cells are continuously subjected to DNA damage, as well as cancer cells, they possess several pathways that lead to their resistance [65]. The identification of these pathways could provide us with an insight into the mechanism of increasing the cytotoxicity of bleomycin in specific cancer cells.

1.2.1 DNA repair pathways

Bleomycin is a radiomimetic agent that generates mostly the same types of DNA damages (DSB and AP sites) caused by ionizing radiations (IR) [66]. Its cytotoxicity is particularly related to its potency to induce DSB [67].

Unlike the IR, where the ratio of DSB to SSB is 1/20 [68], each molecule of bleomycin generates in vivo and in vitro 8 to 10 DNA breaks [50], and for 6-10 SSB, 1 DSB is produced [69]. In recent studies on Chinese hamster lung fibroblasts, it has

been demonstrated that bleomycin induced 2-3 times more DSB in G1 or G2/M phases than in S-phase [70].

In yeast, depending on the type of damage generated by bleomycin, whether it is single or double cleavage, cells can select the appropriate pathway to repair its DNA damage. When the double strand breaks (DSB) occur, the DNA repair predominantly proceeds through the homologuous recombination pathway [71] where Rad52 epistasis group proteins (Rad51, Rad52, Rad54, Rad55/57 and Rad59) are involved [72, 73].

In the case of a single strand break and oxidized AP site, the DNA repair process is carried out by base excision repair (BER) pathway [74], which required the major apurinic/apyrimidinic endonuclease 1(Apn1) [75, 76] homologous to APN1 in human cells.This pathway consists of the following: first of all, the BER pathway is initiated by the glycosylase enzyme necessary for the recognition and the excision of a damaged base that generates an AP site [77, 78], which in turn recruits the endonuclease Apn1. This latter cleaves the 5' phosphodiester bonds to the AP site yielding free 3′-hydroxyl and 5′-deoxyribose phosphate moieties [79, 80] that will be subsequently removed by DNA deoxyribophosphodiesterase (dRpase). Depending on the total number of nucleotides that are excised through the repair process, the resulting gap is filled either by one single nucleotide (through a short-patch sub-pathway), or by two or more new synthesized nucleotides. These are incorporated by DNA polymerase through long-patch sub-pathway and sealed with DNA ligase [81], (Fig. 5).

Recent studies demonstrated that the expression level of APN1 determined by immunohistochemical analysis is increased in a number of human cancer cells, such as the prostate and the lung cancers [82-85]. It has also been supported that the increased level of APN1 observed in testicular cancer correlates with bleomycin resistance by providing a 3-fold increase in protection against bleomycin [86].

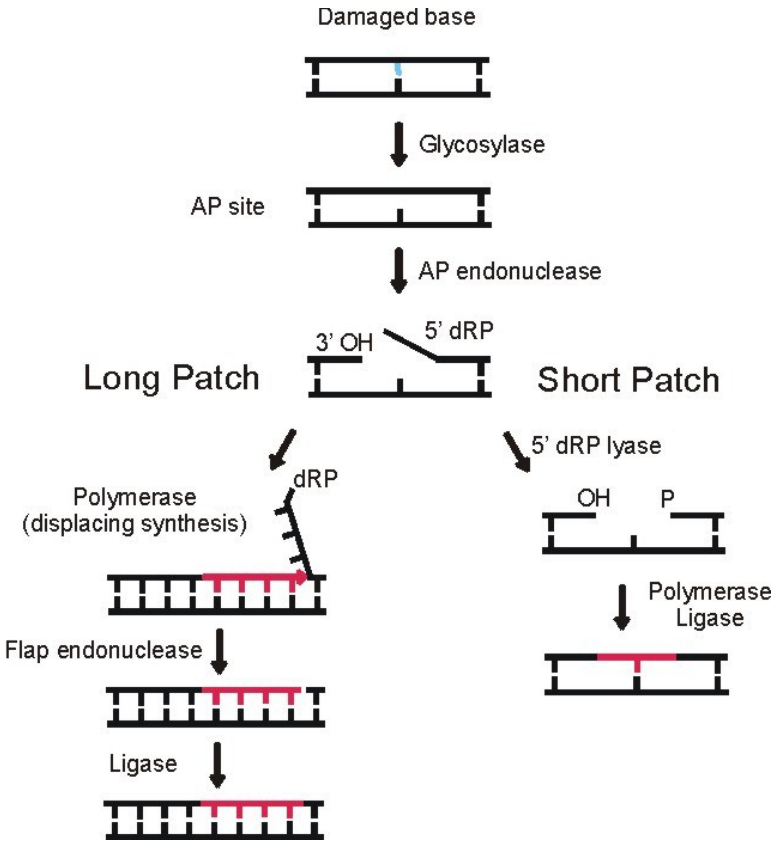


Figure 5. Base excision repair pathway
During the BER pathway, the glycolase recognizes and excises the DNA damage generating an AP site that is cleaved by Apn1 endonuclease, then a DNA polymerase is recruited to fill the gap by synthesizing one or more nucleotides through the short pach or the long patch. *J Biol Chem.* [81].

1.2.2 Major mechanisms leading to resistance

Current findings from Ramotar's lab raised the possibility that the resistance to bleomycin can be related to drug efflux as well as drug uptake. As it has been demonstrated, when the *AGP2* gene – a transporter of bleomycin – was mutated, it reduced the drug uptake, and hence induced an (~3000-fold) increase in resistance with high efficiency [87].

A similar effect is induced by the overexpression of an efflux pump that extrudes the drug from the cytoplasm, and thus, reduces the effective intracellular concentration [88, 89]. In agreement, it has been shown recently that the principal mechanism leading to the resistance of stem cells to chemotherapy is related to the expression of multi-functional efflux transporters in human stem cells [90].

1.3 Transcriptional co-activator Imp2 provides resistance

IMP2 is a well known gene that provides resistance against bleomycin among the *S.cerevisiae* genes. It was characterized as a gene encoding a transcriptional co-activator that turns on the gene expression of maltase permease (MALS), maltose permease (MALT) and galactose permease (GAL2) [91, 92].

It has been demonstrated that the *IMP2* gene is required to mediate cellular resistance to oxidative DNA damaging agents that generate free radicals such as H_2O_2 and bleomycin. When *IMP2* is deleted, mutants become 15-fold hypersensitive to bleomycin [93]. Recent findings suggest that the hypersensitivity exhibited by *imp2Δ* mutants towards bleomycin, is not due to the expression deficiency of drug efflux

pumps such as Snq2, Yor1, Flr1, Atr1 [94-100] or defect in antioxidant activity [101]. To date, there is no evidence indicating that Imp2 directly regulates genes implicated in oxidative stress response [102]. It could manifest via a specific gene target that regulates by itself the expression of an efflux pump responsible for expelling bleomycin.

1.4 Polyamines

In spite of the fact that bleomycin is a hydrophilic molecule unable to diffuse passively across the cell membrane [6], its spermidine substituent, which is positively charged, might have a role in the cellular uptake. In fact, as bleomycin has an affinity to a specific transporter (Agp2) responsible for its uptake [87], probably through the recognition of the polyamine region, then it must have an affinity to efflux pumps, that are specific to polyamines such as spermine, spermidine and putreschine. These polyamines are natural and small molecules; they are water soluble and have an aliphatic carbon chain that is positively charged [103].

1.4.1 Polyamine synthesis pathway

The polyamine synthesis pathway is initiated by the conversion of arginine – resulting from the urea cycle – into ornithine. The latter is decarboxylated to form putrescine through the action of the ornithine decarboxylase (ODC) enzyme. Meanwhile, S-adenosyl methionine (SAM), known to be involved in methyl group transfers, is converted to a decarboxylated form (DAM) by the adenosylmethionine decarboxylase enzyme (SAMD). The decarboxylated S-Adenosyl methionine donates

its aminopropyl group to putrescine to produce spermidine and spermine, by spermidine and spermine synthetase, respectively [104] .

In the polyamine catabolism pathway, spermine and spermidine are acetylated to N-acetyl SPM and N-acetyl SPD, respectively, via SPM and SPD acethyltransferase enzyme (SSAT). These two acetylated polyamines could be converted back to putrescine through a catalyzation reaction mediated by flavin-dependent polyamine oxidase [104] .

1.4.2 The importance of polyamine in the cell

Since polyamines are known to be positively charged, they bind to DNA, RNA and phospholipid macromolecules [105] that carry a negative charge, then get involved in many cellular processes including cell growth and proliferation, differentiation, chromatin structure, gene expression, transcription, signal transduction, membrane stability, ion transport, and cell signalling [106-108] .

In recent studies, it has been reported that when polyamines bind to chromatin, they alter DNA and RNA synthesis by increasing or decreasing the accessibility of genomic sites for DNA and RNA synthetases. However, when polyamines are depleted, chromatin becomes more accessible to DNA digestion. Other studies supported that polyamines like spermidine and spermine play a role in DNA condensation and segregation, and induce DNA conformational transition [103].

1.4.3 Polyamines related to cancer

Polyamine levels are tightly regulated by their influx and efflux across the plasma membrane, in such a way that their level is never extremely low or high, which would result in either failure in supporting cell growth or toxicity [108-111].

Many studies focused on cancer research, have explored the polyamine biosynthetic pathway for the development of agents that inhibit tumor growth. It has been suggested that molecules such as methylglyoxal bis-guanylhydrazone (MGBG), known as a potent inhibitor of polyamine biosynthesis [112], inhibit the proliferation of 99% of many types of cancer cells in culture, and more than 95 % of transplanted cancers in animals. This occurs by the inhibition of the S-adenosyl methionine decarboxylase enzyme (SAMDC) that is involved in the polyamine pathway [103]. Thereby, the cell is depleted from its natural polyamines, and the toxic analogue of polyamine is then accumulated into the cell resulting in cell death.

1.5 The transcriptional factor Yap1

To search for possible efflux pumps, a study was carried out that consisted of creating a multi-copy plasmid bearing the whole genomic DNA that derived from *imp2* null mutant, and introduced into *imp2Δ* [102]. The main aim was to identify multi-copy suppressor genes that would restore the normal resistance to the *imp2Δ* mutant in response to bleomycin. This study showed that only 3 clones restored the normal phenotype, and all of them were carrying the entire *YAP1* gene (Ramotar D., unpublished data), while no efflux pump gene was reported.

1.5.1 Yap1 structure

Yap1 was isolated based on its ability to recognize and to bind to the recognition element TTAGTCA of virus 40 (Simian vacuolating virus) [113, 114]. Yap1 possesses 3 major domains: a basic leucine zipper DNA binding domain (bZip), a C-terminal cysteine-rich region (c-CRD) and an N-terminal cysteine-rich region (n-CRD) [115] (Fig. 6). The bZip domain contains a leucine zipper domain necessary for dimerization of DNA, and a basic DNA binding domain, that share a homology with members of the mammalian Jun family of transcription factors, AP1.

Because its bZip domain contains a DNA-binding domain, Yap1 was characterized as a member of the Jun family based on transcription factors [116].

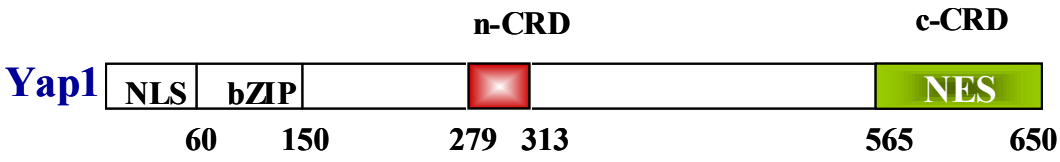


Figure 6. Schematic structure of Yap1
Yap1 comprises three conserved domains: a basic leucine zipper DNA binding domain (bZIP), a C-terminal cysteine-rich region (c-CRD) and an N-terminal cysteine-rich region (n-CRD).The Nuclear Localization Signal (NLS) is located at the N-terminal whereas the leucine-rich Nuclear Export Signal (NES) is located at C-terminal. Adapted from *Nature review* [115].

1.5.2 Role and mechanism of action of Yap1

In *Saccharomyces cerevisiae*, the basic leucine-zipper transcription factor Yap1 [116] regulates the gene expression of antioxidant enzymes like the reactive oxygen species (ROS) – removing enzymes such as: *SOD1-2 CTT1* and *TSA1*, and also the components of the cellular thiol-reducing pathways that include the REDOX group *TRR1-2 GLR1* and *GRX1*, necessary for maintaining the cytosol in a reduced state by NADPH [117]. In addition, Yap1 plays a major role in response to H_2O_2 [118, 119], and many electrophilic thiol-reactive chemicals such as N-ethylmaleimide (NEM), and the lipid peroxidation by-products 4-hydroxynonenal [120] and malondialdehyde [119].

Under non stress conditions, Yap1 is freely imported and exported from the nuclear compartiment [121, 122], whereas upon activation by oxidative stress – mainly caused by H_2O_2 [123], –Yap1 is rapidely redistributed in the nucleus because of the inhibition of its nuclear exportation [121, 124].

In response to oxidative stress, the Gpx3p Peroxidase (GPX)-like enzyme initially perceives the hydrogen peroxide signal, then transduces it to Yap1p resulting in the formation of two intramolecular disulfide bonds: one is between theYap1 N-terminal CRD Cys^{303} and the C-terminal CRD Cys^{598} [117], and the other one is between the Cys^{310} and Cys^{629}, it stabilize the activated form of Yap1 [125]. These two conformational changes mediate interactions with a conserved amino-terminal α-helix [115], and therefore, mask the nuclear export signal sequence in the c-CRD (C-terminal rich domain) of Yap1. This then becomes non-accessible to the Crm1

protein (β-karyopherin-like nuclear exporter), known to export Yap1 from the nucleus to the cytoplasm [121].

As a result, the oxidized Yap1 accumulates in the nucleus, where it regulates the expression of up to 71 genes (one half is regulated by Yap1 alone, whereas the second half depends on both Yap1 and the transcription factor Skn7) [123, 126, 127], through its binding to the recognition element YRE: T (T/G) ACTAA [128-130] that is located within the promoter of target genes.

1.6 Yap1 regulates the expression of efflux pumps

It has been found that Yap1 does not regulate only antioxidants, but mediates drug resistance in *Saccharomyces cerevisiae* by regulating the expression of efflux pumps such as: Flr1p and Ycf1p [94, 131] which are involved in diazaborine resistance.

1.6.1 Major Classes of efflux pumps

Efflux pumps are subdivided into two main classes of pumps depending on their source of energy: ATP binding cassette (ABC) transporters and multifacilitator superfamily MFS. The ABC proteins are energized by ATP hydrolysis, while MFS proteins use the proton-motive force across the plasma membrane to translocate compounds [132-138].

Most of the efflux pumps specific to polyamines are part of the MFS superfamily. They are classified in 2 sub-families: DHA1 (the drug), H+ antiporter

families have 12 putative membrane-spanning helices; whereas the DHA2 family has 14 predicted spanners [139]. Although it has been shown that many efflux pumps confer resistance to a range of structurally dissimilar compounds (including antibiotics and drugs) [140-142], the molecular mechanisms remain not well elucidated [141-146].

1.6.2 Polyamines efflux pumps confer multi-drug resistance

Among the 28 MFS [89] pumps identified to date in the *Saccharomyces cerevisiae,* 5 have been described: Tpo1, Tpo2, Tpo3, Tpo4 [147-150], Qdr3 [151, 152], and also Qdr2 [150].

The *QDR3* gene encodes an MFS efflux pump that belongs to the DHA1 family [140]. It is located in the yeast plasma membrane [151, 152], and provides resistance to a variety of drugs including the polyamines [149], the antimalarial drug quinidine, the cisplatin, the bleomycin and the herbicide barban [152]. Its homologue – *QDR2* – produces similar effects. A genome wide screen revealed that *QDR3* also provides resistance to the antifungal drug fluconazole [153], selenomethionine [154], the antiarrhythmic drug amiodarone [155], and magnesium dichloride [156]. This screen was carried out by testing the sensitivity of the *Qdr3*Δ mutant to the drugs mentioned above. Unlike Qdr3, Qdr2 is involved in potassium uptake and might be capable of coupling K+ ion transport with the export of specific substrates such as quinidine, that have been shown to interfere with K+ uptake [157].

Besides Qdr2 and Qdr3, other efflux pumps have been described. Tpo1, Tpo2 Tpo3 and Tpo4 are also members of the DHA1 family as drug: H^+ antiporters.

All of them share a similarity in their amino acid sequences [149], and thus have the same specificity to polyamine substrates like spermine, except Tpo1 and Tpo4 that are specific also to putrescine and spermidine, in addition to spermine [158]. Igarashi'group reported that *TPO1, TPO2, TPO3* and *TPO4* encoded specific polyamine transporters. They overexpressed each one of them, and then examined their role in response to methylglyoxal bis-guanylhydrazone (MGBG) [112], $Co^{2}+$ – an inorganic bivalent cation – was used as a control since it does not have any effect on polyamine transport.The four overexpressed efflux pumps demonstrated resistance to 1 mM MGBG compared to the wild type, but not to Co2+, indicating that Tpo1, Tpo2, Tpo3 and Tpo4 transport specific polyamines [149].

This was confirmed by a northern blot experiment showing that cells overexpressing *TPO* genes have an increased level of their *TPO* mRNA expression, in response to 0.3 mM of spermine, but not as much as *TPO1* mRNA, which was the most expressed compared to *TPO2 TPO3* and *TPO4* mRNA (Fig. 7).

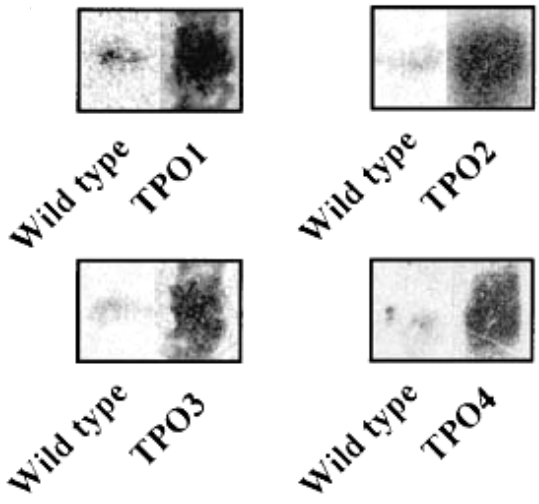


Figure 7. Northern blot analysis
Each *TPO* gene was overexpressed and transformed into wild type strain. Wild types and transformed strains were cultured in the presence of 0.3mM of spermine. The expression level of *TPO1, TPO2, TPO3* and *TPO4* mRNA in wild types was compared to those overexpressing *TPO* genes by northern blot analysis *Biochem Journal* [149].

1.6.3 Characterisation of the MDR efflux pump Tpo1

Although these proteins have been shown to confer resistance to a range of structurally dissimilar compounds (including antibiotics and drugs) [140-142], besides polyamines, Tpo1 [148], remains the best characterized efflux pump. It is involved in the extrusion of the artesunate [159], the herbicide 2, 4-D [160, 161], and the antimalarial drugs quinidine [162].

The *TPO1* gene is located on chromosome XII and encodes a membrane protein consisting of 586-amino-acid residues [163], specific to putrescine, spermidine, and spermine.

In its structure, Tpo1 possesses (Fig. 8):

- 3 glutamate acids Glu-207, Glu-324 and Glu-574, necessary for polyamine transport [149, 164].

- A long hydrophilic N-terminal region rich in serine and threonine residues, including serine 19, Threonine 52. These have been shown to enhance the activity of Tpo1 when phosphorylated by the protein kinase C [150].

- Ser 342, that is located in the cytoplasmic loop between the VI and VII transmembrane segment, helps releasing Tpo1 from the endoplasmic reticulum to the plasma membrane once it is phospohrylated by cAMP-dependent protein kinases [150].

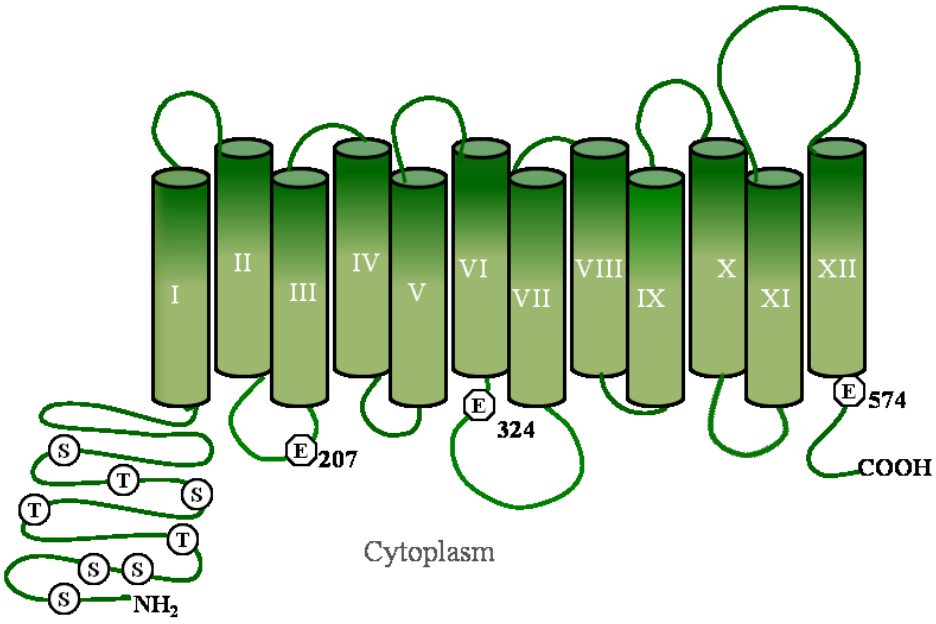


Figure 8. Model of Tpo1 structure
The DHA1 efflux pump Tpo1 has in its N-terminal glutamic acid, serine and threonine residues, necessary for its transport activity. Adapted from *Biochem Journal.* [164].

Tpo1 is mainly located on the yeast plasma membrane [147], and it catalyzes the efflux of polyamines at acidic pH, however when it is overexpressed, it is found in both plasma and vacuolar membrane. This has been demonstrated by a study showing that when Tpo1 is expressed from a single copy plasmid, it is detected only on the plasma membrane, whereas when it is overexpressed using a multi copy plasmid, Tpo1 is produced in many copies that will be located on plasma and vacuole membranes [150].

1.6.4 The regulation of *TPO1* by transcriptions factors

The *TPO1* gene includes within its promoter region many known specific binding sites that are recognized by specific transcription factors such as Pdr1, Pdr3, Skn7, Msn2, Msn4, Wrr1, and War1 (Fig. 9). These are involved in the transcriptional regulation of the gene depending on the type of stress that the cell undergoes [165].

For example, in response to the stress induced by the antimalarial drug artesunate, *TPO1* is dependent on the zinc finger Pdr1p for its transcriptional control [159]. It also requires Pdr3 – that shares 36% of its amino acid identity with Pdr1p – and Pdr1p, in case cells are under stress, caused by the chlorophenoxy herbicides 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-D [159, 160].

Based on the YEASTRACT database (www.yeastract.com), the transcription factor Yap1 is found to activate the transcription of the *TPO1* gene by binding to its recognition site YRE [159], when cells are exposed to oxidative stress (H_2O_2).

It has been shown by Sá-Correia's group that Yap1 is involved in resistance to polyamine, since *yap1Δ* mutant exhibits an extreme sensitivity to spermine, spermidine and putrescine compared to the wild type [166]. Consistent with this finding, another study focused especially on the role of polyamine, and demonstrated that in presence of free iron, polyamines function as pro-oxidants able to induce oxidative stress [167]. This explains why Yap1 confers resistance to polyamine as it does to H_2O_2.

Conferring resistance to polyamine does not necessarily mean that only one efflux pump is involved in polyamine detoxification. This might result from the transcriptional regulation of a set of genes specific to polyamine substrate such as *TPO1 TPO2, TPO3, TPO4* and *QDR3* [166].

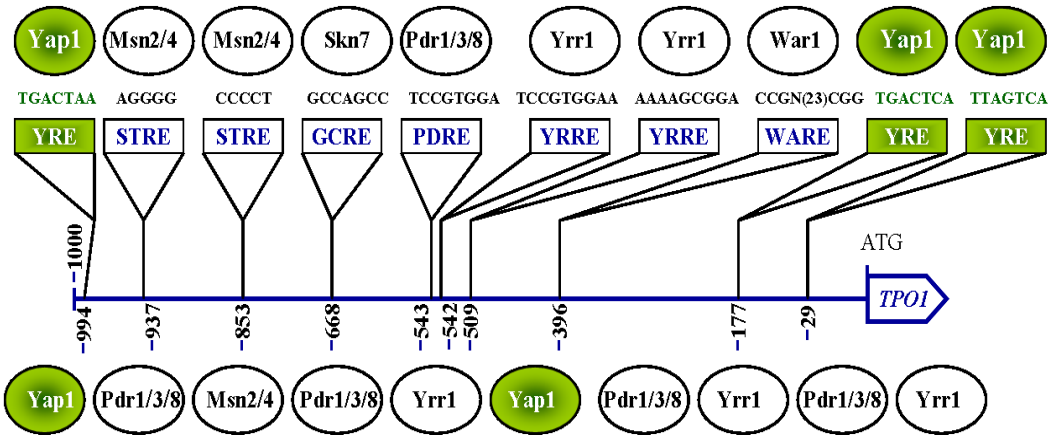


Figure 9. Position of consensus sequences recognized by transcription factors. Transcription fators control the regulation of *TPO1* by binding to specific motifs. Yap1 recognized and binds to a concensus sequence located in 3 positions on the *TPO1* gene: 994 (Forward strand), 29, 177 (Reverse strand). Adapted from *Biotechnol.Review* [159].

1.6.5 The orthologue of *TPO1* in human cells

To date, no efflux pumps specific to bleomycin have been identified in mammalian cells. In the human genome, 48 ABC efflux pumps have been identified [168]. However, none of them have been shown yet to confer resistance to bleomycin, as Tpo1 does in *saccharomyces cerevisiae.*

Based on blast search, a tetracycline transporter-like protein (TETRAN) has been the first MFS protein characterized in human cells as an orthologue to *TPO1* [169]. Since TETRAN shares some similarity with *TPO1*, it is that which is believed to expel specifically indomethacin (non-steroidal anti-inflammatory drugs (NSAIDs) in yeast. It is supported that TETRAN functions probably as an efflux pump in the same way as Tpo1 and confers resistance to some NSAID when overexpressed [169]. Such a protein could lead us in further studies to check its specificity for other substrates and eventually bleomycin.

Since no efflux pump specific to bleomycin has been identified in mammalian cells, we decided to use the powerful genetic system of *saccharomyces cerevisiae*, the simplest and most well-known representative of eukaryotic cells, for evaluating toxic effects of bleomycin and characterizing potential efflux pumps that probably have their homologuous in mammalian cells and function similarly as in yeast.

The main goal from this work is first to explore and understand how cells could develop resistance to the drug in yeast, then extrapolate the information from yeast to human. This will provide an insight into how cancer cells treated with bleomycin develop cancer.

In this work, we provide evidence that Yap1 regulates the expression of the multidrug efflux pump *TPO1* that in turn provides resistance to *imp2Δ* mutants toward bleomycin. However, we still do not know if this regulation is direct, or mediated by other proteins such as the transcription factor Skn7p, since we know that half of the 71 proteins induced by oxidative stress depend on both Yap1p and Skn7p [127].

Chapter 2: Material and methods

2 Material and methods

2.1 Yeast strains and growth media

The yeast strains used in this work are listed in Table 1. Cells were grown either in YPD medium containing 1% Yeast Extract (MULTICELL) peptone (BIO BASIC INC) and 2% dextrose (BIO BASIC INC), or selective medias contained Yeast Nitrogen Base 10x (DIFCO), 2% dextrose (BIO BASIC INC), supplemented with the amino acids necessary for cells growth depending on the genotype.

Solid media was obtained by the addition of 1.5% Agar (MULTICELL).

Table 1. List of strains and their genotype

Strains used in the present study:

Strain	Genotype	Source
By4741 *(imp2Δ)*	Isogenic to BY4741, except *imp2Δ*::LEU2	Lab strains collection
By4741 (*agp2Δ)*	Isogenic to BY4741, except *agp2Δ*::URA3	
By4741 (*tpo1Δ)*	Isogenic to BY4741, except *tpo1Δ::*KAN	
By4741 (*tpo2Δ)*	Isogenic to BY4741, except *tpo2Δ*::KAN	
By4741 (*qdr3 Δ)*	Isogenic to BY4741, except *qdr3Δ*::KAN	
By4741 (*yap1Δ)*	Isogenic to BY4741, except *yap1Δ::*HIS3	
By4742 (parent)	*MATα; his3Δ* 1; *leu2Δ* 0; *lys2Δ* 0; *ura3Δ* 0	
By4741 (parent)	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	

Strains made for the present study:

Strains	Genotype	Source
Wt (parent) / pYCP50	BY4741 / pYCP50	This work
Wt (parent) / pYAP1	BY4741 / pYAP1	
imp2Δ / pYCP50	Isogenic to BY4741,except *imp2Δ*::LEU2 / pYCP50	
imp2Δ / pYAP1	Isogenic to BY4741,except *imp2Δ*::LEU2 / pYAP1	
tpo1Δ / pYCP50	Isogenic to BY4741, except *tpo1Δ*::KAN / pYCP50	
tpo2Δ / pYCP50	Isogenic to BY4741, except *tpo2Δ*::KAN / pYCP50	
qdr3 Δ / pYCP50	Isogenic to BY4741, except *qdr3Δ*::KAN / pYCP50	
tpo1Δ / pYAP1	Isogenic to BY4741, except *tpo1Δ::*KAN / pYAP1	
tpo2Δ / pYAP1	Isogenic to BY4741, except *tpo2Δ*::KAN / pYAP1	
qdr3 Δ / pYAP1	Isogenic to BY4741, except *qdr3Δ*::KAN / pYAP1	
tpo1Δimp2Δ / pYCP50	Isogenic to BY4741, except *tpo1 Δ* ::KAN *Imp2Δ*::LEU2 / pYCP50	
tpo2Δimp2Δ / pYCP50	Isogenic to BY4741, except *tpo2Δ*::KAN *Imp2Δ*::LEU2 / pYCP50	
qdr3Δimp2Δ / pYCP50	Isogenic to BY4741, except *qdr3Δ*::KAN *Imp2Δ*::LEU2 / pYCP50	
tpo1Δimp2Δ / pYAP1	Isogenic to BY4741, except *tpo1 Δ* ::KAN *Imp2Δ*::LEU2 / pYAP1	
tpo2Δimp2Δ / pYAP1	Isogenic to BY4741, except *tpo2Δ*::KAN *Imp2Δ*::LEU2 / pYAP1	
qdr3Δimp2Δ / pYAP1	Isogenic to BY4741, except *qdr3Δ*::KAN *Imp2Δ*::LEU2 / pYAP1	
Wt (parent) / pQDR3	By4741 / pQDR3	This work
Wt (parent) / pTPO1	By4741 / pTPO1	
Wt (parent) / pTW438	By4741 / pTW438	
imp2Δ/ pTW438	Isogenic to BY4741, except *imp2Δ*::LEU2 / pTW438	
imp2Δ/ pTPO1	Isogenic to BY4741, except *imp2Δ*::LEU2 / pTPO1	
imp2Δ/ pMYC-TPO1	Isogenic to BY4741, except *imp2Δ*::LEU2 / p MYC-TPO1	
imp2Δ/ pQDR3	Isogenic to BY4741, except *imp2Δ*::LEU2 / pQDR3	
tpo1ΔImp2Δ / pTW438	Isogenic to BY4742, except *tpo1 Δ* ::KAN *imp2Δ*::LEU2 / pTW438	
tpo1ΔImp2Δ / pTPO1	Isogenic to BY4742, except *tpo1 Δ* ::KAN *imp2Δ*::LEU2 / pTPO1	
tpo1ΔImp2Δ / pQDR3	Isogenic to BY4742, except *tpo1 Δ* ::KAN *imp2Δ*::LEU2 / pQDR3	
Wt(parent)/ pYAP1-GFP	BY4741 / pYAP1-GFP	This work
imp2Δ/ pYAP1-GFP	Isogenic to BY4741, except *imp2Δ*::LEU2 /pYAP1-GFP	
agp2Δ/ pYAP1-GFP	Isogenic to BY4741, except *agp2Δ*::URA3 /pYAP1-GFP	

Wt: Wild type BY 4741 (parent)

Table 2. List of plasmids

Plasmid	Description and use	Source
pYCP50	-*URA3* plasmid used as an empty vector control.	Lab plasmids collection
pYAP1	-*URA3* plasmid containing *YAP1* gene used for complementation of mutants lacking *IMP2* gene.	
pYAP1-GFP	-*URA3* plasmid with *YAP1*-GFP used for monitoring the localization of Yap1.	
pTW438	-URA3 plasmid gap repair containing ADH promoter, MYC tag sequence.	
pTPO1	-*TPO1* gene cloned into *URA3* plasmid (pTW438) used for restoring resistance to polyamines and bleomycin.	This work
pQDR3	-Same as above.	
pMYC-TPO1	-Same as above, but the restriction enzymes used are not the same.	

2.2 Spot test

Yeast strains were inoculated in 1 ml of YPD liquid media and were grown overnight at 30 °C with agitation. The next day, cells were subcultured in 2 ml at 30 °C on the rotary shaker. After 3-4 hours of subculture, cells reached exponential phase (OD: 0.6). Serial dilutions were performed, and from each one 4µl or 5 µl were spotted on agar plates contained or not different concentration of drugs: spermidine, spermine, bleomycin, 4NQO, and H_2O_2, depending on the experiment. Plates were incubated for two days at 30°C before being photographed by the ALPHA imager robot.

2.3 Spermidine excretion assay

The strains By4741, *imp2Δ* were harvested at A_{600}=0.5, then washed 2 times with a buffer containing 20mM Na/MES, pH 5.0 and 10 mM glucose, and resuspended in the same buffer. The excretion assay was initiated by the addition of ^{3}H spermidine (16.6 Ci /mmol Perkin Elmer) in 2ml of cells. Suspended cells were incubated at 30 °C with shaking for 0, 2, 4 and 6 min. After each incubation, cells were washed 3 times with the buffer described above supplemented with 1 mM of cold spermidine and resuspended in 400 µl of water. The radioactivity content was measured by adding 5 ml of scintillation liquid (Amersham Biosciences) to each sample, and using liquid scintillation spectrometry.

2.4 Fluorescence microscopy

We overexpressed the *YAP1* gene as a GFP fusion gene into a wild type, *imp2Δ* and *agp2Δ* strains. Transformed cells were grown in 2 ml of selective liquid media –URA and harvested at OD_{600} 0.5. Next, the cultures were diluted and incubated in the absence and presence of 0.5mM H_2O_2, and 18 mM spermidine for 10 min and 30 min respectively. Cells were first fixed in methanol 50%, then stained with DAPI (4',6-diamidino-2-phenylindoledihydrochloride) (DAPI; 2.5 pg/ml), and viewed using a Leica DMRE microscope.

2.5 Yeast transformation

Depending on the genotype, strains were grown either in 1 ml of YPD liquid or 1 ml of selective media, over night at 30 °C. The following day, cells were sub-cultured in a fresh media, for 3-4 hours. After incubation, they were washed twice with sterilized water followed by a quick spin (30 sec) at 1000 rpm. The pellet was washed with 600 ul of TE/LiAc solution (10 mM Tris - pH 7.5, 1mM EDTA, 100 mM LiAc - pH 7.5), then centrifuged at room temperature and resuspended in 30 ul of the same solution which was sequentially supplemented with a list of components listed in the Table 2, below.

Table 3. List of the components used for transformation

Samples	**Volume**		
	Wild type or mutant strain	Controls	
Cell culture	30 µl	30 µl	30 µl
SS carrier DNA	5 µl	5 µl	5 µl
Plasmid	1-2 µl	-	-
Empty vector	-	1-2 µl	-
PEG solution*	150 µl	150 µl	150 µl

*PEG solution includes TE/LiAc 1 X and 50 % PEG (polyethylene glycol).

Cells were incubated at 30°C for one hour, followed by a heat shock in a water bath at 42°C for 15 min. Next, we microcentrifuged them at 3000 rpm for 1 min, removed the supernatant and resuspended their pellet in 100 µl of sterilized water by pipeting up and down. This volume was plated on YPD or appropriate selective agar plates that were then incubated at 30°C. The single colonies detected after 2-3 days, were patched on new agar plates.

2.6 Bacterial transformation

2.6.1 Chemical transformation using $CaCl_2$

2.6.1.1 Preparation of competent cells

A single bacterial DH5α colony inoculated in 2 ml of LB liquid, was grown over night at 37°C with moderate shaking. The following day, cells were subcultured in 50 ml LB for 4-5 hours at 37°C, followed by a spin down at 4°C for 10 min at 4000 rpm. The supernatant was discarded while the pellet was washed twice with sterilized water, resuspended in 100 mM of cold $CaCl_2$ and then chilled on ice for 1 hour. After ice incubation, cells were spun down. The supernatant was then spun down and the pellet recovered. At this step, one can either place the tube containing suspension cells on ice for an immediate use or can add 100 mM $CaCl_2$ and 50 % glycerol, then aliquote 50 µl of cells into 1.5 ml eppendorf tubes and store them at -80 °C.

Frozen competent cells will not be efficient beyond 4-6 months.

2.6.1.2 Transformation of competent cells

Competent cells already stored at -80 ^{o}C were thawed gently on ice. For each transformation, 25 µl was aliquoted into pre-chilled 1.5 ml eppendorf tubes, and then 1-2 µl of DNA (plasmid of genomic DNA) was added to the aliquot. At this step, 50 µl can also be used instead of 25 µl.

The tube containing the mix of the DNA and cells was incubated on ice for 30 min followed by a heat shock in a water bath at 42 °C for 90 sec.

After that, the tube was left on ice to cool for 1-2 min, then 1 ml of LB was added to the culture and incubated in the shaker at 37 °C for 1-2 hours.

After incubation, cells were spun down at high speed for 5 min and the pellet was recovered only with 200 µl of its supernatant. This was then plated onto LB agar media supplemented with 100 µg/ml Ampicillin. Plates were inverted and incubated at 37 °C over night. After 14-16 hours bacterial colonies appeared.

2.6.2 The electroporation

2.6.2.1 Preparation of electrocompetent cells

A single bacterial DH5α colony picked from the LB plate or from the frozen glycerol stock of bacterial cells, was inoculated into 3 ml LB media liquid, and incubated over night at 37°C in the shaker. The next day, cells were subcultured in 30 ml of fresh LB media at 37°C. After 4-5 hours of incubation, cells were washed with MiliQ water 3 times and centrifuged at 4°C for 10 min at 4000 rpm. The pellet was re-suspended in 20 µl of miliQ water for immediate use.

2.6.2.2 Electroporation of electrocompetent cells

0.5 -1 µl of transforming DNA was added to 20 µl of electrocompetent cells, the content was mixed gently, then placed along walls of a 0.1 cm prechilled cuvet on ice for 30 min. After the incubation, cells were electroshocked according to the conditions below:

-The voltage: 2.5 kV

-The capacitance: 25 µF

-The resistance: 200 ohm

-Time constant: 2.5-2.6

Once cells are electroporated, 0.5 ml of LB media is added immediately, and the transformed cells are transferred to a 1.5 ml eppendorf tube then incubated at 37 °C for 30-60 min. After the incubation, cells were spread on LB agar media + 100ug/ml Ampicillin, and incubated at 37 °C over night. After 14-16 hours bacterial colonies appeared.

2.7. Plasmid extraction from *E.Coli*

One single colony picked from a LB/Amp transformation plate, was inoculated into 4 ml LB liquid containing 100ug/ml Ampicillin, and grown over night at 37°C in the shaking incubator. The next day; the sample was centrifuged at high speed (4000 rpm) for 10 min. The supernatant was carefully poured-off and the bacterial pellet was resuspended in 0.1 ml of solution 1 (50mM glucose, 25 mM Tris-HCl - pH 8.0 and 10 M EDTA - pH 8.0).

Next, 0.2 ml of solution 2 (1% SDS, 0.2 N NaOH) was added, and the contents was mixed by inverting the tube gently 5 times, then the tube was left to stand at room temperature. After 5 min of incubation, 0.15 ml of solution III (3 M potassium acetate K+, 5M glacial acetic acid) was added, and then the tube was incubated on ice for 10 min, and centrifuged at 12000 rpm for 5 min.

In order to get rid of cell debris, the supernatant was transferred to a new eppendorf, to which was added 300 µl phenols twice, followed by adding the same volume of chloroform, twice also. The DNA was pelleted by centrifugation at 12000 rpm for 5 min, washed with ethanol 95%, and then span down at the same speed for 3 min.

After this step, the ethanol was poured out and the pellet was dried in the fume hood. Once the ethanol had evaporated, we dissolved the pellet in 50 µl of dH_2O, then stored it at -20 °C.If necessary, before storing, the DNA can be incubated with RNase for 30 min at 37 °C, however this step is not required in case we use the plasmid mini-prep kit.

2.8. Yeast Genomic DNA extraction

2.8.1 The procedure for extracting genomic DNA

Yeast cells were grown in 5 ml of appropriate media over night at 30°C. Next, they were centrifuged in 15 ml conical tubes at 4000 rpm for 1 min at 4 °C and then resuspended in 200 µl of breaking buffer (TE, 100 mM $CaCL_2$ and 0.1 % SDS) by vortexing. We added sequentially 200 µl phenol: chloroform: IAA (12:24:1) followed

by 200 µl of acid washed glass beads (425-600 micron). We capped the tubes tightly, and vortexed them for 2 min by placing them inside a mini bead beater. After repeating this step 2 times more, we spun the cells for 5 min at 15000 rpm to separate the lysed cells from the upper phase that we collected in a new tube.

300 µl of 6M NaI and 10 µl glassmilk were added and mixed with 100 µl of supernatant. After 5 min of vortexing at high speed, the sample was pelleted by centrifugation and washed 3 times with 400 µl of new wash buffer. After the last wash, we let the tube drain at room temperature on a clean paper towel until the pellet get dried , then resuspended in 10-100 µl of dH_2O.

At this point, the sample could be centrifuged to separate the glassmilk from the supernatant containing dissolved DNA then used immediately, or alternatively stored at -20 °C.

2.8.2 Purification of extracted genomic DNA

In order to purify the extracted genomic DNA, 1/10 volume of 3 M NaAc and 2 volumes of 100% ethanol were added and mixed with the sample by inverting the tube many times, before incubation at -80°C for 30 min.

Next, the sample was spun down at 12000 rpm for 15 min, and the pellet was washed with ethanol (70%) followed by a short spin down (1 min). Finally, the pellet was air-dried and the DNA was resuspended in 20 µl of dH_2O and stored at -20 °C.

2.9 RT-PCR

2.9.1 RNA extraction

RNA was extracted using a RiboPure-Yeast extraction kit (Ambion). 3 ml of yeast culture, grown overnight at 30 °C, was centrifuged at high speed. We discarded the supernatant while resuspending the pellet in 480 µl Lysis Buffer followed by 48 µl 10% SDS and 480 µl Phenol: Chloroform: IAA. After adding these lysis reageants, we transferred the cell mixture to the tubes already filled with 750 µl of cold zircona beads. Tubes were capped tightly then vortexed at high speed by using the bead beater. After 10 min of vortexing, cells were spun down at 16,000xg for 6 min to separate the pellet and the beads from the aqueous phase containing RNA that we transferred to 15 conical tubes. 1.9 ml binding buffer, followed by 1.25 ml ethanol (100%) were added and mixed thoroughly with the partially purified RNA. This RNA mixture was drawn through the filter cartridge, collected in new tubes, then centrifuged for 1-2 min at high speed. Once done, we washed the filters with wash solutions (700 µl of wash solution 1, 500 µl of wash solution 2/3, twice). They were then transferred to new tubes in which we eluted the RNA in 25-50 µl of elution solution.

Once the RNA is isolated, it is recommended to treat it with DNase since RNA contains chromosomal DNA. In order to remove contaminating DNA, we incubated 50-100 µl of the RNA sample with 4 µl DNase I (8 U) in 1/10 volume 10X DNase 1 buffer at 37 °C in a water bath. After 30 min of digestion, we inactivated the DNase by adding 0.1 volume DNase inactivation reagent, and then we let the reaction

stand at room temperature for 5 min. The sample was centrifuged for 2-3 min at 10,000 xg. Finally the total RNA was transferred to a new tube and its absorbance was measured at 260 nm.

2.9.2 RNA denaturing agarose gel electrophoresis

10 ml 10X MOPS running buffer (0.3 M MOPS - pH 7.0, 0.1 M sodium acetate,0.01 M EDTA) and 18 ml of 37% formaldehyde were added to 1 g agarose already dissolved in 72 ml water. The gel was poured, then covered by 1X MOPS running buffer. For RNA samples, we added 0.5-3X volumes of formaldehyde load dye to 1-3 µg RNA, before heating the sample to 65-70°C for 5 min to denaturate RNA, and then loading it.

2.9.3 The synthesis of cDNA

For cDNA synthesis, we added 0.2 µl of random primers and 1 µl of 10 mM dNTP to each RNase free tube containing 2 -5 µg of total RNA and we filled it to 12 µl with sterilized distilled water. The mixture was heated to 65 °C for 5 min, and immediatly cooled on ice. We spun down the sample then added 4 µl 5X First-Strand Buffer, 2 µl 0.1 M DTT and 1 µl RNase OUT^{TM} (40 units/µl). We mixed gently the sample that we placed in a water bath at 37 °C for 2 min. After 2 min of incubation, 1 µl (200 units) of M-MLV RT (Reverse transcriptase) was gently mixed with the contents of the tube that we incubated for 10 min at 25 °C, 50 min at 37°C, and 15 min at 70 °C. At this step cDNA is ready to use as a template for PCR reactions.

2.9.4 PCR reaction

We designed the following primers using Gene Runner software, to assess the expression level of *TPO1* and *QDR3*:

-RT-PCR-*TPO1*-F: 5'- TTATATCGCACAAAGAACTACC-3'

-RT-PCR-*TPO1*-R: 5'- GCATAATGATAGGCCTCGTC-3'

-RT-PCR-*QDR3*-F: 5'- ATGCAAGCCCAAGGTTCAC-3'

-RT-PCR-*QDR3*-R: 5'- TCCTCACTCTTCAACCTAC-3'

We used the following primers already designed by a previous student, to detect the expression of *AGP2,* by using the *ACTIN* as a control:

-RT-PCR-*AGP2*-F 5'-TTGATTGCTATTTCCGGTGTCA-3'

-RT-PCR-*AGP2*-R 5'GGGCCCTCCACGGTATAAAG-3'

-RT-PCR-*ACT1*-F1 5'- GTTTTGCCGGTGACGACGCTCCTCGTGCTG-3'

-RT-PCR-*ACT1*-R1 5'-CGGCTTGGATGGAAACGTAGAAGGCTGGAACG-3'

The master mix was prepared by adding these reagents: 1-4 µl cDNA, 5 µl 10 X taq polymerase buffer, 1 µl of forward primer, 1 µl of reverse primer, 1 µl of taq polymerase (5U/µl) and 5 µl of 10mM dNTP. We filled up the tube with sterilised water to 50 µl.

<u>The PCR designed programs are:</u>

-*TPO1*: 1- 95 ºC for 2 minutes, 2- 94 ºC for 0.5 minute, 3- 58 ºC for 0.5 minute, 4- 72 ºC for 2.20 minutes, 5- 22 times repeat of steps 2-4, 6- 72 ºC for 7 minutes, and 7- 4 ºC.

-*QDR3*: 1- 95 ºC for 2 minutes, 2- 94 ºC for 0.5 minute, 3- 51 ºC for 0.5 minute, 4- 72 ºC for 2.20 minutes, 22 times repeat of steps 2-4, 6- 72º C for 7 minutes, and 7- 4 ºC.

-*AGP2*:1- 95 ºC for 2 minutes, 2- 94 ºC for 0.5 minute, 3- 55 ºC for 0.5 minute, 4- 72 ºC for 2.30 minutes, 22-30 times repeat of steps 2-4, 6- 72 ºC for 2:30 minutes, and 7- 4 ºC.

2.10 Plasmid constructions

2.10.1 Primers design

We used the plasmid pTW438 as a substrate in the gap repair assay. It contains a Myc tag, URA cassette and an ADH promoter.

It was digested with HindIII and SmaI. The linear DNA was gel purified. The *TPO1* and the *QDR3* genes were amplified by PCR using genomic DNA of the strain BY4741 as a template, then purified. The following PCR primers designed for untagged *TPO1* and *QDR3* are:

Table 4. Design of PCR primers for *TPO1* and *QDR3* genes

Name	Sequence
***TPO1*-F**	5'-GCACAATATTTCAAGCTATACCAAGCATACAATAAGCTTCTCACC ATGTCGGATCATTCTCCCAT-3'
***TPO1*-R**	5'-TATGTAACGTTATAGATATGAAGGATTTCATTCGTCTGTCGACCCT TAAGCGGCGTAAGCATACT-3'
***QDR3*-F**	5'-GCACAATATTTCAAGCTATACCAAGCATACAATAAGCTTCTCACC ATGCAAGCCCAAGGTTCACA-3'
***QDR3*-R**	5'-TATGTAACGTTATAGATATGAAGGATTTCATTCGTCTGTCGACCC TTAATCAATTTTGTCGTACA-3'

Primers for *TPO1*: The product size is 1.7 kbp

Primers for *QDR3*: The product size is 2 kbp

Table 5. Design of PCR primers for *TPO1* gene tagged with MYC tag

Gene	Sequence
MYC-*TPO1*-F	5'-ACCATGGCGTCCGAGCAAAAGCTCATTTCTGAAGAGGACTTGCGG TCGGATCATTCTCCCATTTC-3'
MYC-*TPO1*-R	5'-TATGTAACGTTATAGATATGAAGGATTTCATTCGTCTGTCGACCC TTAAGCGGCGTAAGCATACT-3'

The same procedure was followed to amplify *TPO1* as MYC tagged gene (Table 4), but instead of digesting pTW438 with HindIII and SmaI (Fig. 10 and 11); we used the restriction enzymes BamHI and SmaI (Fig 12).

The PCR fragments were inserted into pTW438 between the 2 restriction sites mentioned above, near to the ADH1 promoter.

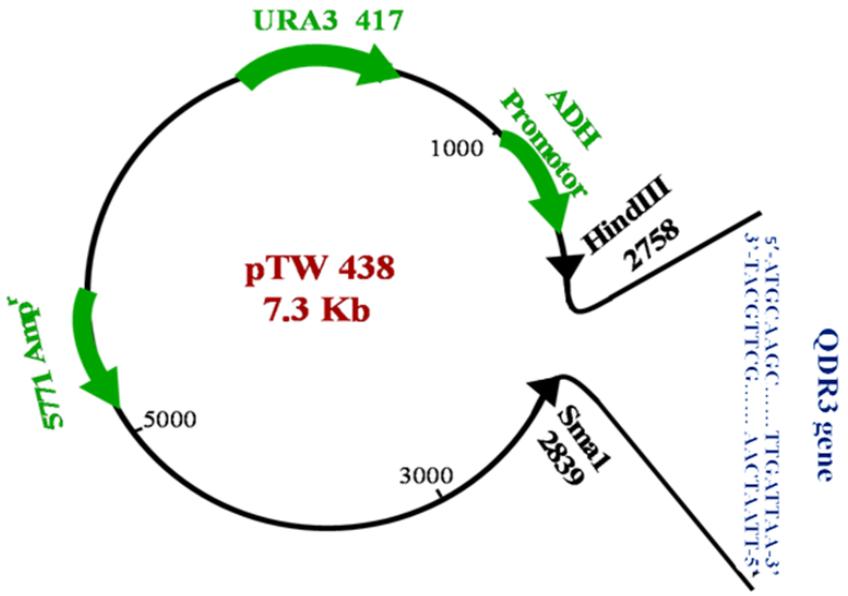


Figure 10. Design of plasmid carrying *QDR3* gene
QDR3 gene was amplified and inserted into the digested plasmid pTW438, between the two restricted sites recognized specially by HindIII and Sma1.

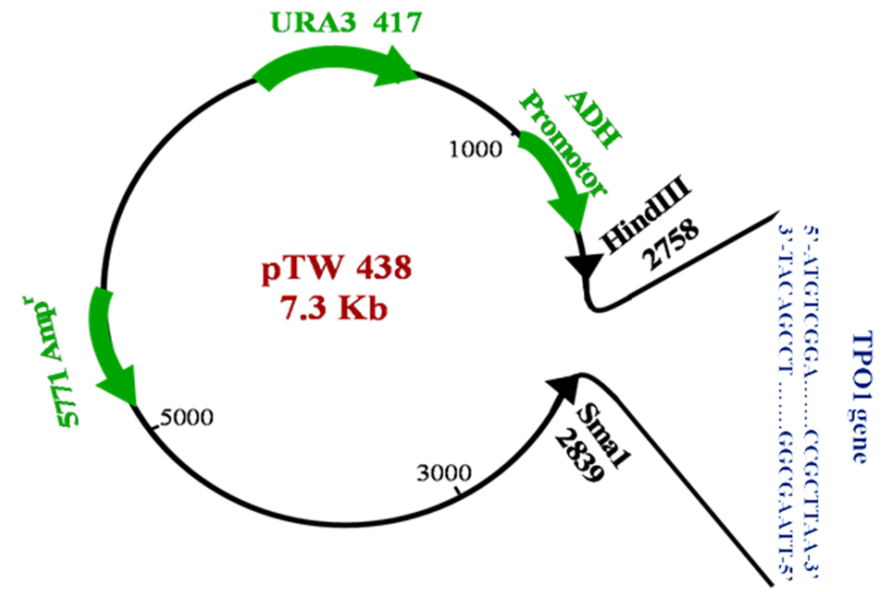


Figure 11. Design of plasmid carrying *TPO1* gene
TPO1 gene was amplified and inserted into the digested plasmid pTW438, between the two restricted sites recognized specially by HindIII and Sma1.

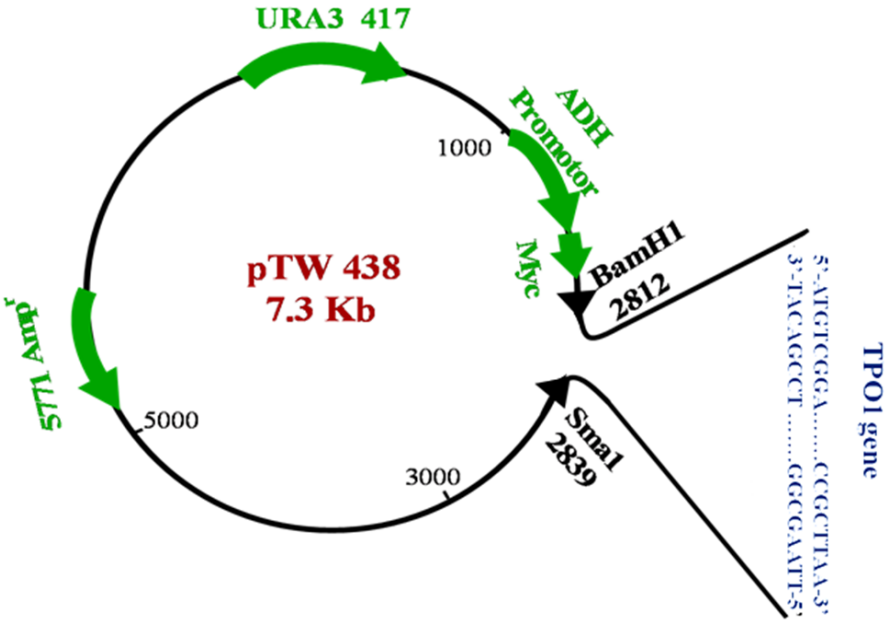


Figure 12. Design of plasmid carrying *TPO1* gene tagged with MYC tag.
TPO1 gene was amplified and inserted into the digested plasmid pTW438, between the two restricted sites recognized specially by BamH1 and Sma1.

2.11 Dissection of tetrads

By4742 strain carrying pTPO1 was crossed with the double mutant *tpo1Δimp2Δ* that has an opposite mating type, by patching and mixing them onto the same YPD agar plate. The plate was incubated at 30 °C. After 24 hours, we picked grown colonies from the center of the cross, streaked them onto selective media plate, and placed them in the incubator at 30 °C. 1-2 days later, single colonies that resulted from the mating mixture had grown. They were inoculated in 3 ml of sporulation media (5 g KAc, 0.5 g yeast extract, 450 ml water), supplemented with the amino acids necessary for cell growth, and then incubated in the shaker at 30 °C for 3-5 days. At the third-fourth day, cells were checked under the microscope to look for tetrads. Once diploid cells sporulated, we spun down 100-200 µl of cultures resuspended the pellet in 100 µl of 1M sorbitol and added 2 µl lyticase, required for the digestion of the ascus cell wall.

After 15-20 min incubation at room temperature, sporulated cells were spread onto an inoculum area of predried selective media plate, then tetrads were dissected under the microscope using a Singer micro-manipulator robot. Once done, the dissection plate was placed at 30 °C then printed after 2-3 days of incubation to different selective media in order to determine the genotype of each grown colony.

Chapter 3: Results

3 Results

3.1 *imp2Δ* mutants are hypersensitive to bleomycin and polyamines

It has been recently reported that strains lacking *IMP2* gene exhibit parental resistance to UV light and methyl methane sulfonate. This resistance is not apparent for bleomycin, which induces strand breaks resulting in 15-fold increases in the sensitivity of *imp2Δ* mutants compared to the wild type [93]. The *IMP2* gene does not possess any binding domain but it has a C-terminus leucine- rich repeat (LRR) domain, believed to mediate protein-protein interactions [170, 171], once *IMP2* gets activated by phosphorylation under stress conditions. According to these finding, it has been suggested that Imp2 functions as a transcriptional factor that regulates the expression of genes required for preventing oxidative damage [93]. However the mechanism through which Imp2 confers resistance remains unclear.

In this work, we decided to check whether the *imp2Δ* mutant, derived from By4741 background, presents sensitivity to bleomycin as it was seen in the case of *imp2Δ* isogenic to W303α [102]. Besides bleomycin, we used polyamines, since we know that they might act as pro-oxidants in the presence of free iron [104], and therefore induce oxidative stress as bleomycin does by generating free radicals. The main goal from using these two drugs is because both are related to cancer [13-15, 104].

Spot tests revealed that the *imp2Δ* mutant was extremely sensitive to bleomycin (1.5 μg/ml) as well as spermidine (15 mM), compared to the wild type. However, in response to 4-nitoquinoline-1-oxide (4NQO), a drug that normally causes bulky lesions onto the DNA, the mutant presented normal sensitivity, comparable to the wild type (Fig. 13). A similar result has been observed when the *imp2Δ* mutant was treated by DNA damaging agents such as the alkylating agents MMS, UV light and 4NQO [93], thus we exclude any role of Imp2 in DNA repair. Based on these results, the *imp2Δ* mutant appears to be hypersensitive only to oxidative DNA damage resulting in the accumulation of DNA breaks, suggesting that Imp2 is required especially for the detoxification of bleomycin and polyamines and not for DNA repair.

Next, we checked whether the sensitivity displayed by *imp2Δ* toward drugs, is indeed due to the accumulation of polyamine and bleomycin. In this case we monitored the excretion of radiolabelled spermidine in the *imp2Δ* mutant during a time course from 0 to 6 min. As shown in figure 14, *imp2Δ* accumulated rapidly more labeled polyamines than the wild type and did not expel the excess as the parent strain does. This data is consistent with the previous result observed in figure 13, and indicates that the increased sensitivity to polyamine might occur at the efflux level.

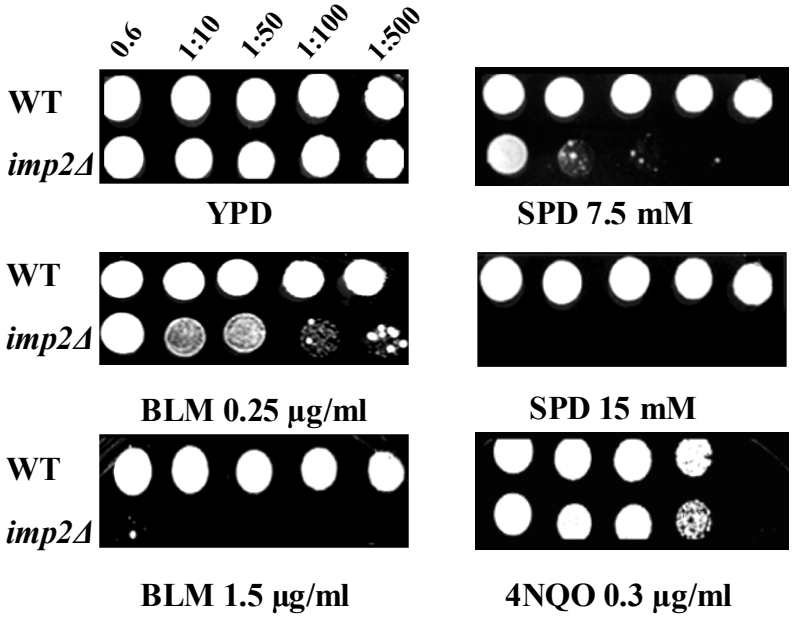


Figure 13. *imp2* null mutants displayed more sensitivity to bleomycin and spermidine compared to the wild type.
Cells were grown to exponential phase, and then serially diluted as indicated. 5 µl from each culture was spotted onto YPD agar media with and without different concentrations of BLM, 4NQO and SPD. After 2 days of incubation at 30°C, plates were photographed using an alpha imager machine. This result is representative of 3 independant spot tests.

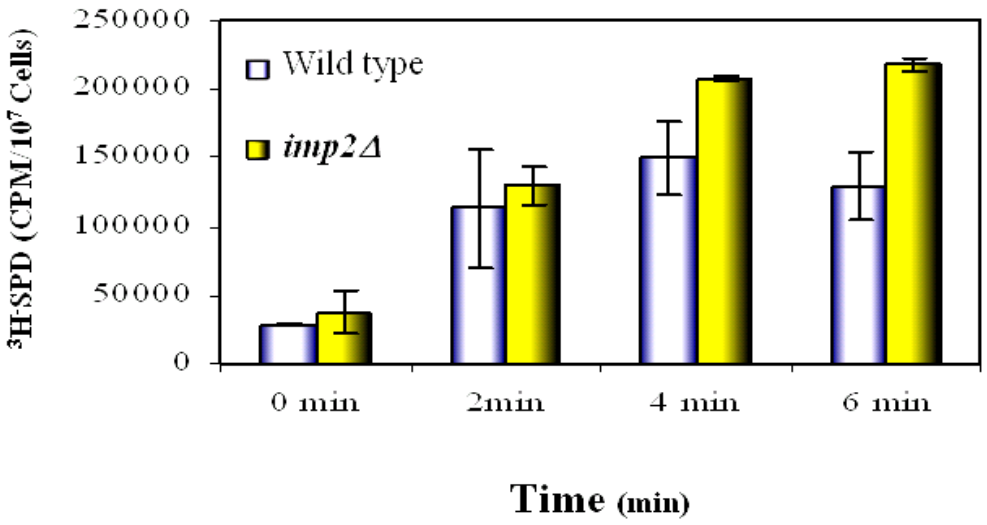


Figure 14. The excretion assay of ^{3}H spermidine during the time course 0-6 min. Exponentially growing cells were diluted to OD: 0.5 then incubated with ^{3}H spermidine for 0, 2, 4 and 6 min. After each incubation, cells were washed three times, and then resuspended in a specific buffer, as described in materials and methods.The radioactive content was measured by liquid scintillation spectrometry. This result is representative of 2 independant assays.

3.2 The multicopy plasmid bearing *YAP1* gene restores bleomycin and polyamine resistance to *imp2Δ* mutant

To confirm that Imp2 is required for polyamine and bleomycin detoxification, we decided to use it as a tool in order to look for their potential target genes. It was already shown that the *IMP2* gene is not involved in the DNA repair mechanism, since the mutant displayed normal sensitivity to the DNA damaging agent 4NQO, in the same way as the wild type. It might therefore be expected that the *IMP2* gene will regulate the expression of an efflux pump that can reduce the accumulation of the bleomycin and polyamines by increasing their efflux from the cell. Such a mechanism has been identified as the major one that provides resistance to cancer

stem cells in human [90].

To search for possible efflux pumps, an experiment has been done by Dindial's group that consists of cutting the whole genome of *imp2* null mutant to fragments inserted into multi-copy plasmids [102]. The plasmids carrying these fragments were subsequently transformed into *imp2Δ* mutants, in order to check whether they will restore bleomycin resistance or not. As a result, three clones exhibited resistance to bleomycin, resulting from the overexpression of a gene that does not encode an efflux pump in the way one might expect. Instead, it encodes a transcriptional activator, Yap1p.

Yap1p plays a major role in the regulation of most known cellular antioxidant genes [117], and it is involved in mediating multidrug resistance, by regulating the expression of efflux pumps such as the membrane pump Ycf 1p, the vacuolar pump Flr1p (that has been shown to confer resistance to diazaborine) [94, 131], and the Atr1 efflux pump [95] (that provides resistance to aminotriazole) [172].

To confirm whether Yap1p is responsible for restoring BLM resistance to *imp2Δ* mutant, we used the multicopy plasmid carrying the entire sequence of the *YAP1* gene and then we introduced it into the mutant to check for any complementation. In parallel, we introduced an empty vector into the same strain as a control.As shown in figure 15, pYAP1 plasmid restored a partial bleomycin and spermidine resistance to the *imp2Δ* mutant compared to the wild type (+/- Ycp50), whereas in response to spermine, the rescue was more than partial at 0.2 mM , but not as significant at high doses.

As a control, we used 4NQO again, to make sure that the resistance observed in spot tests, is not due to the inability to repair DNA damages, but is due to the expression of efflux pumps.

In response to 0.5µg/ml of 4NQO, we observed that the overexpression of Yap1 conferred resistance to strains carrying pYAP1, while the wild type (+/- Ycp50) was extremely sensitive (Fig. 15). This could be explained by the fact that Yap1p stimulates the expression of the ABC efflux pump Snq2 [173], that has been shown to be involved in the resistance to 4NQO [96, 174]. These findings provide evidence that Yap1 overexpression might activate an efflux pump in the *imp2Δ* mutant that expels both bleomycin and polyamines.

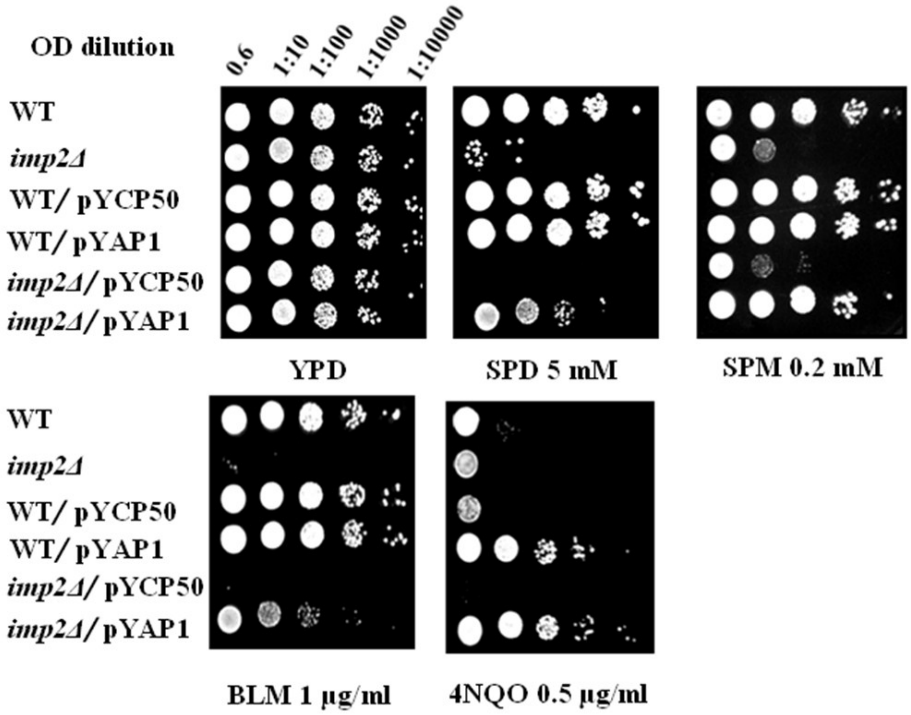


Figure 15. The complementation with pYAP1 restored resistance to *imp2Δ*.
Cells were serially diluted after reaching the exponential phase, and then 4 µl from each culture, was spotted on YPD solid media with and without different concentrations of drugs as indicated above. After 48 hours of incubation at 30 °C, plates were photographed. This data represents 3 independent spot tests analysis.

3.3 The single mutants *tpo2Δ* and *qdr3Δ* show parental resistance to polyamines and bleomycin, except *tpo1Δ*

In *saccharomyces cerevisiae*, 28 MFS efflux pumps have been identified [89]. Most of them have been shown to pump out a variety of toxic coumpounds [140-142]. Such transporters are associated with multiple drug resistance (MDR), among them, Tpo1, Tpo2, [147-150] and qdr3 [151, 152] have been characterized.

It has been reported recently that these pumps are required for polyamine

detoxification, and when they are deleted, cells exhibite sensitivity [166], and since bleomyicn includes in its N-terminal a chemical structrure similar to polyamine, we focused on the 3 pumps mentioned above, that might expel bleomycin besides polyamine.

So based on these findings, we decided to mutate each one of these efflux, then check whether *tpo1Δ, tpo2Δ,* or *qdr3Δ* mutants would be sensitive to bleomycin as well as to polyamine. As shown in figure 16, none of the 3 single mutants showed sensitivity to spermidine even at high dose (30 mM). This result is in opposition to recent data [166] showing that in response to 10 mM of spermidine, *tpo1Δ, tpo2Δ and qdr3Δ* single mutants – that derived from the same background, By4741 – were extremely sensitive. In response to bleomycin, all mutants exhibited parental resistance, same as the wild type, except *tpo1Δ* that showed partial resistance. This suggests that Tpo1 might have a major role in bleomycin detoxification.

However, this does not exclude the fact that Tpo1 alone cannot be enough to maintain optimal polyamine concentration in the cell, when cells are exposed to high and toxic doses. This means that the polyamine resistance observed in single mutants, results from the effect of multiple efflux pumps that compensate the lack of one efflux.

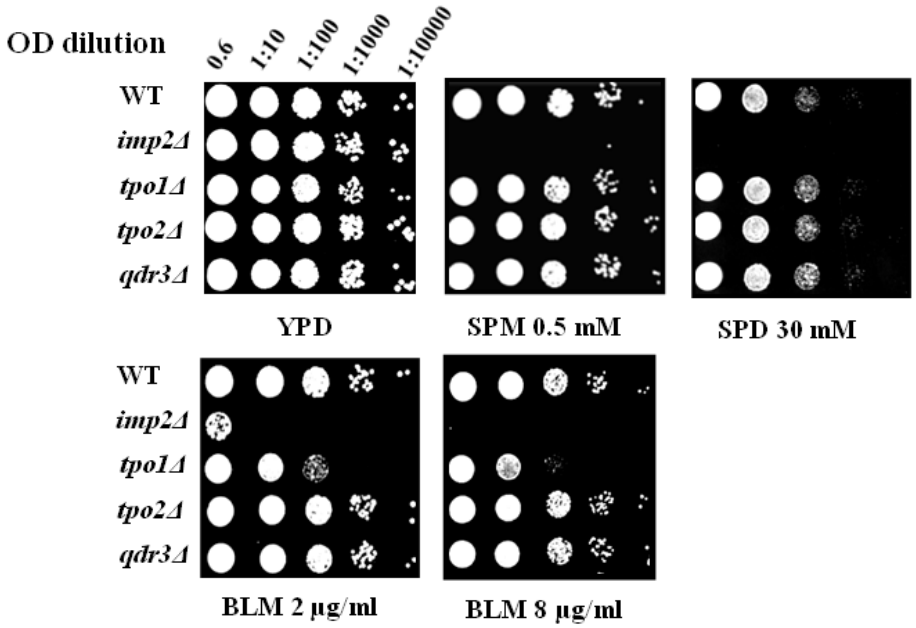


Figure 16. *tpo1Δ* exhibited partial resistance to bleomycin compared to *tpo2Δ* and *qdr3Δ.*
At OD: 0.6, cells were diluted and then spotted on YPD agar media with and without different doses of drug, as indicated above. Plates were photographed after 48 hours of incubation as was done in previous spot tests. These data are representative of 4 independant experiments.

3.4 The activation of *TPO1* via the overexpression of Yap1, confers bleomycin and spermidine resistance to *imp2Δ* mutant

Based on the Yeastract database, *TPO1, TPO2* and *QDR3* have been found to have specific domain located within their promoter region that is recognized especially by Yap1p. This suggests that these efflux pump genes might be upregulated by the transcriptional factor yap1 in response to oxidative stress.

To test whether *TPO1, TPO2* and *QDR3* genes are dependent on Yap1p, we constructed double-mutants *tpo1Δimp2Δ*, *tpo2Δimp2Δ* and *qdr3Δimp2Δ*, and then we checked whether pYAP1 would rescue them as it does in the previous spot test experiment, when it restored bleomycine and polyamine resistance to the *imp2Δ* mutant. The double mutants *tpo2Δimp2Δ* and *qdr3Δimp2Δ* displayed similar sensitivity to that observed in *imp2Δ* in response to 7.5 mM of spermidine, whereas *tpo1Δ imp2Δ* showed higher sensitivity (Fig. 17). This suggests that among these efflux pumps, Tpo1 plays a major role in the detoxification of bleomycin and polyamines.

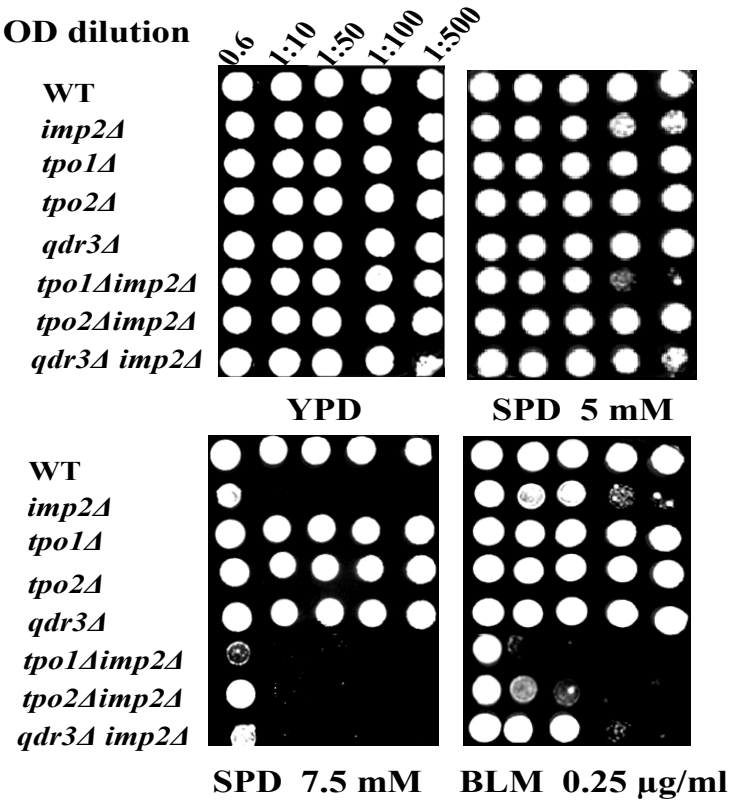


Figure 17. *tpo1Δimp2Δ* exhibited higher sensitivity toward drugs compared to other double mutants.
Cells were adjusted to OD: 0.6 then 5 µl was spotted onto YPD agar media, with and without different doses of SPD and BLM, as indicated above. Plates were incubated at 30 °C for 48 hours, and then photographed as was done in previous spot tests. Data are representative of 3 independent experiments.

The introduction of pYAP1 into *tpo2Δimp2Δ* and *qdr3Δimp2Δ* mutants restored completely the resistance to spermidine, as compared to *imp2Δ* carrying pYAP1, but only partially restored resistance for *tpo1Δimp2Δ* (Fig. 18).

We can reasoned that the deletion of *TPO1* into *tpo1Δ imp2Δ* that resulted in a partial resistance to spermidine, was probably provided by other efflux pumps, maybe Tpo2 and Qdr3, or possibly others with/(out) a dependence on Yap1.

The full resistance displayed by *tpo2Δimp2Δ* and *qdr3Δimp2Δ* double mutants, is mainly due to the role of Tpo1 in pumping out the drug.

These data raise the possibility that Tpo1 is indeed a potential candidate required for bleomycin and spermidine efflux, probably in the dependency of the transcriptional factor Yap1 since this has been shown to be the major determinant of polyamine (spermidine) stress resistance [166].

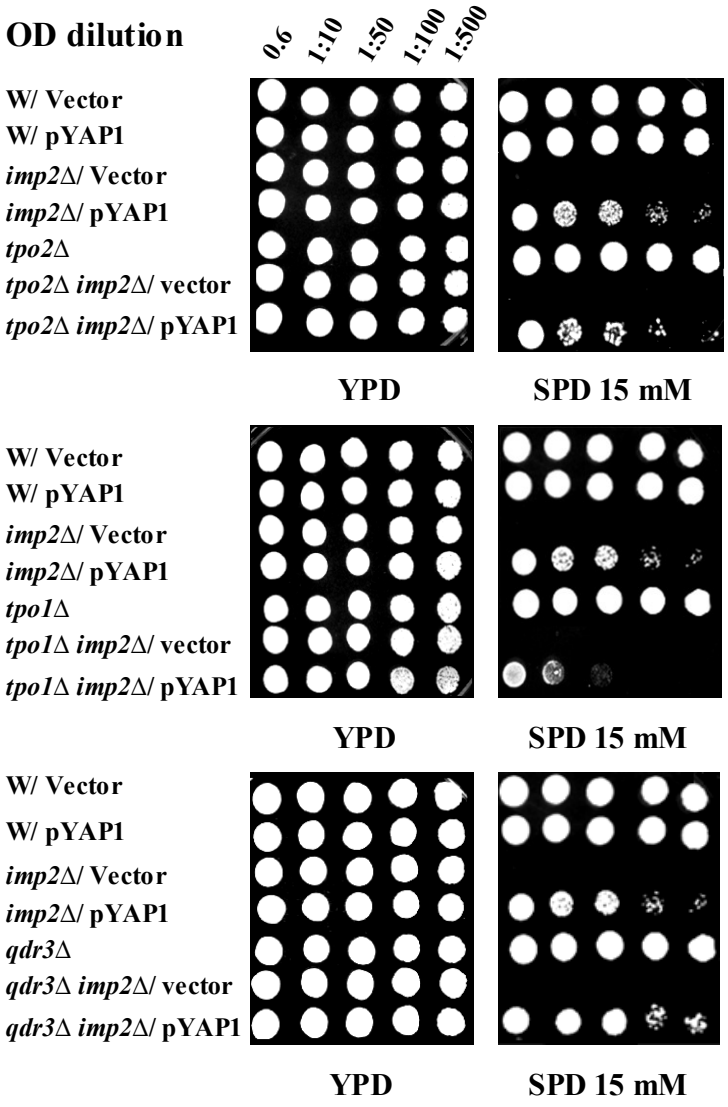


Figure 18. pYAP1 rescues partially *tpo1Δimp2Δ* but completely *tpo2Δimp2Δ* and *qdr3Δ imp2Δ*

As described in previous spot tests, diluted cells were spotted onto YPD agar media, with and without SPD and BLM. Plates were incubated at 30°C for 48 hours, and then photographed. These data are representative of 3 independent analyses.

3.5 The overexpression of Tpo1 confers resistance to *imp2Δ* mutant toward bleomycin and polyamine

If it is true that Yap1 restored both bleomycin and polyamine resistance to the *imp2Δ* mutant through the activation of *TPO1*, then one might speculate that the overexpression of Tpo1 would substitute for Yap1.

The *TPO1* gene was cloned into multi-copy plasmid pTW438 bearing the strongest promoter ADH, leading to Tpo1 overexpression. The plasmid carrying *TPO1* was then introduced into the single mutant *imp2Δ,* to check whether it would restore resistance as pYAP1 did in the previous results. As a control, we introduced an empty vector pTW438 into the same mutant. In parallel, we engineered the same construct to overexpress QDR3 as a control, since it has been found to be under the regulation of a second transcriptional factor, Yap1, besides Gcn4 when, cells are exposed to polyamines [166].

The overexpression of Tpo1 provides more resistance to *imp2Δ* in response to bleomycin and polyamines as compared to the *imp2Δ* mutant carrying p*QDR3*, and the control carrying the empty vector does not show any resistance to the drug, as it would be expected (Fig. 19). On the basis of these findings, it seems that Tpo1 efflux is more critical than Qdr3 to the detoxification process.

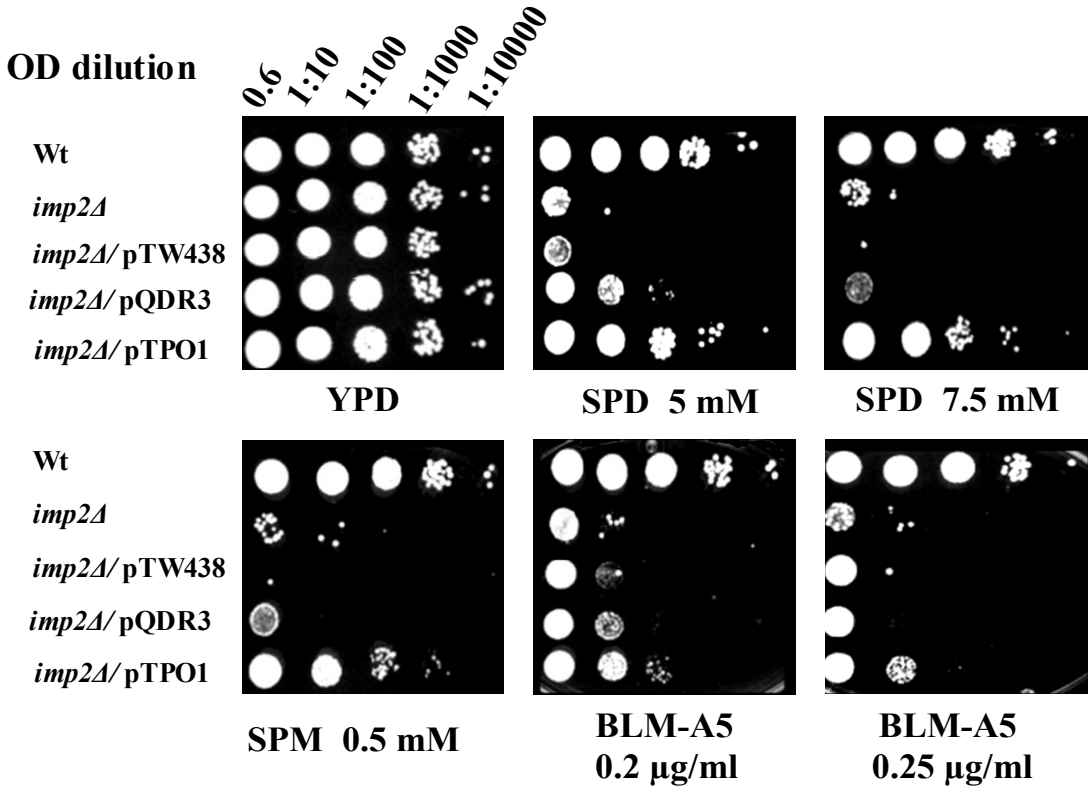


Figure 19. pTPO1 restores resistance to *imp2Δ* mutant towards drugs.
0.4 µl of diluted cells was spotted onto YPD agar media, with and without the indicated concentrations of SPD, BLM and SPM. Plates were incubated at 30 °C for 48 hours, and then photographed. These data are representative of 3 independent analyses.

We expected that in response to bleomycin, pTPO1 would confer to *imp2Δ* similar resistance to that provided by pYAP1 as shown in figure 15, but this was not the case. A first possible explanation is that the partial resistance displayed by *imp2Δ* /pTPO1 and *imp2Δ* was due to the effect of BLM-A5, which is more toxic than the 2 major components A2 and B2 of the blenoxane [175].

A second explanation could be that the endogenous polyamines might compete with the bleomycin by reducing its efflux, resulting then in the accumulation of the drug in the cell rather than its efflux and degradation. Although pTPO1 did not confer the same level of resistance, this does not exclude that *TPO1* could be dependent on Yap1 in response to bleomycin and polyamines.

Next, we tried to overexpress Tpo1 into the double mutant *tpo1Δ imp2Δ*, by using the LiAC transformation method to introduce the gene as we did before. Surprisingly no clones were grown in the media. Since we know that Imp2p is involved in maintaining cation homeostasis, including Na+ and Li+ [102], the failure of cell transformations could be due to the sensitivity of strains to lithium resulting in their growth inhibition. So we exposed the wild type, *imp2Δ*, *tpo1Δimp2Δ and qdr3Δ imp2Δ* strains to different concentrations of lithium starting from 100 mM which is the same dose used in the transformation method (material and methods).

As shown in the figure 20, strains lacking *IMP2* gene displayed resistance to 100 mM of lithium compared to the wild type, and even at high concentrations, the resistance was maintained through the expression of genes that act independently of Imp2p, such as *HAL3* and *ENA1*. This is supported by the fact that the pHal3 protein promotes Li+ resistance to *imp2Δ* by stimulating the expression of the ATPase efflux Ena1p [102]. Based on these data, it appears that lithium was not the main cause of cell growth inhibition, even after modifying the yeast transformation parameters, the result remained the same. Subsequently, we decided to use instead the dissection of tetrad as an alternative method, and check the expression of the pTPO1 by RT PCR (Fig. 21).

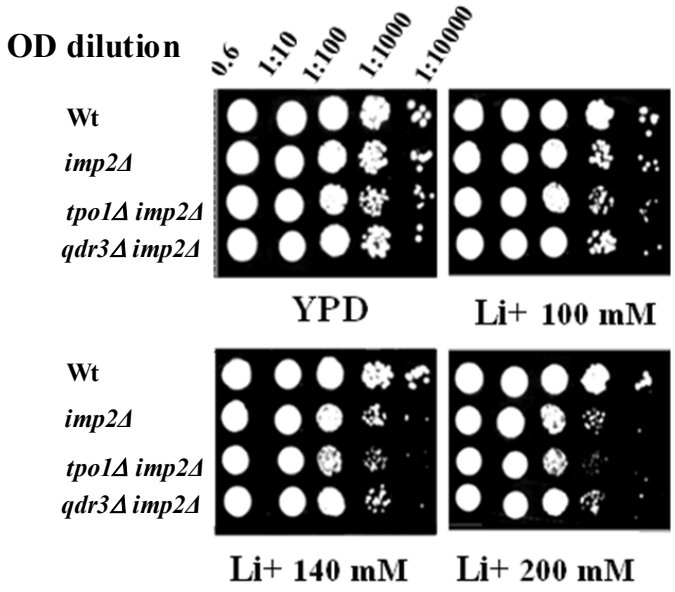


Figure 20. Lithium does not affect the phenotype of the wild type and mutants.
0.4 µl of serially diluted cells was spotted onto YPD agar media, with and without different concentrations of lithium. Plates were incubated at 30 °C for 48 hours, and then photographed.

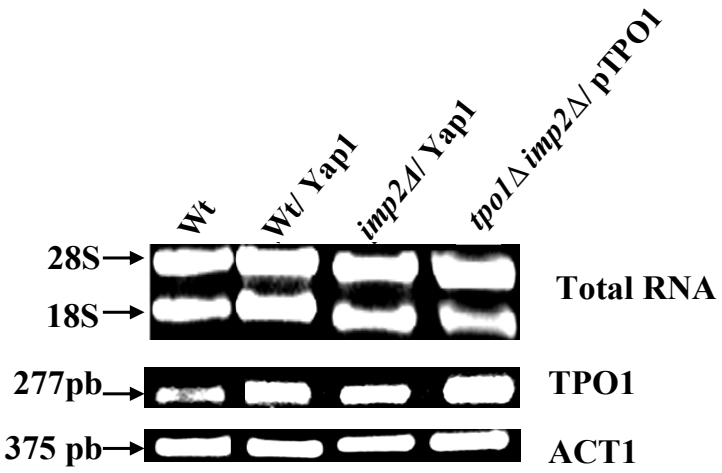


Figure 21. Expression of pTPO1 and the endogenous *TPO1* gene.
RT PCR analysis indicates the expression level of the endogenous *TPO1* compared to that overexpressed

The Introduction of the pTPO1 plasmid into the double mutant restored parental resistance to both bleomycin and polyamines compared to the wild type. In contrast, although Qdr3 is believed to be involved in SPD efflux, its overexpression did not provide resistance to *tpo1Δ imp2Δ* (Fig. 22), meaning that Qdr3 is not a potential efflux pump like Tpo1.

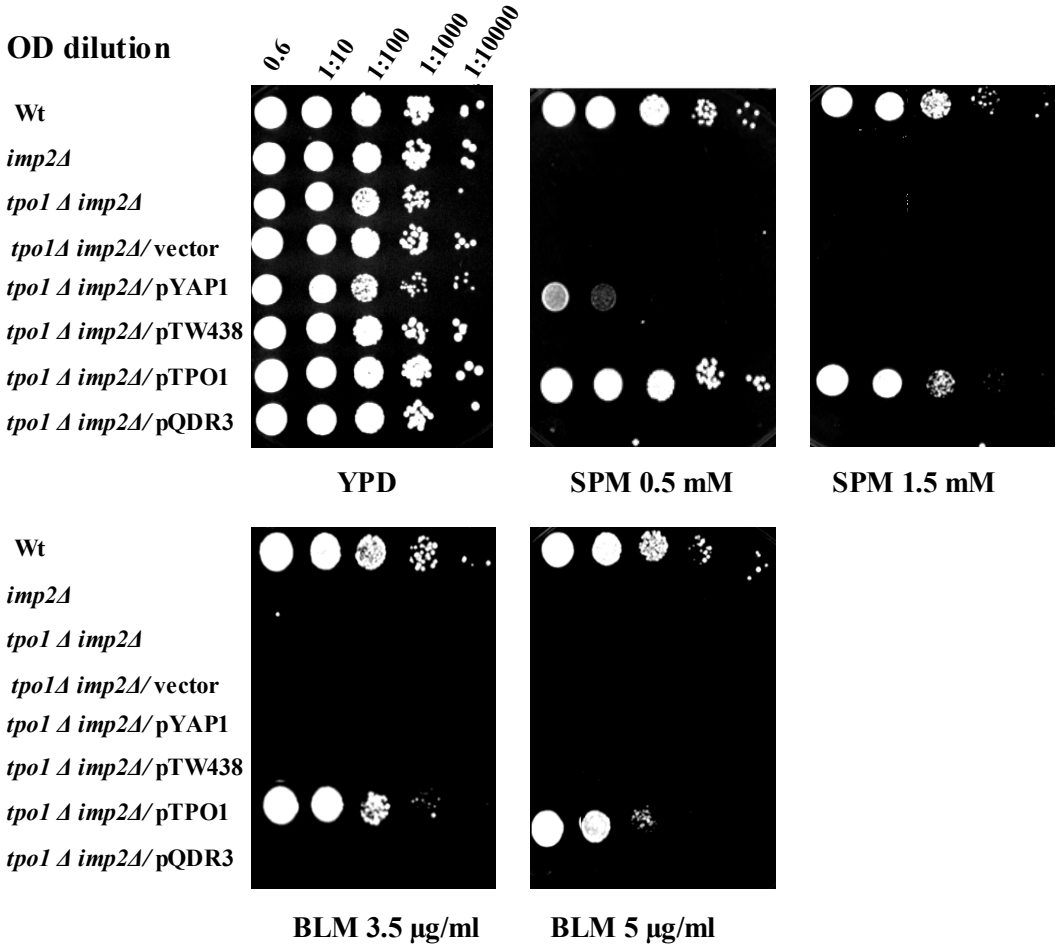


Figure 22. Overexpression of *TPO1* restored full resistance to *tpo1Δimp2Δ* towards BLM and SPD.
Cells were spotted onto YPD agar media, with and without the indicated concentrations of BLM and SPM. Plates were incubated at 30 °C for 48 hours, and then photographed. These data are representative of 3 independent analyses.

Based on the result 19, it is likely that the resistance conferred to *imp2Δ/*pQDR3 is mainly due to the expression of Tpo1, which is consistent with the result 22 showing that the overexpression of Qdr3 does not rescue *tpo1Δimp2Δ*.

In addition, we believe that unlike pYAP1, the overexpression of Tpo1 conferred parental resistance to *imp2Δ tpo1Δ* because in this case, Tpo1 could be located in both plasma and vacuolar membranes as it has been demonstrated previously [150]. This means that the drug would not only be pumped out from the cell, but it would be also degraded into the vacuole once it is transported across the vacuolar membrane by Tpo1, resulting in higher resistance towards bleomycin and polyamines.

We postulated that Tpo1 behaves as a polyamine efflux that is dependent on Yap1 in stressful conditions; however there is no evidence indicating that it is the only transcription factor required. We found that cells lacking the YAP1 gene exhibited parental resistance to spermine as well as bleomycin (Fig. 23). This is contradictory to recent data showing that the same cells were not resistant in the way we have shown, but instead they were extremely sensitive to higher concentrations of polyamines (4mM spermine and 10 mM spermidine) [166]. If Yap1 was the only factor required fot *TPO1*, *yap1Δ* mutants would be sensitive not only to high doses, but also to low doses of polyamines, and since no data have shown this, we suggest that other transcriptions factors could be involved in the oxidative stress besides Yap1.

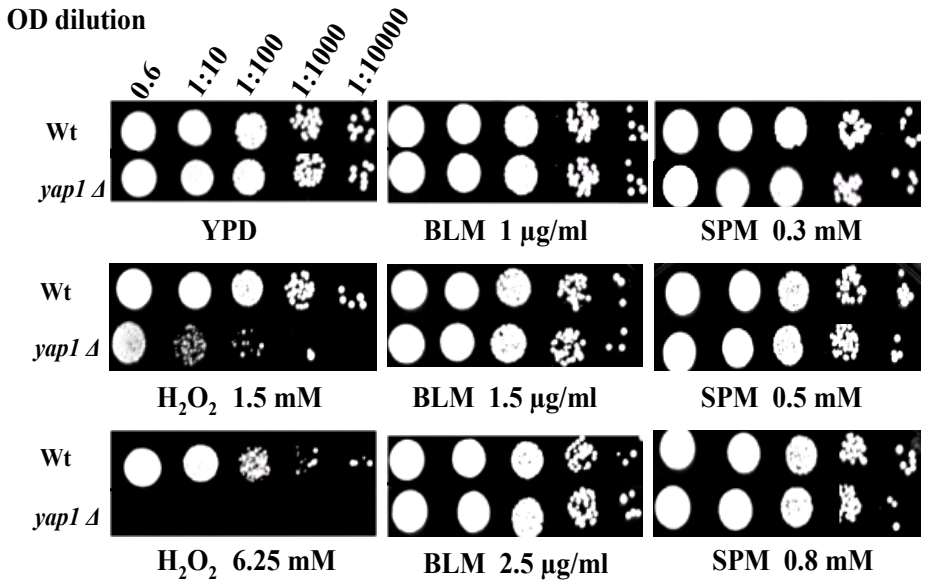


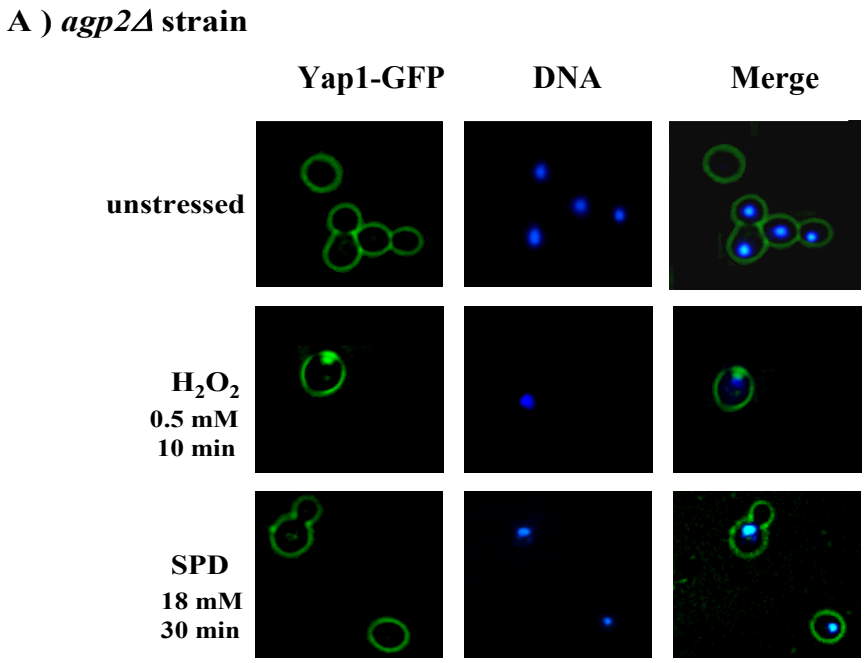
Figure 23. Cells lacking the *YAP1* gene displayed parental resistance towards BLM and SPM.
Exponentially growing cells were serially diluted, then 0.4 µl of cells were spotted onto YPD agar media, containing different concentrations of BLM, SPM and, as a drug control, H_2O_2. Plates were incubated at 30 °C for 48 hours, and then photographed.

3.6 Yap1-GFP does not localize to the nucleus of *agp2Δ* in response to spermidine

In a separate experiment, Yap1 was overexpressed as a GFP fusion protein into the wild type, *imp2Δ and agp2Δ* in order to monitor its cellular localization in response to spermidine. Since it has been reported that Yap1-GFP accumulates in the nucleus of yeast cells after 30 min of spermidine exposure [166], we decided to check its localization in response to the same conditions and as a control, we treated cells with 0.5 mM of H_2O_2 for 10 min [176].

As shown in figures 24B-C, under spermidine stress, Yap1-GFP is mainly located in the nucleus of *imp2Δ and* wild type strains. The same results were observed in response to H_2O_2. In contrast, we noticed that no Yap1-GFP protein was detected in *agp2 Δ* in response to spermidine, and in response to H_2O_2 few Yap1-GFP were detected (Fig. 24-A).

Because of the deletion of *AGP2* resulting in a decrease of spermdine uptake, Yap1-GFP cannot be located in the nucleus in response to spermidine treatment, as long as the polyamine content is maintained. Instead, Yap1 would accumulate in the cytoplasm. These finding arise the possibility that Agp2 might be related to Yap1.



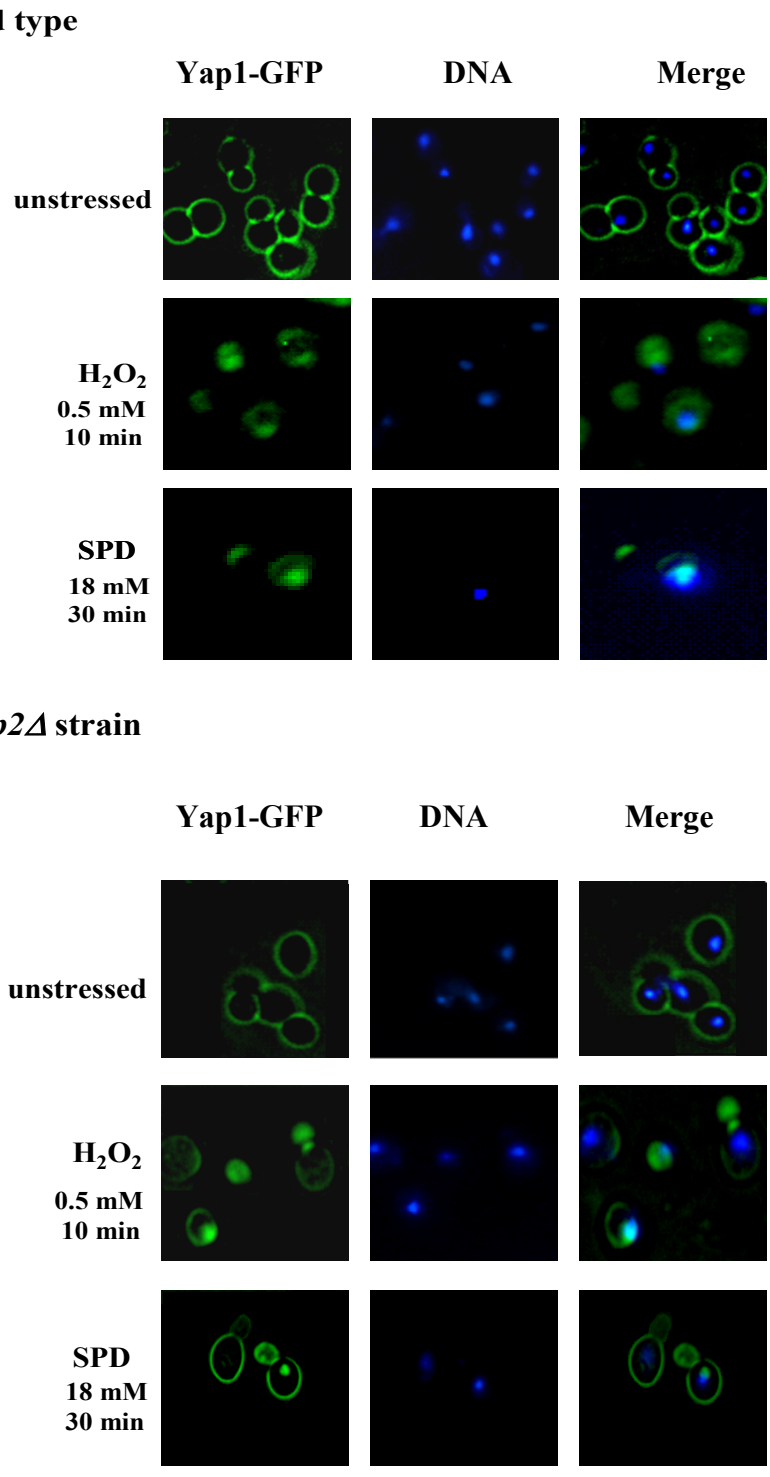


Figure 24. Cellular localization of Yap1-GFP in reponse to H_2O_2 and spermidine in the wild type, *imp2Δ* and *agp2 Δ*.
Cells transformed with a plasmid bearing YAP1-GFP were treated (or not) with 0.5 mM H_2O_2 for 10 min and 18 mM of spermidine for 30 min. Transformed cells are presented in this order: A) *agp2 Δ* mutant; B) Wild type; C) *imp2Δ* mutant.

In addition, we performed an RT PCR (Fig. 25) to check the level of expression of Agp2 in strains overexpressing *YAP1*.

Interestingly, strains harboring pYAP1 showed an important expression level of Agp2 under normal condition as compared to the wild type. A possible explanation is that as Agp2 imports endogenous polyamine molecules, the cell becomes concentrated in polyamine. This excess would therefore stimulate Yap1 to activate the polyamine efflux, in order to regulate the polyamine homeostasis. Although Yap1 is not a transcription factor required for *AGP2*, the relation between Agp2 and Yap1 needs to be further explored.

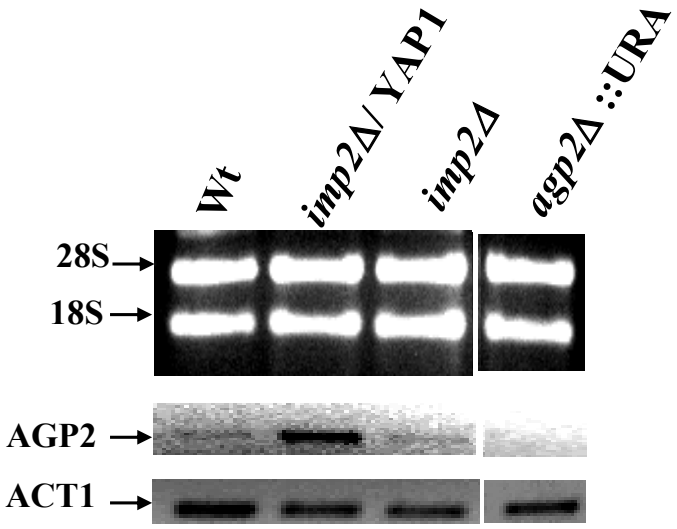


Figure 25. Expression of endogenous *AGP2* gene.
RT PCR analysis indicates the expression level of the endogenous *AGP2* in cells overexpressing *YAP1* compared to the wild type and *imp2Δ*.

Chapter 4: Discussion

4 Discussion

In this study, we used the powerful genetic system of *saccharomyces cerevisiae* as a tool to search for determinants of resistance to bleomycin and to characterize the molecular transport machinery of Tpo1.

TPO1 encodes a yeast plasma membrane multi-drug efflux pump that belongs to the major multi-facilitator superfamily (MFS) [89]. As shown in the result section, Tpo1 is mainly required to strengthen the polyamines as well as bleomycin.

Herein, the purpose of using this efflux pump is that because we know that bleomycin includes a spermidine substituent that has an affinity to polyamine efflux pumps including the well characterized pump Tpo1. However, it should be noted that this finding is not a reason to exclude the other efflux pumps such as Qdr3 and Tpo2. Thus, we proceeded by mutating each one of these pumps and then assessed whether *tpo1Δ*, *tpo2Δ*, or *qdr3Δ* mutants would be sensitive to bleomycin or not. Interestingly, all the mutants exhibited parental resistance similar to the wild type towards bleomycin and spermidine except *tpo1Δ,* which was sensitive only to bleomycin. These results suggest that Tpo1 might be a specific efflux pump that is required for expelling bleomycin out of the cell, even if it is enough to maintain alone the optimal polyamine concentration without the contribution of other pumps.

To verify this, we first looked for a gene that when deleted, induces sensitivity to bleomycin in *qdr3Δ*, *tpo1Δ* and *tpo2Δ* mutants. In this way, we would be able to check whether the bleomycin resistance would be restored by overexpressing the gene *TPO1*. Recent findings showed that the deletion of *S. cerevisiae IMP2* gene, which encodes a transcriptional co-activator, (required for mediating cellular resistance to oxidative DNA damaging agents) induces hypersensitivity to bleomycin by 15-fold [93].

Based on this finding, we used *IMP2* as a tool to make double mutants. Our data revealed that when *TPO1*, *TPO2* and *QDR3* are mutated in combination with *IMP2*, cells become extremely sensitive to bleomycin and polyamines, as well as the *imp2Δ* mutant alone. This led us to propose that Imp2 might regulate the expression of an efflux pump whether or not it is the only Tpo1. In contrast with this hypothesis, other reports suggest that the hypersensitivity exhibited by the *imp2Δ* mutant towards bleomycin, does not result from the deficiency of efflux pumps expression [94-100, 131] as we thought. Nor is it a consequence of a defect in antioxidant activity [101], and it is not due to unrepaired DNA strand breaks [40], since *imp2Δ* mutants are known to be insensitive to ionizing radiations and MMS [92, 93].

As we know, Imp2 controls several genes under stress conditions, so a plausible mechanism is that Imp2 might control the expression of target genes that in turn, regulate the expression of these efflux pumps. This might explain why the *imp2* null mutant uptakes more labelled spermidine during the time course (0-2-4 min) in the excretion spermidine assay, while the wild type uptakes less during the first 4 min

and then starts expelling the H^3-spermidine in the next 2 min. This is probably due to the lack of a gene expression that normally requires Imp2 as a regulator.

Over the years, many genes were identified by their ability to bind and activate efflux pumps, by enhancing cell resistance to stress conditions, among them; Yap1 remains the most known activating factor. Yap1 is a transcriptional factor involved in oxidative stress [118, 119, 123]. It regulates the expression of up to 71 genes [126, 127] including efflux pumps genes –such as *FLR1, YCF1, SNQ2* and *PDR5*- [94, 131, 177] upon exposure to stress. Further analysis demonstrated that Yap1 found in multicopy transformants, confers resistance to the iron chelators 1,10-phenantroline and 1-nitroso-2-naphtol, to cycloheximide [178], as well as the anticancer drug bleomycin.

According to these findings, we suspected that Yap1 could be probably the major regulator of *TPO1*, *TPO2* and *QDR3* genes, and if it is the case, it would restore bleomycin resistance to double mutants. Indeed, Yap1 restored BLM and spermidine resistance to *tpo2Δimp2Δ* and *qdr3Δimp2Δ* double mutant, except for *tpo1Δimp2Δ* that showed partial resistance to the drug.

Based on the Yeastract database, the *TPO1* gene includes in its promoter region three potential Yap1p response elements (YREs) [159] to which Yap1 binds, in order to activate the gene expression. Althoug *QDR3* and *TPO2* also include YRE within their promoter region, we reasoned that Tpo1 could be a major efflux pump required for Yap1 to restore drug resistance, since we believe that the resistance observed in both *tpo2Δimp2Δ* and *qdr3Δimp2Δ* resulted from the activation of *TPO1* via Yap1.

If we assume that Yap1 restores bleomycin and polyamine resistance to the double mutants through the activation of *TPO1* gene, then we expect that the overexpression of Tpo1 would substitute for Yap1. With this in mind, we designed a multi-copy plasmid pTPO1 in such a way to express *TPO1* as a gene tagged with MYC tag in its N terminal. Spot test analysis revealed that the expression of the Myc-Tpo1 fusion protein in the *imp2Δ* mutant did not confer resistance to bleomycin and polyamines (data not shown) as we were expecting. This could be explained by the targeting of Tpo1 to the vacuolar membrane when it is overexpressed and thus, stimulates the uptake of polyamines and BLM into the vacuole for detoxification.

It has been reported that N-terminal of Tpo1 includes 2 major amino acids – serine [19] and threonine[52] – that are essential for the regulation of Tpo1. The phosphorylation of Ser [19] and Thr [52] is necessary for enhancing the activity of Tpo1 [150]. So, as it was tagged with Myc protein in its N-terminal, Ser [19], Thr [52] would not be phosphorylated. Therefore, as far as the drug is transported into the cell, it will accumulate causing toxicity, and that explains why *imp2Δ* mutant overexpressing the tagged Tpo1 was extremely sensitive. Additionally, we decided to use an untagged *TPO1* as long as it does not interfere with the function. When pTPO1 is overexpressed, it restored bleomycin and polyamine resistance to the *imp2Δ* mutant while in *tpo1Δimp2Δ*, it conferred parental resistance.

Because Tpo1 is located in both plasma and vacuolar membrane once it is overexpressed [150], it expels the bleomycin out of the cell, then the drug is transported across the vacuolar membrane via Tpo1 and gets degraded into the vacuole. This explains why at high doses of bleomycin, pTPO1 was still rescuing the

double mutant. Similar results would be observed in the single mutant if we used BLM instead of BLM-A5.

It is noteworthy that unlike pTPO1, Yap1 overexpression conferred resistance but not as much as the overexpressed Tpo1 does. Recent reports showed that when *TPO1* is overexpressed in a single copy plasmid, it is found mainly on the plasma membrane [150]. This correspond to the normal level of Tpo1 expression in the cell once it get activated, however, by using a multi-copy plasmid, Tpo1 is located on the plasma membrane and also the vacuolar membrane, so the more drugs entering the cell, the more are expelled. In turn there is transportation into the vacuole and degradation. This explains why double mutants were extremely resistant to the drug when Tpo1 was overexpressed.

Since the resistance was restored in double mutants by overexpressing either Tpo1 or Yap1, we can conclude that Tpo1 is required for the detoxification process in dependency of Yap1p. This was supported by a recent study showing that the deletion of *YAP1* gene induced a hypersensitivity to high doses of polyamines [166], in other word, the efflux pump Tpo1 would not be activated in cells lacking the transcriptional factor Yap1. This result was contradictory with our previous data, showing that *yap1Δ* mutants become resistant in response to polyamine and bleomycin rather than sensitive, suggesting that other transcription factors might be required to confer resistance to polyamine and bleomycin besides Yap1.

In the cell, under stressed conditions, we believe that Yap1 plays its role by providing resistance to oxidative stress, in the dependency of Agp2 protein. This hypothesis is consistent with the microscopy assay result showing that in response to spermidine, none of Yap1-GFP proteins were detected in the nucleus of *agp2Δ* compared to the wild type, meaning that as long as the polyamine is not imported into the cell because of the lack of *AGP2* gene, Yap1 cannot not be induced, and therefore it would not be redistributed in the nucleus.

In another word, when cells are treated with bleomycin and polyamines, Agp2 transports the drug across the yeast plasma membrane and gets inside. Each one molecule of bleomycin caused damage in a single DNA molecule [179, 180] and generated free radicals, resulting in oxidative stress. Under these conditions, Yap1 is transported from the cytoplasm to the nucleus, binds to the specific motif TGACTAA on the promoter of *TPO1* and subsequently activates the expression of the gene encoding the efflux pump Tpo1 that in turn expels efficiently the drug.

It cleary appears that Agp2 and Yap1 work dependently to maintain polyamine homeostasis when cells are challenged to oxidative stress. Surprisingly, the same observation was noted in the case of non-stressful conditions as shown in the RT PCR result. As we overproduced Yap1 in the wild type (not shown) and the single mutant *imp2Δ* (Fig.25), the expression level of Agp2 became enhanced. A possible explanation could be that Agp2 requires Yap1, even if it is not its transcription factor (YEASTRACT database). Therefore under normal conditions, the endogenous polyamine levels are tightly regulated by their influx and efflux across the plasma membrane, so when their level is low, Agp2 transports polyamines into

the cell in order to keep the intracellular concentration of polyamines optimal, however, as long as Agp2 keeps importing these compounds to the cell, their level become high even toxic, in this case, cells would be subjected to an oxidative stress, that in turn induces the Yap1 activity. Once Yap1 is stimulated, it will activate the expression of specific efflux pumps able to expel the excess of polyamines out of the cell.

According to our recent data mentioned above and our interpretations, Yap1 might be involved in the regulation of Agp2 in such a way that when Agp2 transports more bleomycin or polyamines to the cell, Yap1 gets involved to negatively control the *AGP2* in order to prevent toxicity. Such a possibility needs to be further investigated.

5 Conclusion

In this work, we demonstrated that unlike Tpo2 and Qdr3, Tpo1 is a major MFS-MDR protein required for the polyamine and bleomycin detoxification process, with dependency on Yap1, and perhaps other transcription factors also.

When cells are exposed to bleomycin or to high doses of polyamines, Yap1 binds to its specific region onto the *TPO1* gene, and then activates its transcription, resulting in the overexpression of the efflux proteins, that in turn, pump out either the toxic bleomycin or the excess of the exogenous polyamines (Schema 27) in order to maintain an optimal concentration of polyamine inside the cell.

The identification of Tpo1 as a potential candidate has emerged from our results showing that when Tpo1 is overexpressed, it confers resistance to *imp2Δ* as well as to the double mutant. However this does not exclude the fact that other efflux pumps might contribute to the efflux process in order to maintain cell homeostasis. Even if Qdr3 did not provide any resistance, its role could be minor compared to other efflux pumps. In addition, no evidence indicates that Tpo1 functions as a polyamine transporter only under the regulatory control of the transcription factor Yap1. Although we have shown that the overexpression of Yap1 provides resistance to *imp2Δ* and double mutants, by acting on the *TPO1* gene, Yap1 seems not to be the only factor involved.

When we deleted the gene *YAP1* in a separate experiment, we found that cells become resistant rather than sensitive, suggesting that other transcription factors are required to confer resistance besides Yap1.

Under normal conditions, Yap1 is found in the cytoplasm, whereas in response to oxidative stress caused either by H_2O_2 or a high dose of spermidine, it localizes in the nucleus of the wild type and *imp2Δ* mutant strains, but not in the *agp2Δ* mutant. Since Agp2 is known to behave as a polyamine and bleomycin transporter, it seems logical to expect that when deleted, no exogenous polyamines enter to the cell, thus the intracellular concentration of polyamine remains optimal and none influenced. Such conditions, similar to unstressed conditions, do not stimulate Yap1 expression.

All of this has helped to a better understanding of the functioning of Yap1 and Tpo1 in response to bleomycin. Although no *TPO1* homologue has been identified in human cells as a bleomycin efflux, other efflux pumps could behave similarly to Tpo1. Once they are activated and overexpressed, they would decrease the intracellular concentration of chemotherapeutic agents by limiting their cytotoxic effect on healthy cells as well as cancer cells, these latters would continue growing instead of dying, and consequently invade other healthy tissue, and this explains why cancer cells are resistant to anti-cancer agents.

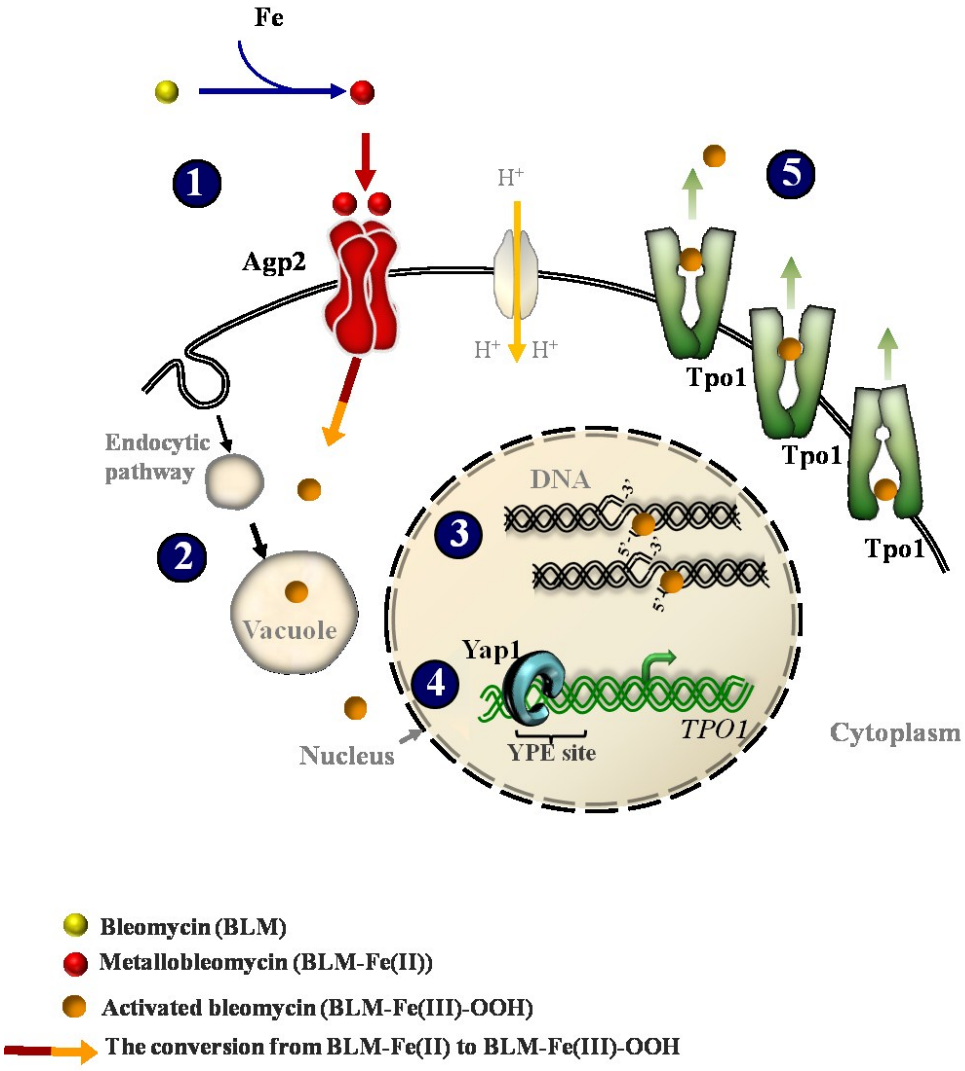


Figure 26. Predicted Model showing how the resistance to bleomycin can occur

1) When cells are subjected to bleomycin treatment, Agp2 (polyamine and bleomycin transporter) transports the drug (metallobleomycin BLM-Fe^{2+}) in the cell. In the presence of oxygen, the mettalobleomycin is converted to an activated form (BLM-Fe^{3+}-OOH). **2)** The drug is then sequestreted into the vacuole as a mechanism of detoxification [181, 182]. **3)** Once accumulated, some bleomycin molecules leak to the cytoplasm [183], and then reach the nucleus where their DNA target is located. Each molecule of bleomycin caused damage in a single DNA molecule [179, 180] and generated free radicals resulting in oxidative stress. **4)** Under this stress condtion, Yap1 is transported from the cytoplasm to the nucleus, and then activates the expression of *TPO1* gene, by binding to its YPE site located on the promoter of *TPO1* gene. **5)** The activation of *TPO1* transcription leads to the overexpression of Tpo1 efflux pumps, which will pump the drug out of the cell.

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