A PROTEOMIC ANALYSIS OF THE EFFECTS OF YESSOTOXIN AND DI-DESULFOYESSOTOXIN ON YEAST AND MAMMALIAN CELLS

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“You can’t rush science, Gibbs. You can yell at it and scream at it, but you can’t rush it.”

~ Abby Sciuto; NCIS season 1, episode 14

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"Perseverance conquers all."

ABSTRACT

Yessotoxin (YTX) is a disulfated polycyclic polyether, produced by dinoflagellate algae. It is known to accumulate in edible shellfish, raising concerns about its potential risk to human health. YTX was initially classified as a diarrhetic shellfish poisoning toxin, due to commonly being extracted alongside toxins of this variety. However, YTX does not induce any of the effects characteristic of this group. A separate category for YTXs was established by the European Commission in 2002 and a limit of 1 mg/kg of shellfish meat was established.

YTX has been shown to be an apoptosis inducer in a variety of cell lines *in vitro*. It has also been shown to be lethal to mice when administered by intra-peritoneal injection. However, when administered orally only limited toxicity is observed. The di-desulfated derivative (dsYTX) has also been shown to be lethal to mice following intra-peritoneal injection. However it causes damage mainly to the liver, whereas YTX appears to target the heart. The mechanism of action of YTX is still unknown.

The goals of this project were to use proteomic techniques, to examine the effects of YTX and dsYTX on *Saccharomyces cerevisiae* and human promyelocytic leukemic blood leukocyte (HL60) cells. Young *et al.* (2009) showed that the major proteins affected by YTX in HepG2 cells were heterogeneous ribonucleoproteins (hnRNPs), lamins, cathepsins and heat shock proteins. HnRNPs had not previously been identified as possible targets of YTX. Alterations of hnRNP levels were also seen in HL60 cells treated with microtubule stabilising agents, peloruside A or paclitaxel (Wilmes *et al.*, 2011, 2012).

No differences in cell morphology or significant changes in protein abundance were observed when *S. cerevisiae* cells were exposed to YTX. A small number of significant changes in abundance were detected when these cells were exposed to dsYTX. The small number of protein changes seen is possibly due to poor toxin entrance into the cell through the yeast cell wall, lack of protein targets structurally homologous to those found in mammalian cells, or fast removal of the toxin through export pumps.

Twenty-four hour incubation of HL60 cells with YTX resulted in increased cell death but no change in cell morphology. Treatment with dsYTX caused cells to aggregate into clusters and a 24% decrease in the number of live cells. Increases were found in the abundance of β -actin, hnRNP A and BiP proteins in response to dsYTX treatment. Decreases in these proteins were seen in HepG2 cells treated with YTX for 24 hours. As seen in *S. cerevisiae* cells, dsYTX had a greater effect in HL60 cells compared with YTX.

Overall, the results provide some support for the previously identified effect on hnRNPs in mammalian cells exposed to YTX.

ABBREVIATIONS

1DE	one-dimensional gel electrophoresis
2DE	two-dimensional gel electrophoresis
ABC	adenosine triphosphate binding cassette
BiP	binding immunoglobulin protein
BSA	bovine serum albumin
b.w.	body weight
CHCA	α -cyano-4-hydroxycinnamic acid
DIGE	differential in gel electrophoresis
DSP	diarrhetic shellfish poisoning
dsYTX	di-desulfoyessotoxin
DTT	dithiothreitol
DTX	dinophysistoxin
FCS	fetal calf serum
FDR	false discovery rate
GRP	glucose-regulated protein
hnRNP	heterogeneous nuclear ribonucleoprotein
HSP	heat shock protein
IAA	iodoacetamide
IEF	isoelectric focusing
i.p.	intraperitoneal

IPA	Ingenuity Pathways Analysis
LC-MS	liquid chromatography – mass spectrometry
LSP	ladder-shaped polyether
MALDI-TOF MS	matrix assisted laser desorption/ionisation – time of flight mass-spectrometry
MALDI-TOF/TOF MS/MS	matrix assisted laser desorption/ionisation - time of flight tandem mass spectrometry
MTX	maitotoxin
OA	okadaic acid
PARP	poly(ADP-ribose)polymerase
PBS	phosphate buffered saline
Pel A	peloruside A
PI	propidium iodide
PTP	permeability transition pore
PTX	paclitaxel
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TCR	T-cell receptor
TFA	trifluoroacetic acid
YPD	yeast peptone dextrose
YTX	yessotoxin

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1 INTRODUCTION

1.1 Yessotoxins

Yessotoxin (YTX) is a disulfated polycyclic polyether (Fig. 1), produced by dinoflagellate algae. It was first isolated from the digestive glands of the scallops *Patinopecten yessoensis* collected in Japan (Murata *et al.*, 1987). YTX was originally classed as a diarrhetic shellfish poisoning toxin (DSP) as it is often extracted with DSP toxins such as okadaic acid (OA) and dinophysistoxin (DTX) (Fig. 2). Subsequent studies have shown that it does not display any of the typical DSP characteristics, such as inducing diarrhea (Murata *et al.*, 1987, Ogino *et al.*, 1997) or inhibition of protein phosphatase 2A (PP2A) (Ogino *et al.*, 1997). In 2002 a separate group was established for YTXs by the European Commission (directive 2002/225/EC). The presence of YTX in New Zealand mussels was first detected in 1997 (Yasumoto *et al.*, 1997). The current limit of YTXs allowed in shellfish meat for human consumption is 1 mg YTXs/kg of mollusc (Regulation (EC) No. 853/2004). The exact mechanism of action of YTX has yet to be determined.

1.2 Algal production and shellfish metabolism

There are currently three species of dinoflagellate algae confirmed to be producers of YTX. These are: *Protoceratium reticulatum*, *Lingulodinium polyedrum* and *Gonyaulax spinifera*. A phylogenetic analysis found that all YTX producing species of dinoflagellate belong to the order *Gonyaulacales* (Howard *et al.*, 2009). However, there is evidence that some analogues of YTX are produced by metabolism of the toxin by the shellfish that feed on toxic algae as opposed to being produced by the algae themselves. Cultures of *P. reticulatum* have been found to contain YTX, but not 45-hydroxy-YTX (45-OH-YTX), 45, 46, 47-trinor YTX or homo-YTX (Fig. 1) (Satake *et al.*, 1997). Another study using LC-MS found indications of 93 YTXs in extracts from *P. reticulatum* (Miles *et al.*, 2005).

In contaminated Greenshell™ mussels (*Perna canaliculis*) collected around the coast of the upper South Island of New Zealand in 2001, YTX was found to be the major toxin

present. 45-OH-YTX was also detected although at a lesser concentration (Mackenzie *et al.*, 2002). There was also evidence from liquid chromatography work, in the same study, for the presence of three other YTXs, thought to be most likely carboxy-YTX, a hydroxylated carboxy-YTX analogue and aldehyde metabolite intermediates (Fig. 1). YTX was still detectable in the shellfish meat for some time after exposure to toxic algae ended, taking approximately 100 days to decline from an initial concentration of 326 µg/100 g of shellfish meat, to the EU limit of 100 µg/100 g. This gives an estimated half-life of the toxin in Greenshell™ mussels of about 49 days. Interestingly while the amount of YTXs decreased over time, the ratio of 45-OH-YTX to YTX remained constant ($12.3\% \pm 3.3\%$).

A study of Norwegian Blue mussels (*Mytilus edulis*) (Aasen *et al.*, 2005), found that the main toxin present was carboxy-YTX, with YTX being only a minor component, except for the time just after an algal bloom. Half-lives for the YTXs detected in the mussels were determined to be 20 days for YTX, 22 days for 45-OH-YTX, 60 days for carboxy-YTX and 33 days for 46-OH-carboxy-YTX. This led to the conclusion that in Norwegian Blue mussels, YTX was converted rapidly to carboxy-YTX and, more slowly, to 45-OH-YTX.

YTX was found to be the dominant toxin in contaminated French mussels (*Mytilus galloprovincialis*). The detoxification profile was similar to that of the Norwegian blue mussels (Amzil *et al.*, 2008). Toxicology profiles from the French mussels indicated that YTX was being converted to homo-YTX as well, as this analogue had a very low initial concentration which increased during the detoxification stage.

Pacific oysters (*Crassostrea gigas*) were found to undergo a much faster detoxification of YTXs than mussels (*M. edulis*) (Roder *et al.*, 2011). Four days after feeding on YTX producing algae ended, *C. gigas* contained only approximately 15% of the initial amount of YTX and < 5% of the initial amount of 45-OH-YTX. Two mono-desulfated analogues were detected in both shellfish, one of which was found to increase during the feeding and detoxification stages, indicating that it is possibly an end product of the metabolism process.

Name	R ₁	R ₂	R ₃	n
Yessotoxin (YTX) ^a	SO ₃ Na	SO ₃ Na		1
Homo-YTX ^b	SO ₃ Na	SO ₃ Na		2
Desulfo-yessotoxin (dsYTX) ^c	H	H		1
Carboxy-YTX ^d	SO ₃ Na	SO ₃ Na		1
Carboxy-homo-YTX ^d	SO ₃ Na	SO ₃ Na		2
1-desulfo-carboxy-homo-YTX ^d	H	SO ₃ Na		2
4-desulfo-carboxy-homo-YTX ^d	SO ₃ Na	H		2
45-hydroxy-YTX (45-OH-YTX) ^b	SO ₃ Na	SO ₃ Na		1

Figure 1 The chemical structure of yessotoxin (YTX), its di-desulfated derivative (dsYTX) and selected other naturally occurring derivatives.

^a Young *et al.*, 2009; ^b Dominguez *et al.*, 2010; ^c Inoue *et al.*, 2003; ^d Ciminiello *et al.*, 2007

Figure 2 Chemical structures of toxic polycyclic polyethers produced by dinoflagellates. **A** okadaic acid (OA), **B** brevetoxin B, **C** pectenotoxin 1, **D** ciguatoxin, **E** dinophysistoxin 1 (DTX 1), **F** yessotoxin (YTX), **G** maitotoxin (MTX), **H** azaspiracid 1 (AZA 1)

¹ Dominguez *et al.*, 2010; ² Inoue *et al.*, 2007; ³ García *et al.*, 2005; ⁴ Young *et al.*, 2009; ⁵ Nicolau *et al.*, 2010; ⁶ Evans *et al.*, 2007

1.3 Yessotoxin

1.3.1 *In vivo* effects

1.3.1.1 Lethality

YTX has been found to be lethal over a range of dose levels in murine studies, but only when administered by intraperitoneal (i.p) injection (Table 1). The differences in LD₅₀ values between these studies may be due to the different strains and sexes of mice used (Aune *et al.*, 2008). LD₅₀ values may also be influenced by mice fasting or receiving food *ad libitum* prior to YTX treatment. Death following YTX administration appears to always occur within 24 h or not at all (Terao *et al.*, 1990; Ogino *et al.*, 1997; Aune *et al.*, 2002; Tubaro *et al.*, 2003; Franchini *et al.*, 2004).

Table 1 LD₅₀ values by i.p injection found in mice. * Doses that are lethal but not necessarily an LD₅₀

Study	Strain	Sex	LD ₅₀ (µg/kg)
Murata <i>et al.</i> , 1987	-	-	100*
Terao <i>et al.</i> , 1990	IRC	M	286 (at 3 h)
Ogino <i>et al.</i> , 1997	ddY	M	80-100*
Tubaro <i>et al.</i> , 2003	CD-1	F	512
Aune <i>et al.</i> , 2008	ICR (CD-1)	F	380
		M	462
	Swiss (CFW-1)	F	269
		M	328
	NMRI	F	314
		M	412

Oral administration of YTX is non-lethal in mice, even at doses as high as 1 - 10 mg/kg body weight (b.w) (Ogino *et al.*, 1997; Tubaro *et al.*, 2003 and 2008; Aasen *et al.*, 2011) - more than 10 times the i.p. lethal dose. This may be due to YTX being unable to cross membrane barriers and therefore being poorly absorbed following oral ingestion.

However YTX has been detected in the blood of mice at a concentration of 3.12 ng/mL, following repeated oral administration (Tubaro *et al.*, 2008).

YTX is structurally similar to brevetoxin B (Fig. 2B) which is lethal to zebra fish (*Brachydanio rerio*) at a concentration of 18 nM (Lin *et al.*, 1981). YTX was not found to be lethal to killifish (*Oryzias latipes*) at a concentration of 420 nM or 840 nM, within 24 h (Ogino *et al.*, 1997).

1.3.1.2 Symptoms

Mice appeared to behave normally for the first hour or so following i.p administration of YTX (Terao *et al.*, 1990). They then appeared restless, displayed jumping and muscle cramps, before dyspnea set in followed by death (Terao *et al.*, 1990, Aune *et al.*, 2002, Tubaro *et al.*, 2003, Franchini *et al.*, 2004a).

No difference in behaviour from controls was observed in mice following oral administration of YTX (Ogino *et al.*, 1997, Aune *et al.*, 2002, Tubaro *et al.*, 2003)

1.3.1.3 Morphological and histological analysis

In mice, YTX appears to mainly affect the heart (Terao *et al.*, 1990; Aune *et al.*, 2002; Tubaro *et al.*, 2003 and 2008), but also causes damage to the intestine and some areas of the brain (Franchini *et al.*, 2004a and 2004b). In the heart it causes rounding and swelling of myocardial cells, rounding of the mitochondria and disrupts the organisation of myofibrils, particularly in the region around the capillaries. Little morphological or histological changes have been observed in the intestine, other than an apparent inflammation response observed in the duodenum (Franchini *et al.*, 2004b). YTX does not cause fluid accumulation in the intestine of suckling mice (Murata *et al.*, 1987; Ogino *et al.*, 1997), unlike DSP toxins such as OA.

In the brain, disruption of neuron arrangement and shrinkage and increased hematoxylin and eosin staining of the cytoplasm of Purkinje cells was observed following i.p administration of a lethal dose of YTX (Franchini *et al.*, 2004a). An increased amount of S-100 protein and a decreased amount of calbindin D-28K, both Ca^{2+} binding proteins, was also observed. Decreased amounts of β -tubulin,

neurofilaments and neurotubules, and disruption to the organisation of these proteins were also detected. The authors postulated that damage to the cytoskeleton of Purkinje cells, was due to the action of increased amounts of S-100 protein. Changes to the cell organisation and an increase in the number of apoptotic cells were also seen in the thymus following i.p administration of lethal and sub-lethal doses of YTX (Franchini *et al.*, 2004b). The same study also found an increase in the number of cells positive for the cytokines IL-6 and TNF- α , and a decrease in the number of IL-1 α AND IL-8 positive cells.

No changes (Ogino *et al.*, 1997, Aasen *et al.*, 2011) or changes similar to, but less pronounced than, those seen following i.p injection (Aune *et al.*, 2002, Tubaro *et al.*, 2008) were observed with oral administration of YTX.

1.3.2 *In vitro* effects

1.3.2.1 Anti-microbial action

YTX inhibits growth of yeast and fungi. It has no effect on bacterial growth (Ogino *et al.*, 1997).

1.3.2.2 Cell death and morphological changes

YTX induced cell death has been observed in a range of cell types (Leira *et al.*, 2002, Malaguti *et al.*, 2002, Korsnes *et al.*, 2006, Pérez-Gómez *et al.*, 2006, Young *et al.*, 2009, Pang *et al.*, 2011, Korsnes *et al.*, 2011). Changes observed accompanying cell death include DNA and chromatin fragmentation and chromatin condensation, decreased membrane potential of mitochondria, caspase activation and poly(ADP-ribose)polymerase (PARP) fragmentation. These changes are typically characteristic of apoptosis rather than necrosis. That YTX is inducing apoptosis is supported by Annexin V/propidium iodide (PI) staining (Leira *et al.*, 2002, Young *et al.*, 2009). One of the hallmarks of apoptosis is translocation of phosphatidylserine from the inner leaflet to the external surface of the cell membrane. Annexin V preferentially binds to phospholipids, such as phosphatidylserine. The externalisation of phosphatidylserine

also occurs in necrotic cells, but is also accompanied by loss of integrity of the cell membrane. The use of PI as a counterstain with Annexin V allows necrotic cells to be distinguished from those undergoing apoptosis using flow cytometry, as it can only enter cells with damaged membranes (Vermes *et al.*, 1995).

Paraptosis is a form of programmed cell death which does not meet the criteria for apoptosis or necrosis (Sperandio *et al.*, 2000). It has recently been suggested as the form of cell death induced by YTX in myoblast cells (Korsnes *et al.*, 2011). This is due to the observation of rampant cytoplasmic vacuolisation, swelling of the ER and mitochondria, lack of DNA fragmentation and increased levels of JNK/SAPK1, which are not typically associated with apoptosis or necrosis (Korsnes *et al.*, 2011).

Alterations to cell shape, both rounding and membrane blebbing, and detachment from adhesion surfaces were also observed (Aune *et al.*, 1991, Leira *et al.*, 2002, Malaguti *et al.*, 2002, Pang *et al.*, 2011, Korsnes *et al.*, 2011). Effects appear to be concentration and time dependent, with higher concentrations inducing more evident damage earlier (Aune *et al.*, 1991, Pérez-Gómez *et al.*, 2006). The concentration of YTX required for an observable alteration in morphology also seems to depend on the type of cell used (Aune *et al.*, 1991, Leira *et al.*, 2002, Malaguti *et al.*, 2002, Korsnes *et al.*, 2006, Pérez-Gómez *et al.*, 2006, Pang *et al.*, 2011).

1.3.2.3 Immune system

Yessotoxin has been found to affect cells of the immune system such as macrophages (Orsi *et al.*, 2010) and T-lymphocytes (López *et al.*, 2011). YTX was found to inhibit macrophage maturation and their ability to internalise the yeast *Candida albicans* at 1 and 10 nM concentrations but not at 0.1 nM (Orsi *et al.*, 2010). The same study also found changes in morphology, with macrophages becoming smaller and more rounded. The organisation of F-actin in the cytoplasm was also disrupted although no difference in the level of F-actin was detected. Production of the cytokines TNF- α , MIP-1 α and MIP-2 was also found to be increased in response to YTX treatment.

In T-lymphocytes, a rapid down-regulation of the T cell receptor (TCR) followed by a late up-regulation after 48 h incubation with YTX is seen. YTX was able to induce this

effect at a concentration of 1 nM (López *et al.*, 2011). The expression of TCR returned to basal levels following removal of the toxin. This down regulatory effect was found to be partially blocked in a dose-dependent manner by staurosporine, indicating that the down-regulation of the TCR by YTX is partially modulated by the protein kinase C pathway and a second unknown pathway. The expression of other cell surface molecules such as CD90.2 and CD40L were unaffected.

1.3.2.4 Effect on intra-cellular calcium

YTX has been found to cause an increase in intracellular Ca^{2+} levels in human peripheral blood lymphocytes (de la Rosa *et al.*, 2001a, b) and mussel immunocytes (Malagoli *et al.*, 2006b). However this effect is only observed if Ca^{2+} is present in the extracellular matrix. Compared to maitotoxin (MTX) (Fig. 2G), another polycyclic marine toxin, YTX was not a potent inducer of Ca^{2+} influx (de la Rosa *et al.*, 2001b). YTX caused intermittent influx of Ca^{2+} whereas MTX had a more sustained effect. MTX also affected a greater number of cells than YTX. Incubation of cells with YTX or MTX and various calcium channel blockers and agonists suggested that YTX and MTX are not acting on the same Ca^{2+} mediated pathway and are possibly activating more than one influx pathway (Malagoli *et al.*, 2006a and b). YTX and dsYTX do not strongly interact with sodium channel in membranes (Inoue *et al.*, 2003). In neuronal cells, blocking voltage sensitive calcium channels prevented calcium influx but did not stop cell death (Pérez-Gómez *et al.*, 2006). Blocking voltage sensitive sodium channels, in the same study, also failed to prevent cell death.

The permeability transition pore (PTP) of mitochondria was also found to be affected by YTX (Bianchi *et al.*, 2004). YTX was found to cause an increase in the rate of state 4 respiration in mitochondria isolated from rat liver. However this effect was only observed when Ca^{2+} was present in the media, implicating opening of the PTP. The proposed mechanism behind this effect is that Ca^{2+} binds to the sulphate groups of YTX at physiological pH 7.4, allowing YTX to then interact directly with the PTP or increase the hydrophobicity of YTX making it more membrane soluble. The opening of the PTP may cause activation of the mitochondrial pro-apoptotic pathway. The effect of YTX on the PTP, in addition to the increase in mitochondrial enzyme activity in

cardiomyocytes (Dell'Ovo *et al.*, 2008) and the mitochondrial rounding seen *in vivo* (Terao *et al.*, 1990, Aune *et al.*, 2002, Tubaro *et al.*, 2008) suggest that mitochondria may be one of the main targets of YTX.

1.3.2.5 Effect on Cyclic AMP

Changes in the levels of intracellular cAMP have also been seen in human peripheral lymphocytes (Alfonso *et al.*, 2003) and in primary cultures of rat cardiomyocytes (Dell'Ovo *et al.*, 2008). In lymphocytes, YTX caused a decrease in the level of cAMP which was dependent on the presence of extracellular Ca^{2+} (Alfonso *et al.*, 2003). The authors suggest that YTX is increasing the rate of cAMP hydrolysis by activating phosphodiesterases. A decrease in cAMP accumulation was also observed in rat cardiomyocytes (Dell'Ovo *et al.*, 2008). However, this was only seen when cAMP synthesis had been stimulated by prior incubation with forskolin. YTX was not found to have any effect on basal or unstimulated levels of cAMP. A decrease in cell viability and the beat frequency of the cardiac cells was also observed. These effects are not thought to be related to the effect of YTX on cAMP as they are observed at lower concentrations than those required for a cAMP effect. A transient, but significant, increase in the activity of mitochondrial enzymes was also observed in cardiomyocytes at low concentrations (first seen at 0.1 nM) of YTX.

1.3.2.6 Effect on the cytoskeleton

YTX appears to cause disruption of the cytoskeleton. In MCF epithelial cells, no difference was observed in the level or structure of β or γ -catenins, however the amount of intact E-cadherin decreased after treatment with YTX (Ronzitti *et al.*, 2004, Callegari *et al.*, 2006). Cell detachment was also observed. Cell growth stopped 1-2 days following treatment; however widespread cell death was not observed (Ronzitti *et al.*, 2004). *In vivo*, however stabilisation of E-cadherin was observed in mouse colon, following repeated oral administration of YTX (Callegari *et al.*, 2006). This effect appears to be reversible as no difference was detected between YTX treated and control mice 30 or 90 days after treatment. YTX did not appear to affect E-cadherin in the lungs or kidneys, or N-cadherin. YTX was found to disrupt the F-actin cytoskeleton

within 72 hours in the IPLB-LdFB insect cell line, mouse fibroblasts and primary cultures of myoblast and cerebellar neuronal cells (Malagoli *et al.*, 2006a, Pérez-Gómez *et al.*, 2006, Korsnes *et al.*, 2007). Decreased levels of native tensin and alterations to its distribution within cells were also observed (Korsnes *et al.*, 2007). The changes caused to the cytoskeleton by YTX may account for the changes to cell morphology observed in the studies mentioned in section 1.3.3 and in *in vivo* studies.

1.3.2.7 Proteomic studies

Young *et al.* (2009) showed that the major proteins affected by YTX in HepG2 liver cells were heterogeneous nuclear ribonucleoproteins (hnRNPs), lamins, cathepsins and heat shock proteins (HSPs). Apoptosis was also observed in YTX treated cells. Ingenuity pathways analysis (IPA) provided a possible network for the interaction between the proteins affected (Fig. 3A). HnRNPs had not previously been identified as possible targets of YTX. Alterations of hnRNP levels were also seen in HL60 cells treated with the microtubule stabilising agents; Pel A or PTX (Fig. 4) (Wilmes *et al.*, 2011, 2012). In HL60 cells treated with Pel A, IPA identified a protein network affected which was similar to that identified for YTX treated HepG2 cells (Fig. 3B).

Comparison of protein changes identified in HepG2 cells treated with YTX (Young *et al.*, 2009) and those identified in HL60 cells in response to microtubule stabilising agents (Wilmes *et al.*, 2011, 2012) revealed a number of proteins in common, although these were not always similarly affected (Fig. 4). BAG2, BiP (HSP70), and hnRNP A were affected in both cell lines. Lamin B1 had decreased levels in paclitaxel (PTX) treated HL60 cells and HepG2 cells treated with YTX. Actin (β), β -tubulin, HSP90B1, and hnRNP K were affected by Pel A in HL60 cells and YTX in HepG2 cells, although changes in abundance were usually in opposite directions.

1.4 Desulfo-yessotoxin

1.4.1 *In vivo effects*

1.4.1.1 Lethality

It has been suggested that di-desulfoyessotoxin (dsYTX, Fig. 1) may be formed through metabolism of YTX by micro-organisms in the intestine of humans following ingestion (Ogino *et al.*, 1997). Chemically prepared dsYTX, has an i.p LD₅₀ of 301 µg/kg b.w at 48 h (Terao *et al.*, 1990). In the same study YTX had an LD₅₀ of 286 µg/kg b.w at 3 h.

Mono-desulfated analogues, 1-desulfocarboxy-homo-YTX and 4-desulfocarboxy-homo-YTX, have an i.p LD₅₀ > 0.5 mg/kg b.w at 24 h, compared to YTX which was lethal to two out of three mice at a concentration of 0.1 mg/kg b.w at 24 h in female mice (Ciminiello *et al.*, 2007).

dsYTX is lethal to killifish (*Oryzias latipes*) at a concentration of 0.5 ppm (510 nM) after 6 h (Ogino *et al.*, 1997). YTX was found to be non-lethal in the same study.

1.4.1.2 Histopathology

Histopathological analysis following administration of dsYTX to mice found changes in the heart, liver and pancreas, but not in the intestine (small or large), adrenal glands, kidneys, spleen or thymus (Terao *et al.*, 1990). The main effect observed was accumulation of fat droplets within cells of the affected organs. The accumulation was slight in cardiac cells, but more pronounced in the liver, which became yellow and swollen within 12 h of i.p administration of 300 µg/kg b.w of dsYTX, and pancreatic acinar cells.

Electron microscopy of the liver cells showed approximately 1 fat droplet formed per cell, in the cytoplasm, within 10 min of dsYTX administration. However this increased to numerous small fat droplets within 24 h. By 10 min after administration, autophagosomes were observed near the bile capillary, associated with the rough endoplasmic reticulum (ER). The cytoplasm was full of large and small autophagosomes by 24 h. The contents of these were found to be degenerated ER, vesicles and other cell debris. At later stages after administration, autophagosomes

were also detected in Kupffer and Ito cells. Mitochondria were also noted to be swollen and have reduced electron density (Terao *et al.*, 1990).

The pancreatic acinar cells contained several fat droplets in their cytoplasm. Complete disappearance of zymogen granules was also observed, as well as disarrangement of the rough ER. Other pancreatic cells were not affected (Terao *et al.*, 1990).

Lipid analysis from the same study showed that, following the administration of dsYTX, there was an increase in the total fat content of the liver, but not of the kidneys or blood. Livers of mice treated with dsYTX contained 60 times the amount of triglycerides of controls or YTX treated mice, and twice the amount of phospholipids. A lipid peroxidation assay using thiobarbituric acid, showed double the amount in dsYTX mice livers compared to YTX. This suggests the increase in fat content is due to an increased degradation of membrane structures within the cell.

Oral administration of 500 µg dsYTX/kg b.w to mice 21 h prior to sacrifice, produced similar, but less severe, effects as i.p injection (Terao *et al.*, 1990).

The differences in effects of dsYTX compared with the effect of YTX *in vivo* (section 1.3.1), indicates that the two toxins have different targets and mechanisms of action.

1.4.2 *In vitro* effects

1.4.2.1 Anti-microbial action

Like YTX, dsYTX inhibits the growth of fungi and yeast but not bacteria (Ogino *et al.*, 1997). It was found to be a more potent inhibitor of the fungus *Penicillium funiculosum* than YTX, causing inhibition at 10 µg/disc compared to YTX which caused inhibition at 50 µg/disc.

1.4.2.2 Interactions with proteins

dsYTX has been found to enhance the hemolysis caused by alamethicin I and melittin, α-helix containing peptides, in a dose-dependent manner (Mori *et al.*, 2005). In the same study it was also shown to decrease the amount of oligomers of glycophorin A

and its transmembrane peptide (both containing an α -helix), while increasing the number of dimers and monomers. This suggests that the toxin has the ability to interact with the α -helix of these proteins and peptides. YTX and brevetoxin B (Fig. 2B) were observed to have similar efficacy as dsYTX. The tested ladder shaped polyethers (LSPs) were found to have 10-fold higher affinity than artificial polyethers in the glycophorin dissociation assay. The authors concluded that the LSP structure can generally interact with α -helix motifs.

1.5 Aims

The aims of this project were to use the proteomic techniques of differential in gel electrophoresis (DIGE) and peptide mass fingerprinting to examine the effects of YTX and dsYTX on *Saccharomyces cerevisiae* and HL60 cells, in order to elucidate the mechanism of action of these toxins, by identifying potential targets – either proteins or pathways.

Proteomics is the study of the proteins present in cells at a given time. Comparison of protein profiles from cells under different conditions can identify those proteins or pathways which are most affected. These may be primary targets or a secondary effect.

For this study *S. cerevisiae* was selected as a model organism. As many basic biological structures and processes have been preserved in eukaryotes, it is possible for yeast to serve as indicators for the function of human genes (Kolkman *et al.*, 2005). *S. cerevisiae* can be readily genetically manipulated, allowing for the generation of gene knock-out mutant strains. Examining the response of a mutant strain to a compound of interest, and comparing it with the response of the wild type strain can give an indication of the target or mechanism of action of that compound.

As similarities in the proteins affected in HepG2 cells treated with YTX (Young *et al.*, 2009) and HL60 cells treated with microtubule stabilising agents (Wilmes *et al.*, 2011, 2012) were observed (section 1.3.2.7), HL60 cells were also exposed to YTX, in order to identify which changes seen in YTX treated HepG2 cells are toxin or cell line specific, or a general cell response to stress.

Figure 4 Venn diagram comparing the protein changes seen in HepG2 cells treated with YTX (Young *et al.*, 2009) and HL60 cells treated with peloruside A (Pel A) or paclitaxel (PTX) (Wilmes *et al.*, 2011, 2012). Upwards pointing arrows indicate proteins which showed an increase in abundance in response to treatment. Downwards pointing arrows indicate proteins which decreased in abundance in response to treatment.

2 METHODS

2.1 Yessotoxins

Both YTX and dsYTX were provided by C. Miles and A.D. Hawkes, AgResearch Limited, Ruakura Research Centre, Hamilton, New Zealand. These compounds were each dissolved in methanol ($\geq 99.8\%$ pure), giving stock concentrations of 10 $\mu\text{g}/\mu\text{L}$ and 5 $\mu\text{g}/\mu\text{L}$ respectively (Appendix B).

2.2 Yeast strains

The *Saccharomyces cerevisiae* strains used were the haploid BY4741 mat a wild type strain and an ADA pump knock-out mutant which lacks seven of the ABC transporters (Lamping *et al.*, 2007). The latter was provided by Dr. Brian C. Monk, Department of Oral Sciences, University of Otago, Dunedin, New Zealand. The ADA strain was received on agar slants. Upon arrival stocks were made up in 10% glycerol YPD media. These were stored at -80°C until required.

2.3 Mammalian cell lines

HL-60 human promyelocytic leukemic blood leukocyte cells were used for mammalian cell work, and were acquired from Professor John H. Miller's group, School of Biological Sciences, Victoria University of Wellington, Wellington, New Zealand.

2.4 Yeast cell culture and protein extraction

2.4.1 Plate culture

S. cerevisiae strains were plated onto YPD agar plates using sterile technique. Plates were incubated at 30°C for 2 days. They were then stored at 4°C until required.

2.4.2 Liquid culture

Single colonies were selected from plates and transferred into 3 mL of 2% glucose YPD media. These were incubated at 30°C, 225 rpm (Bioline Shaker, Edwards Instrument Company, Australia), until cells reached stationary phase. Cultures were considered to be at stationary phase when they reached a cell density of approximately 2×10^8 cells/mL.

An aliquot (typically 10 μ L) from these cultures was then taken and transferred into 10 mL of 2% glucose YPD media, and incubated until cells reached either growth or stationary phase as required. Cultures were considered to be at stationary phase when they had reached a density of approximately 2×10^8 cells/mL or an absorbance of 0.8-1 at 595 nm. Cultures were considered to be at log phase when they had reached an absorbance of 0.4-0.5 at 595 nm.

2.4.3 Determination of cell density

The cell density of cultures was determined by cell counting using a hemocytometer. Ten μ L of a yeast sample was diluted 1:100 with Milli-Q or High-Q water, and 10 μ L of this was then loaded onto the hemocytometer, and observed with a phase contrast microscope

For absorbance measurements, triplicate 100 μ L aliquots of each sample were pipetted into a 96 well plate. Absorbance readings were measured at 595 nm using a Molecular Devices, VERSAmax tunable microplate reader (Applied Biosystems, Melbourne, Australia). YPD media containing 2% glucose was used as a blank.

2.4.4 Toxin treatment

Ten mL *S. cerevisiae* cultures were divided into 1.5 mL aliquots once cells had reached either log or stationary phase as required. There were three conditions tested: untreated, methanol treated, and toxin treated (n=2). Two μ L methanol (0.13% v/v final concentration) was added to methanol treated controls. Toxin treated samples

were treated with 2 or 20 µg of YTX or 2 µg of dsYTX. All cultures were then incubated at 30°C for 24 h.

S. cerevisiae cells from liquid cultures were harvested by centrifugation at 2300 *g* for 5 min. The supernatants were discarded and the pellets were resuspended in 1.5 mL Milli-Q or High-Q water before being centrifuged as before. The supernatants were removed and the wash step repeated. The supernatants were removed and the pellets were resuspended in 1 mL protease inhibitor solution (1 complete mini EDTA free protease inhibitor cocktail tablet (Catalogue number: 11 836 170 001, Roche Diagnostics GmbH, Mannheim, Germany) dissolved in 10 mL Milli-Q or High-Q water). The cell suspensions were then centrifuged as before, the supernatants were removed and cell pellets were stored at -80°C until required.

2.4.5 Morphological analysis

Ten µL of sample culture was diluted 1:100 with Milli-Q or High-Q distilled water. Fifty µL of the diluted sample was then observed using a phase contrast microscope.

2.4.6 Cell lysis and protein extraction

Cell pellets were resuspended in 100 µL cell lysis buffer (30 mM TrisCl (pH 8.8), 7 M urea, 2 M thiourea, 4% w/v CHAPS) and transferred to 1.5 mL microcentrifuge tubes containing approximately 50 µL white quartz sand (50+70 mesh, SIGMA-ALDRICH Inc., St. Louis, Missouri). The samples were then ground using mini pestles. During this process the samples were kept on ice. Following grinding, the samples were vortexed for 30 min at 4°C. They were then centrifuged at 16,000 *g* for 15 min at 4°C. The supernatants were transferred to 0.6 mL microcentrifuge tubes and stored at -20°C until required. On average, 1×10^7 cells yielded approximately 20 µg of protein.

2.5 HL-60 cell culture and protein extraction

2.5.1 Mammalian cell culture

HL-60 cells were cultured in GIBCO® RPMI-1640 media containing L-glutamine and phenol red indicator (Invitrogen). The media was supplemented with 10% FCS, 100 units/mL penicillin and 100 units/mL streptomycin, at 37°C with 5% CO₂ in air. When cells reached approximately 80% confluency they were passaged by diluting the culture 1:10 or 1:20 with media.

2.5.2 Determination of cell density

The density of HL-60 cells was determined by a trypan blue live/dead exclusion assay. Ten µL of HL-60 cell samples were diluted 1:2 with 0.4% trypan blue. Ten µL of this was then loaded onto a hemocytometer. Live cells were considered to be those which were white or colourless. Cells which displayed blue colouration were considered to be dead. Only live cells were considered when calculating cell density.

2.5.3 Toxin treatment

Three x 20 mL cultures of HL-60 cells were grown to 80% confluency then combined, and 9×10^6 cells were then seeded into cultures of 10 mL total volume. Ten mL cultures (n=4) were then treated with methanol (0.02% v/v final concentration) or 1 µM of YTX or dsYTX. The cultures were then incubated at 37°C with 5% CO₂ in air for 24 h.

HL-60 replicates were then transferred to 15 mL screw top test tubes and centrifuged at 300 g for 5 min. The supernatants were discarded and the pellets were resuspended in 10 mL ice cold PBS before being centrifuged as before. The supernatants were removed and discarded. The pellets were resuspended in 1 mL of PBS and transferred to 1.5 mL microcentrifuge tubes and centrifuged at 300 rpm (SIGMA 1-14 Laboratory Table Top Microcentrifuge). The supernatants were discarded

and the pellets resuspended in 1 mL PBS/protease inhibitor solution (one complete mini EDTA free protease inhibitor cocktail tablet (Catalogue number: 11 836 170 001, Roche) in 10 mL PBS and centrifuged again. The supernatant was removed from each sample as much as possible. The pellets were then stored at -80°C until required.

2.5.4 Morphological analysis

HL60 cells were observed in culture using phase contrast microscopy.

Images of cells were acquired using a CC12 Soft Imaging System camera connected to an Olympus IX51 microscope, and Olympus Cell A Imaging software.

2.5.5 HL-60 cell lysis and protein extraction

Lysis buffer was added to pellets in a ratio of 60 µL per 3.6×10^6 cells. The pellets were resuspended in lysis buffer and vortexed for 30 min at 4°C. They were then centrifuged at 16,000 *g* for 15 min at 4°C. The supernatants were then transferred to 0.6 mL microcentrifuge tubes and stored at -20°C until required. On average, approximately 960 µg of protein were extracted per 1×10^7 cells.

2.5.6 Preparation of samples for analysis by gel electrophoresis

2.5.6.1 Protein assays

Triplicate 1 µL aliquots from each yeast or HL-60 sample were pipetted into a 96 well plate. Two hundred µL of Protein Assay, Dye Reagent concentrate (Bio-Rad, Hercules, California) diluted with 4 volume equivalents of Milli-Q or High-Q water was then added to each well. Absorbance readings were measured at 595 nm using a Molecular Devices, VERSAmax tunable microplate reader (Applied Biosystems). The protein concentration of samples was determined by comparison to a BSA standard curve.

2.5.6.2 Protein precipitation

Proteins were precipitated using Calbiochem ProteoExtract™ Protein precipitation kit (Merck, Darmstadt, Germany). One hundred µL of precipitant was added to 50-100 µL protein extracts from yeast samples. For HL-60 protein extracts 4 x volume equivalents of precipitant were added. The samples were then incubated at -20°C for at least 1 h. Following this they were centrifuged at 13,000 *g* for 10 min. The supernatants were discarded and 100 µL of wash buffer was added to each pellet. The samples were vortexed briefly, before being centrifuged at 13,000 *g* for 5 min. The supernatants were removed again and the wash step repeated. The pellets were then allowed to dry at room temperature for 30 min before being resuspended in either lysis buffer or rehydration buffer.

2.5.7 Gel electrophoresis

2.5.7.1 One-dimensional electrophoresis

Stock sample buffer was made up consisting of 2.5 µL LDS sample buffer (4x, NuPAGE®, Invitrogen, Carlsbad, California), 1 µL reducing agent (10x, NuPAGE®, Invitrogen) and 5.5 µL Milli-Q or High-Q water per sample). One µL of sample was added to 9 µL of sample buffer solution. The samples were then vortexed and centrifuged briefly to remove air bubbles. The samples were electrophoresed on 4-12% Bis-Tris gels (NuPAGE®, Invitrogen), at 200V, 400 mA for 55 min, in MOPS running buffer (MOPS SDS running buffer (20x) NuPAGE®, Invitrogen). The buffer in the central chamber had 500 µL of antioxidant (Invitrogen) added before electrophoresis began.

2.5.7.2 Two-dimensional electrophoresis

2.5.7.2.1 Isoelectric focusing

Protein samples were separated by IEF on 7 cm Immoboline™ DryStrips (GE Healthcare Bio-Sciences A.B, Uppsala, Sweden). Following the precipitation procedure pellets were resuspended in either 125 µL of rehydration buffer (2 M thiourea, 7 M urea, 2% DTT, 4% CHAPS, trace bromophenol blue, 2% IPG buffer (GE Healthcare)), for samples

to be focused on pH 3-10, pH 3-10 NL or pH 4-7 DryStrips, or 100 μ L rehydration buffer (2 M thiourea, 7 M urea, 2.5% DTT, 2% CHAPS, 5% glycerol, 10% isopropanol, 1% IPG buffer) for samples to be focused on pH 6-11 DryStrips. Samples focused on pH 3-10, pH 3-10 NL or pH 4-7 DryStrips were loaded onto the strips by passive loading. This was done by rehydrating the strip overnight in 125 μ L of sample containing rehydration buffer. The pH 6-11 DryStrips always had samples loaded onto them by cup-loading, so were rehydrated overnight in 125 μ L of rehydration buffer. Rehydration of DryStrips was done in an Immobiline DryStrip Reswelling tray (GE Healthcare). After being placed into rehydration buffer each strip was covered with 3 mL of mineral oil (PlusOne DryStrip cover fluid, GE Healthcare).

IEF was carried out using a Multiphor II system (Pharmacia) connected to a power supply (electrophoresis power supply EPS 3500 XL, Pharmacia BioTech) and waterbath (Julabo). DryStrips were run on gradient setting. The first step was at 200 V for 1 h. The second step was 3500 V for 1 h 30 min. The last step was at 3500 V for 1 h 30 min for pH 3-10, and 4-7 strips; and 1 h 5 min for pH 6-11 and 3-10 NL strips. Temperature was kept constant at 20°C. Total current was 2 mA and total power was 5 W. Strips were either equilibrated immediately following IEF, prior to loading on second dimension gels or stored at -80°C until required.

2.5.7.2.2 Equilibration

Strips were equilibrated by 2 x 10 min incubations in 2-2.5 mL equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30 % v/v glycerol, 2% w/v SDS). The first 10 min incubation was in 1% DTT equilibration buffer, the second in 2.5% IAA equilibration buffer.

2.5.7.2.3 Electrophoresis

Second dimension SDS-PAGE was carried out under the same conditions as for 1DE gels on 4-12% Bis-Tris ZOOM™ gels (NuPAGE®, Invitrogen).

2.5.7.2.4 Staining

Gels were stained with Coomassie Brilliant Blue G-250 powder (Bio-Rad). The gels were incubated for at least 30 min in fixing solution (50% ethanol, 3% phosphoric acid) before being washed for 3 x 30 min with Milli-Q or High-Q water. Gels were then incubated in staining solution (34% methanol, 17% ammonium sulphate, 3% phosphoric acid) for at least an hour. A small spatula tip (approximately 10 mg) of Coomassie was added and the gels were left to stain for at least 3 days at room temperature. Gels were washed thoroughly with Milli-Q or High-Q water, at least 3 x 30 min incubations, to remove excess Coomassie prior to image acquisition using a Molecular Dynamics Personal Densitometer SI.

2.5.7.2.5 DIGE

DIGE is proteomics tool which allows direct comparison of samples across multiple gels. Protein samples are labelled with one of three CyDye fluorophores (fluors): Cy2, Cy3 or Cy5. The CyDyes bind to lysine residues present in the proteins. The CyDye fluors are positively charged, to replace the charge on the lysine when bound, so the isoelectric point of the protein is not affected. The CyDyes are also weight matched to each other, so identical proteins will migrate the same distance regardless of which dye they are labelled with. Each CyDye has a different excitation frequency. This allows multiple samples (two) to be run on one gel, alongside an internal standard. An internal standard is a pool of all the samples in a given experiment, and is run on all gels in the experiment. This aids protein spot matching between gels, reducing the effect of gel to gel variation. An overview of the DIGE procedure is shown in fig. 5.

In this project, protein samples were minimally labelled with CyDye fluors. Minimal labelling results in 1-2% of lysine residues being bound by CyDyes (in theory one fluor is bound per protein). This gives a linear relationship between fluorescence and protein concentration, allowing accurate comparison of protein abundances to be made.

Figure 5 Flow diagram of DIGE procedure. Proteins were separated by 2DE. Image analysis and quantitation was carried out using DeCyder software version 6.5. (Image source: *2-D Electrophoresis: Principles and Methods* handbook, GE Healthcare)

The protein concentration of a sample was adjusted to lie in the range 5-10 $\mu\text{g}/\mu\text{L}$ by precipitating the protein, as previously described, and then resuspending the sample in suitable volume of lysis buffer.

The pH of the samples was adjusted to 8.5 by the addition of a small amount (0.1-0.5 μL) of pH 8.8 Tris-HCl buffer.

Eighty pmol of Cy3 or Cy5 CyDyes (GE Healthcare) were added to 20 μg of protein from each treatment condition. Half of the replicates from each condition were labelled with Cy3 and the other half were labelled with Cy5. An internal standard was made consisting of 10 μg of protein from each sample, and 240 pmol of Cy2. The samples were vortexed and centrifuged briefly before being incubated on ice for 30 min in the dark. One μL of 10 mM lysine per 20 μg of protein was added to each solution to quench the reaction. The samples were vortexed and centrifuged briefly, then incubated on ice for a further 15 min in the dark. The samples were then combined so

that sample mixtures contained equal amounts of internal standard, and samples from two different treatment conditions. If not analysed straight away mixed sample solutions were stored in the dark at -20°C. An example of a DIGE experiment design is shown in Appendix C.

Samples underwent first and second dimension separation as described previously. Immediately after second dimension separation had been completed, 2D-DIGE gels were scanned using a Fujifilm Fluorescent Image Analyser FLA-5100 scanner (Fuji Photo Film Co. Ltd., Tokyo, Japan). Cy2 images were scanned using a 473 nm laser and BPB1/530DF20 filter. Cy3 images were scanned using a 532 nm laser and BPG1/570DF20 filter. Cy5 images were scanned using a 635 nm laser and DBR1/R665 filter. All gels were scanned at 50 µm resolution and 16 bits.

2.5.7.2.6 Gel Image Analysis

DIGE gel images were acquired as TIFF files from the scanner. These were converted to .gel files using IQ Tools software, version 5.2 (Molecular Dynamics). The .gel files were then combined to make one multichannel image using Fluorochrome Separation 2.2, Multicolour version (Molecular Dynamics) to make the dataset file. The intensity of each CyDye signal was adjusted using IQ Tools, until it was approximately even between all gels, and protein spots were visible on the display. The image was then cropped, before being imported into DeCyder 2D Software, version 6.5 (GE Healthcare) for DIGE analysis.

The images from one experiment were all imported into DeCyder using the Image Loader function. They were then all processed simultaneously using the Batch Processor function. Estimated number of spots for spot detection was set to 2500. The Batch Processor detects and matches the spots on multiple gels automatically. All gels are matched to a master gel (by default the gel assigned as the master is one on which the most protein spots are detected). Matching is based on a pattern recognition algorithm, where a spot on one gel is matched to a spot on another gel based on the surrounding spots. This process has two parts to it. The first part matches large spots over the whole gel, by comparing the positions and sizes of the spots around it. These matched spots are then used as landmarks. The remaining

spots are matched, moving outwards from the landmarked spots. The second part of the spot matching process involves transforming the spot centre detected on one gel to another gel. The transformation is considered a match if the transformed coordinates are close enough to the spot centre detected on the other gel. After each matching step the algorithm performs a control which removes obvious mismatches.

Following batch processing, the created workspace was opened in the DeCyder Biological Variation Analysis (BVA) module. The spot matching was checked and corrected manually if required. BVA was used to calculate changes in spot volume (average ratio) and statistics for the change (Student's *t*-test, one way ANOVA). Proteins were labelled as being of interest if they had a *t*-test value ≤ 0.05 and an average ratio value ≤ -1.5 or ≥ 1.5 . Changes in protein abundance of 2-fold or greater ($p \leq 0.01$) were considered significant. Two biological replicates per treatment group were used for initial exploratory experiments. Four biological replicates per treatment group were used in the final experiment to give a power of 0.8 (Karp and Lilley, 2007).

To determine if spots of interest were true protein spots or just noise, possibly from dust, spots were also inspected in the 3D-view in DeCyder. Sharp peaks, generally around the edge of a gel, were classed as noise. Round peaks were classed as protein spots (Appendix D). The false discovery rate (FDR) filter was used to control for type I errors. Applying this filter identifies protein spots that have a change in abundance which is more likely to have arisen through chance, than to be a true effect of the treatment.

The average ratio was calculated from the log standardised abundance of the protein spots. The degree of difference in the standardised abundance was the average ratio. The average ratio is expressed as values from $-\infty$ to -1 or 1 to ∞ . The abundance of a protein spot was quantified as a function of the internal standard. This value is the standardised abundance. The $\log_{10}$ of this was the log standardised abundance.

2.5.8 Protein identification

Proteins were identified by peptide mass fingerprinting. Proteins were excised from 2D gels then subjected to tryptic digestion. The resultant peptide fragments were then subjected to MALDI analysis either on a Voyager-DE PRO mass spectrometer or an AB Sciex TOF/TOF 5800 mass spectrometer. The sequences acquired from this process were then searched against the NCBI non-redundant protein sequence database using either the Proteome Discoverer or Mascot algorithm.

2.5.8.1 Preparation of protein spots for MALDI analysis

Protein spots were extracted from the gels using a spot picker (OneTouch Plus Spot/Band picker PDM1.5, The Gel Company, San Francisco, California) or a scalpel and placed in a 96 well v-bottom plate containing 100 μ L destaining solution (50% methanol, 50 mM ammonium bicarbonate) in each well. The plate was then left to destain overnight at 4°C. The plates were then processed using an Ettan Automatic Digester (Amersham Biosciences) with the following programme: 3 cycles in 50% methanol, 50 mM ammonium bicarbonate for 20 min each, followed by air drying for 60 min. Trypsin (Modified Sequencing Grade, Roche); (0.05 μ g/well in 20 mM ammonium bicarbonate) was added for 300 min, followed by the addition of 20 mM ammonium bicarbonate for 20 min. The peptides were transferred to a fresh plate by adding a solution of 50% acetonitrile and 0.1% TFA for 3 x 20 min cycles. The recovered proteins were allowed to air dry overnight in a laminar flow hood. Once dry, the proteins were resuspended in 1.5 μ L of CHCA matrix (10 mg/mL CHCA, 0.25% TFA, 50% acetonitrile, containing 0.5 μ L of Calibration Mixture 2 (AB Sciex, Foster City, California)). Resuspended samples were transferred onto a MALDI plate and left to dry overnight.

2.5.8.2 MALDI-TOF MS Analysis

MALDI TOF analysis was carried out using a Voyager-DE PRO mass spectrometer (Applied Biosystems). The spectra were collected over the mass range 500-3500 m/z, with an approximate laser intensity range of 1450-1600 and 100 shots were collected per spectrum. Analysis of the spectra was carried out using Data Explorer software version 5.1 (Applied Biosystems). The acquired spectra were calibrated internally

using Calibration Mixture 2 (Applied Biosystems, Foster City, CA), containing a angiotensin I (average m/z 1297.51), adrenocorticotrophic hormone (ACTH) clip 1-17 (average m/z 2093.0867) and ACTH clip 18-39 (average m/z 2465.1989). Trypsin peaks (m/z 805.4167, 1153.5740, 2163.0566) were also used for internal calibration if they were present in the spectrum. The peptide masses generated were then searched against the NCBI non-redundant database (2009/09/01) for *S. cerevisiae* using the Profound algorithm. Search criteria included a protein mass in the range of 0-300 kDa, one missed trypsin cleavage, complete cysteine modification by IAA and partial modification of methionine by oxidation, and a mass tolerance of 40 ppm. Match criteria included at least 5 matched peptides, sequence coverage (correct cleavage sites – peptides end with K or R, $\geq 20\%$ coverage), and a difference between first and second ranked identifications of at least 1×10^{-3} .

2.5.8.3 MALDI TOF-TOF MS/MS Analysis

MALDI TOF-TOF MS/MS analysis of samples was carried out using an AB Sciex TOF/TOF 5800 mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a diode pulse laser (355nm, 1kHz). The spectrometer was controlled by TOF/TOF™ Series Explorer™ Software.

2.5.8.3.1 Acquisition of spectra for MS

Spectra for MS analysis were acquired in positive reflector mode with CID off. Peptide fragments were collected in the mass range 800-4000 Da. The laser intensity was fixed at 3000 with a pulse rate of 400 Hz. The laser was fired in a uniform random pattern. A total of 400 laser shots were collected per full spectrum.

2.5.8.3.2 Calibration of spectra for MS

MS spectra were internally calibrated using CalMix2 (Applied Biosystems) monoisotopic peaks for angiotensin I (m/z 1296.6853), adrenocorticotrophic hormone (ACTH) clip 1-17 (m/z 2093.0867), ACTH clip 18-39 (m/z 2465.1989) and ACTH clip 7-38 (m/z 3657.9294). Peak matching criteria for calibration included a signal to noise ratio greater than or equal to 5, mass tolerance of ± 0.3 m/z , a minimum of 3 matched

peaks, and maximum outlier error was 0.8 m/z. A list of reference masses is shown in Appendix E.

2.5.8.3.3 Precursor ion selection for MS/MS

Selection criteria for precursors to undergo MS/MS analysis included a signal to noise ratio greater than or equal to 20, mass within the range 800-4000 Da, and exclusion of precursor ions within a resolution of 200 of the selected precursor ion. Known adduct, calibration and common contaminant peaks, such as trypsin, were excluded. A full list of exclusion masses is shown in Appendix F. A maximum of 10 precursor ions were selected per spot. MS/MS data were acquired from weakest precursor ion first.

2.5.8.3.4 Acquisition of spectra for MS/MS

Spectra for MS/MS analysis were acquired in 1kV positive reflector mode, with CID and metastable suppressor on. The laser intensity was fixed at 3500 with a pulse rate of 1000 Hz. The laser was fired in a random uniform pattern. Acquisition of spectra was stopped after 5 sub-spectra were collected. A total of 1000 laser shots were collected for the final spectrum.

2.5.8.3.5 Calibration of spectra for MS/MS

The monoisotopic angiotensin 1 peak (1296.800 Da) was the selected precursor ion for calibration. MS/MS spectra were then calibrated using known fragment ion masses from this precursor ion (Appendix D). Peak matching criteria for calibration included a signal to noise ratio greater than or equal to 10, mass tolerance of ± 0.3 m/z, a minimum of 3 matched peaks, and maximum outlier error of 20 ppm.

2.5.8.3.6 Comparison of peptide sequences to NCBI database

Peptide fingerprints and MS/MS sequences were imported into Protein Pilot™ software. The MASCOT algorithm was used to search the generated peaks lists against the NCBI non-redundant database (2008/09/02, 21331 sequences) for *S. cerevisiae* (905409 sequences) for proteins obtained from *S. cerevisiae* samples, or for *Homo sapiens* (218446 sequences) for proteins from HL-60 cells. Other search parameters included one missed trypsin cleavage, fixed carbamidomethyl modification of cysteine

by IAA, variable modification by methionine oxidation, MS/MS fragment tolerance of ± 0.1 Da, precursor tolerance of ± 0.05 Da, monoisotopic masses, and a +1 peptide charge.

3 RESULTS

3.1 *S. cerevisiae*

The effects of YTX and dsYTX were initially examined using 2D DIGE. The goal was to identify proteins that changed for comparison with known effects of YTX on HepG2 cells (Young *et al.*, 2009). MALDI mass fingerprinting was used to identify protein spots excised from 2D gels.

3.1.1 *Morphological analysis*

The morphology of *S. cerevisiae* samples was observed at 0 h and 24 h after toxin treatment, using phase contrast microscopy. There were no differences in morphology between toxin treated cells and controls in either the wild type or pump knock-out mutant strain.

3.1.2 *2D-DIGE analysis*

The protein extracts from preliminary tests on yeast (n=2) in stationary and log phase, wild type and pump knock-out mutant yeast strains, treated with either 1.2 μ M or 12 μ M YTX or 1.4 μ M dsYTX were analysed for changes in levels of protein expression by 2D DIGE. The gel images were analysed using DeCyder software (version 6.5), which quantified the fold change and calculated the relevant statistics. A change in protein abundance ≥ 2 -fold, $p \leq 0.01$ was considered significant (Karp *et al.*, 2007). However, as very few protein spots matched this criterion, the decision was made to also take note of proteins which might be changing below the defined significance level. The criteria for these proteins of interest was a fold change ≥ 1.5 , $p \leq 0.05$. Tables 2 - 4 summarise the number of protein spots of interest detected under each experimental condition. These changes were only observed when the false detection rate (FDR) filter was not applied. When the FDR filter was applied, no protein spots met the criteria for significance. DeCyder statistics for all proteins of interest and picked protein spots are reported on the Supplementary Data on CD.

Table 2 Effects of YTX on stationary phase yeast cells. The number of proteins from stationary phase BY4741 and ADA strains, which showed a change in possible change in abundance when analysed using DIGE (≥ 1.5 -fold, $p \leq 0.05$). No protein changes were significant using the criteria ≥ 2 -fold, $p \leq 0.01$. The total spots detected per gel, includes all data point matches detected by DeCyder software, including those which are not necessarily protein spots.

Stationary phase		Comparison of Change							
Strain	pH	YTX/untreated		YTX/MeOH		MeOH/untreated		Total spots	
		1.2 μ M	12 μ M	1.2 μ M	12 μ M	1.2 μ M	12 μ M	1.2 μ M	12 μ M
BY4741	4-7	2	0	1	6	0	0	2530	2355
	6-11	1	0	0	0	0	0	2565	2318
ADA	4-7	0	0	0	0	0	0	2062	2330
	6-11	0	0	0	0	0	0	2458	2564

All detected changes in response to 1.2 μ M YTX were decreases in protein abundance (Table 5, Figs 6 and 7). The two proteins that showed a change in YTX treated cells compared to untreated controls, did not show a change when compared to methanol treated controls. The six detected changes in cells treated with 12 μ M YTX compared to methanol controls were split equally between increasing and decreasing abundances. However no changes in abundance were seen when proteins from treated cells were compared to those from untreated control cells.

Table 3 Effects of 12 μ M YTX on log phase yeast cells. The number of proteins from log phase yeast cells, BY4741 and ADA strains, which were deemed to be of interest when comparing the different experimental treatments (≥ 1.5 -fold, $p \leq 0.05$). No protein changes were significant using the criteria ≥ 2 -fold, $p \leq 0.01$. The total spots detected per gel, includes all data point matches detected by DeCyder software, including those which are not necessarily protein spots.

Log phase		Comparison of Change			
Strain	pH	YTX/untreated	YTX/MeOH	MeOH/untreated	Total spots
BY4741	4-7	6	0	6	2361
	6-11	6	0	7	2514
ADA	4-7	3	6	0	2324
	6-11	0	0	0	2294

Of the six protein spots identified as changing in the pH 4-7 log phase wild type strain cells in response to YTX (Table 3, Fig. 8), four were also detected as changing between controls. In the pH 6-11 gels, two of the six identified as changing were also detected changing between controls. All three of the protein spots identified as undergoing a change in ADA cells in response to YTX compared with untreated cells, were also changing when compared to methanol treated cells (Table 3, Fig. 10).

Table 4 Effects of 1.4 μ M dsYTX on stationary phase yeast cells. Proteins from log phase yeast cells, from BY4741 and ADA strains, which were deemed to be of interest when comparing the different experimental treatments. Numbers not in brackets are the total number of spots which showed a possible change in abundance (≥ 1.5 -fold, $p \leq 0.05$). The number in brackets is the total number of these spots which showed a significant change in abundance (≥ 2 -fold, $p \leq 0.01$). The total spots detected per gel, includes all data point matches detected by DeCyder software, including those which are not necessarily protein spots.

Stationary + dsYTX		Comparison of Change			
Strain	pH	dsYTX/untreated	dsYTX/MeOH	MeOH/untreated	Total spots
BY4741	4-7	6(1)	7(0)	0	2254
	6-11	1(1)	3(0)	0	2475
ADA	4-7	8(2)	26(2)	0	2403
	6-11	2(0)	5(0)	0	2200

Four of the six protein spots identified as undergoing a change in abundance in response to dsYTX (pH 4-7) when compared to untreated cells in wild type BY4741 cells, also changed when compared to methanol treated controls (Table 4, Fig. 9). The protein spot detected as possibly changing on pH 6-11 gels in response to dsYTX also changed when compared to methanol treated cells. The effect of dsYTX appeared to be similar between wild type and pump mutant strains, although it was possibly greater in ADA cells, with more protein spots showing a detectable change in abundance. For ADA cells six of the eight detected in pH 4-7, and both of the protein spots detected in pH 6-11 gel images, changed when compared with methanol treated cells as well (Table 4, Fig. 11).

Figure 6 Coomassie blue stained gels, pH 4-7 and 6-11, of proteins extracted from stationary phase wild type yeast cells treated with 1.2 μ M YTX for 24 h. **Yellow** = not changing. **Pale green** = tending towards a decreasing abundance but not significant (fold change ≥ 1.5 , $p \leq 0.05$).

Figure 7 pH 4-7 Coomassie blue stained gel of proteins from stationary phase wild type yeast cells treated with 12 μ M YTX for 24 h. **Pale green** = tending towards a decrease in response to YTX treatment, but not significant (fold change ≥ 1.5 $p \leq 0.05$). **Pale pink** = tending towards an increase, but not significant (fold change ≥ 1.5 $p \leq 0.05$).

No changes in protein fold were detected between YTX treated and untreated cells. No changes in protein fold were detected between proteins from different treatment conditions when separated on a pH 6-11 linear gradient.

Figure 8 Coomassie blue stained gels, pH 4-7 and 6-11, of proteins extracted from log phase wild type yeast cells treated with 12 μ M YTX for 24 h. **Yellow** = not changing. **Pale green** = tending towards a fold decrease, but not significant (fold change ≥ 1.5 , $p \leq 0.05$). **Pale pink** = tending towards an increase, but not significant (fold change ≥ 1.5 , $p \leq 0.05$). White = proteins which underwent a fold change when methanol and untreated cell extracts were compared.

Figure 9 Coomassie blue stained gels, pH 4-7 and 6-11, of proteins extracted from stationary phase wild type yeast cells treated with 1.4 μ M dsYTX for 24 h. **Yellow** = not changing. **Pale green** = tending towards significance (fold change ≥ 1.5 , $p \leq 0.05$). **Green** = significant decrease (fold change ≥ 2 fold, $p \leq 0.01$). **Pink** = tending towards an increase, but not significant (fold change ≥ 1.5 $p \leq 0.05$).

Figure 10 Coomassie blue stained gels, pH 4-7 of proteins extracted from log phase ADΔ mutant yeast cells, treated with 12 μM YTX for 24 h. **Pale green** = tending towards significance (fold change ≥ 1.5 , $p \leq 0.05$). No proteins of interest were identified amongst proteins separated on a pH 6-11 linear gradient.

Figure 11 Coomassie blue stained gels, pH 4-7 and 6-11, of proteins extracted from stationary phase ADA mutant yeast cells, treated with 1.4 μ M dsYTX for 24 h. **Yellow** = not changing. **Pale green** = tending towards significance (fold change ≥ 1.5 , $p \leq 0.05$). **Pale pink** = tending towards an increase, but not significant (fold change ≥ 1.5 $p \leq 0.05$). **Red** = significant increase (fold change ≥ 2 , $p \leq 0.01$).

3.1.3 Protein identification by peptide mass fingerprinting

In total 76 protein spots which had a fold change in abundance of ≥ 1.5 -fold, $p \leq 0.05$ were detected. Thirty-nine of these were not excised from the gels for MALDI analysis due to low abundance. Of the remaining 37 spots, eight were identified. A further 36 proteins that had not changed in abundance were also identified. These protein spots are numbered in Figs 6 - 11. The DeCyder data and top ranked protein identification for each spot is displayed in Table 5. Identifications which did not reach the level of significance for confident identification of proteins have been included, to allow for comparison to results from HL-60 cells. Further identification data (including gi number, sequence coverage, expectation and lower ranked candidates) are reported in Appendix G and on the Supplementary Data CD.

No stationary phase *S. cerevisiae* proteins showed a significant change in abundance when treated with YTX. Two of the possible (≥ 1.5 -fold, $p \leq 0.05$) changes seen when wild type (BY4741) cells were treated with 1.2 μ M YTX were identified as cytosolic aldehyde dehydrogenase (Spot 10, Fig. 6, Table 5) and phosphoglycerate kinase (Spot 32, Fig. 6, Table 5).

Spot 138 showed a significant 2.37 fold decrease in abundance in wild type cells treated with dsYTX when compared to untreated controls (pH 4-7, Fig. 9, Table 5). This protein spot was identified as GRE1 protein. Mitochondrial aldehyde dehydrogenase was also identified as showing a possible change in abundance (Spot 129, Fig. 9Table 5).

The four significant changes seen when proteins from ADA Δ cells treated with dsYTX (Fig. 11) are compared with proteins from untreated or methanol treated cells correspond to three proteins, two of which only change when compared with one control. Searching acquired spectra using MASCOT was unable to find matches to these spots with significant scores. The top-ranked match for each protein spot returned by the search were YKR101Wp-like protein (Spot 165), Pau9p hypothetical protein (Spot 166), which increased approximately 2.5 and 2-fold respectively, and mitochondrial translational activator (Spot 200), which decreased approximately 2.3 fold, compared to either control. HSP90-Sba1 closed chaperone complex (chain A)

(Spot 168), YLL024Cp-like protein (Spot 173), ATPase involved in protein folding and nuclear transport (Spot 174), alcohol dehydrogenase I (Spot 211), and translational elongation factor 1 α , showed possible changes in abundance (Fig. 11, Table 5). HSP90B and elongation factor 1 δ had altered abundances in HepG2 cells when treated with YTX.

The proteins which were identified with significant Mascot scores, but did not show any change in abundance by DIGE analysis are listed in Appendix H.

A total of 212 MS spectra were collected over all *S. cerevisiae* experiments. Of these 47 were identified with a significant Mascot score. Four of the remaining spectra did not provide a significant identification despite having signals and peak intensities that one would have expected to return a match.

Table 5 *S. cerevisiae* protein abundance changes (Av. Ratio) and associated statistics acquired from DeCyder and protein identifications of proteins of interest and picked protein spots as numbered in Figs. 9 - 11. **Bold** statistics are significant (≥ 2 -fold, $p \leq 0.01$). *N/A* for protein name indicates that a protein spot was not selected for identification, either because it was low abundance or considered not to be due to toxin. Identifications in *italics* are those which did not have a significant match score. An absence of DeCyder statistics is indicative that a protein spot was not present in all protein spot maps. Av. Ratio is the protein volume of the first condition compared to the second in the comparison. For example negative value when comparing dsYTX/methanol (MeOH) means that the protein has undergone a decrease in abundance in response to dsYTX respective to the methanol treated control.

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
1	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.17	-1.3	0.19	Formylglycinamide-ribonucleotide (FGAM)-synthetase
			YTX/MeOH	0.52	-1.1	0.19	
			MeOH/untreated	0.089	-1.18	0.19	
2	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.58	-1.19	0.46	SSE1 protein, ATPase component of Hsp90 chaperone complex
			YTX/MeOH	0.21	-1.39	0.46	
			MeOH/untreated	0.57	1.16	0.46	
5	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.001	-1.57	0.026	<i>Cytoplasmic tryptophanyl-tRNA synthetase</i> (Protein identification score was not significant)
			YTX/MeOH	0.057	-1.59	0.026	
			MeOH/untreated	0.97	1.01	0.026	
9	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.49	-1.32	0.64	Enolase II; Eno2p
			YTX/MeOH	0.46	-1.33	0.64	
			MeOH/untreated	0.98	1.01	0.64	
10	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.019	-1.51	0.085	Cytosolic aldehyde dehydrogenase; Ald6p
			YTX/MeOH	0.14	-1.44	0.085	
			MeOH/untreated	0.73	-1.05	0.085	
11	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.4	-1.21	0.12	cytosolic aldehyde dehydrogenase
			YTX/MeOH	0.13	-1.6	0.12	
			MeOH/untreated	0.037	1.32	0.12	
12	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.55	-1.24	0.57	cytosolic aldehyde dehydrogenase
			YTX/MeOH	0.27	-1.29	0.57	
			MeOH/untreated	0.81	1.05	0.57	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
15	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.42	-1.18	0.41	Threonine synthase; Thr4p
			YTX/MeOH	0.21	-1.24	0.41	
			MeOH/untreated	0.75	1.05	0.41	
16	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.9	-1.03	0.85	Aromatic aminotransferase I; Aro8p
			YTX/MeOH	0.64	-1.15	0.85	
			MeOH/untreated	0.73	1.12	0.85	
17	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.066	-1.27	0.013	<i>Chain D, Crystal Structure Of Yeast Mitochondrial F1-ATPase (Protein identification score was not significant)</i>
			YTX/MeOH	0.0065	-1.5	0.013	
			MeOH/untreated	0.13	1.19	0.013	
18	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.83	-1.07	0.71	Enolase II; Eno2p
			YTX/MeOH	0.42	-1.19	0.71	
			MeOH/untreated	0.6	1.11	0.71	
19	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.75	-1.12	0.74	Enolase II; Eno2p
			YTX/MeOH	0.45	-1.21	0.74	
			MeOH/untreated	0.71	1.08	0.74	
			YTX/untreated	0.79	-1.1	0.77	Enolase II; Eno2p
			YTX/MeOH	0.46	-1.18	0.77	
			MeOH/untreated	0.71	1.07	0.77	
20	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.39	-1.47	0.6	Enolase II; Eno2p
			YTX/MeOH	0.61	-1.31	0.6	
			MeOH/untreated	0.7	-1.12	0.6	
22	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.87	-1.03	0.86	Fructose 1,6-bisphosphate aldolase; Fba1p
			YTX/MeOH	0.66	-1.11	0.86	
			MeOH/untreated	0.69	1.08	0.86	
23	BY4741 1.2 μ M YTX	4-7	YTX/untreated		-1.31		Fructose 1,6-bisphosphate aldolase; Fba1p
			YTX/MeOH		-1.2		
			MeOH/untreated		-1.1		
25	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.15	-1.16	0.085	Subunit d of the five-subunit V0 integral membrane domain of vacuolar H ⁺ -ATPase (V-ATPase); Vma6p
			YTX/MeOH	0.034	-1.2	0.085	
			MeOH/untreated	0.61	1.04	0.085	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
26	BY4741 1.2 μ M YTX	4-7	YTX/untreated	0.86	1.03	0.83	Chain A, Electrophilic Catalysis In Triosephosphate Isomerase: The Role Of Histidine-95
			YTX/MeOH	0.6	-1.07	0.83	
			MeOH/untreated	0.61	1.11	0.83	
27	BY4741 1.2 μ M YTX	6-11	YTX/untreated	0.7	-1.06	0.91	pyruvate kinase
			YTX/MeOH	0.88	-1.03	0.91	
			MeOH/untreated	0.77	-1.03	0.91	
28 & 29	BY4741 1.2 μ M YTX	6-11	YTX/untreated	0.85	-1.03	0.98	Chain A, Circularly Permuted Phosphoglycerate Kinase; Pgk P72
			YTX/MeOH	0.89	-1.07	0.98	
			MeOH/untreated	0.97	1.04	0.98	
32	BY4741 1.2 μ M YTX	6-11	YTX/untreated	0.014	-1.75	0.046	Chain E, Saccharomyces Cerevisiae Phosphoglycerate Mutase
			YTX/MeOH	0.084	-1.64	0.046	
			MeOH/untreated	0.71	-1.06	0.046	
33	BY4741 1.2 μ M YTX	6-11	YTX/untreated	0.93	-1.03	0.93	Protein component of the small (40S) ribosomal subunit; Rps5p
			YTX/MeOH	0.78	1.07	0.93	
			MeOH/untreated	0.74	-1.1	0.93	
34	BY4741 12 μ M YTX (stationary)	4-7	YTX/untreated	0.035	-1.38	0.0061	N/A
			YTX/MeOH	0.0048	-1.71	0.0061	
			MeOH/untreated	0.026	-1.34	0.012	
35	BY4741 12 μ M YTX (stationary)	4-7	YTX/untreated	0.048	1.34	0.016	N/A
			YTX/MeOH	0.032	1.81	0.016	
			MeOH/untreated	0.091	1.24	0.0061	
36	BY4741 12 μ M YTX (stationary)	4-7	YTX/untreated	0.032	1.43	0.0066	N/A
			YTX/MeOH	0.015	1.74	0.0066	
			MeOH/untreated	0.095	-1.27	0.031	
37	BY4741 12 μ M YTX (stationary)	4-7	YTX/untreated	0.086	1.48	0.024	N/A
			YTX/MeOH	0.0036	1.78	0.024	
			MeOH/untreated	0.11	-1.44	0.029	
38	BY4741 12 μ M YTX (stationary)	4-7	YTX/untreated	0.057	-1.42	0.023	N/A
			YTX/MeOH	0.044	-1.53	0.023	
			MeOH/untreated	0.35	1.08	0.012	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
39	BY4741 12 μ M YTX (stationary)	4-7	YTX/untreated	0.13	-1.52	0.048	N/A
			YTX/MeOH	0.046	-1.92	0.048	
			MeOH/untreated	0.27	-1.21	0.024	
41	BY4741 12 μ M YTX (log)	4-7	YTX/untreated	0.025	1.58	0.025	Unknown (Protein identification score was not significant)
			YTX/MeOH	0.86	1.02	0.025	
			MeOH/untreated	0.05	1.55	0.025	
43	BY4741 12 μ M YTX (log)	4-7	YTX/untreated	0.21	1.54	0.13	YLL024Cp-like protein AWRI1631
			YTX/MeOH	0.5	-1.1	0.13	
			MeOH/untreated	0.12	1.7	0.13	
44	BY4741 12 μ M YTX (log)	4-7	YTX/untreated	0.16	1.44	0.077	ATPase; Ssa1p
			YTX/MeOH	0.31	-1.11	0.077	
			MeOH/untreated	0.083	1.6	0.077	
45	BY4741 12 μ M YTX (log)	4-7	YTX/untreated	0.013	1.63	0.0072	Unknown (Protein identification score was not significant)
			YTX/MeOH	0.79	1.02	0.0072	
			MeOH/untreated	0.013	1.59	0.0072	
48	BY4741 12 μ M YTX (log)	4-7	YTX/untreated	0.017	1.5	0.025	N/A
			YTX/MeOH	0.65	-1.06	0.025	
			MeOH/untreated	0.042	1.59	0.025	
49	BY4741 12 μ M YTX (log)	4-7	YTX/untreated	0.0015	1.75	0.0043	Chain A, The Structure Of Tap42 Alpha4 Subunit (Protein identification score was not significant)
			YTX/MeOH	0.55	-1.06	0.0043	
			MeOH/untreated	0.015	1.86	0.0043	
58	BY4741 12 μ M YTX (log)	4-7	YTX/untreated	0.018	1.51	0.013	No hit
			YTX/MeOH	0.99	1	0.013	
			MeOH/untreated	0.022	1.5	0.013	
60	BY4741 12 μ M YTX (log)	4-7	YTX/untreated	0.057	-1.53	0.019	N/A
			YTX/MeOH	0.1	1.09	0.019	
			MeOH/untreated	0.038	-1.66	0.019	
62	BY4741 12 μ M YTX (log)	4-7	YTX/untreated	0.31	-1.13	0.72	YLL024Cp-like protein AWRI1631
			YTX/MeOH	0.92	1.01	0.72	
			MeOH/untreated	0.56	-1.14	0.72	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
66	BY4741 12 μ M YTX (log)	4-7	YTX/untreated	0.026	-1.5	0.027	No hit
			YTX/MeOH	0.54	1.06	0.027	
			MeOH/untreated	0.053	-1.6	0.027	
72	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.047	1.63	0.018	Hypothetical protein identified by homology; Pau9p (Protein identification score was not significant)
			YTX/MeOH	0.85	-1.02	0.018	
			MeOH/untreated	0.015	1.66	0.018	
81	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.096	1.44	0.041	N/A
			YTX/MeOH	0.49	-1.08	0.041	
			MeOH/untreated	0.033	1.55	0.041	
88	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.0057	-1.56	0.0049	Ics2p (Protein identification score was not significant)
			YTX/MeOH	0.19	-1.1	0.0049	
			MeOH/untreated	0.025	-1.42	0.0049	
89	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.023	-1.55	0.036	N/A
			YTX/MeOH	0.68	-1.06	0.036	
			MeOH/untreated	0.059	-1.46	0.036	
92	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.027	-1.52	0.041	YNL022Cp-like protein AWRI1631 (Protein identification score was not significant)
			YTX/MeOH	0.96	-1.01	0.041	
			MeOH/untreated	0.058	-1.51	0.041	
93	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.061	-1.45	0.033	N/A
			YTX/MeOH	0.72	1.04	0.033	
			MeOH/untreated	0.04	-1.51	0.033	
95	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.012	-1.62	0.033	N/A
			YTX/MeOH	0.47	-1.12	0.033	
			MeOH/untreated	0.084	-1.45	0.033	
96	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.066	-1.19	0.077	Nucleoside diphosphate kinase; Ynk1p
			YTX/MeOH	0.84	1.01	0.077	
			MeOH/untreated	0.11	-1.2	0.077	
98	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.063	-1.75	0.026	Thymidylate synthase; Cdc21p (Protein identification score was not significant)
			YTX/MeOH	0.54	1.05	0.026	
			MeOH/untreated	0.048	-1.83	0.026	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
113	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.029	-1.57	0.01	<i>Ydr381wp</i> (Protein identification score was not significant)
			YTX/MeOH	0.11	-1.02	0.01	
			MeOH/untreated	0.033	-1.54	0.01	
114	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.064	-1.55	0.032	N/A
			YTX/MeOH	1	1	0.032	
			MeOH/untreated	0.044	-1.55	0.032	
115	BY4741 12 μ M YTX (log)	6-11	YTX/untreated	0.063	-1.94	0.036	N/A
			YTX/MeOH	0.78	-1.04	0.036	
			MeOH/untreated	0.036	-1.86	0.036	
123	BY4741 dsYTX	4-7	dsYTX/untreated	0.89	-1.01	0.88	Hsp90 cochaperone; Sti1p
			dsYTX/MeOH	0.61	-1.02	0.88	
			MeOH/untreated	0.8	1.01	0.88	
124	BY4741 dsYTX	4-7	dsYTX/untreated	0.032	-1.36	0.03	N/A
			dsYTX/MeOH	0.044	-1.58	0.03	
			MeOH/untreated	0.29	1.16	0.03	
127	BY4741 dsYTX	4-7	dsYTX/untreated	0.0079	1.51	0.0024	<i>Thymidylate synthase; Cdc21p</i> (Protein identification score was not significant)
			dsYTX/MeOH	0.01	1.39	0.0024	
			MeOH/untreated	0.11	1.09	0.0024	
129	BY4741 dsYTX	4-7	YTX/untreated	0.028	-1.59	0.012	Mitochondrial aldehyde dehydrogenase; Ald4p
			YTX/MeOH	0.012	-1.67	0.012	
			MeOH/untreated	0.64	1.05	0.012	
135	BY4741 dsYTX	4-7	dsYTX/untreated	0.077	1.36	0.025	N/A
			dsYTX/MeOH	0.031	1.59	0.025	
			MeOH/untreated	0.17	-1.17	0.025	
136	BY4741 dsYTX	4-7	dsYTX/untreated	0.6	-1.02	0.89	Phosphomannomutase; Sec53p
			dsYTX/MeOH	0.94	-1	0.89	
			MeOH/untreated	0.78	-1.01	0.89	
137	BY4741 dsYTX	4-7	YTX/untreated	0.29	-1.12	0.4	Egd2p
			YTX/MeOH	0.91	1.01	0.4	
			MeOH/untreated	0.25	-1.13	0.4	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
138	BY4741 dsYTX	4-7	dsYTX/untreated	0.0046	-2.37	0.0039	protein GRE1 RM11-1a
			dsYTX/MeOH	0.021	-1.84	0.0039	
			MeOH/untreated	0.11	-1.28	0.0039	
142	BY4741 dsYTX	4-7	dsYTX/untreated	0.0086	-1.52	0.0061	<i>Hypothetical protein identified by homology; Pau9p (Protein identification score was not significant)</i>
			dsYTX/MeOH	0.017	-1.39	0.0061	
			MeOH/untreated	0.26	-1.09	0.0061	
143	BY4741 dsYTX	4-7	dsYTX/untreated	0.056	-1.56	0.027	N/A
			dsYTX/MeOH	0.043	-1.54	0.027	
			MeOH/untreated	0.86	-1.02	0.027	
147	BY4741 dsYTX	4-7	dsYTX/untreated	0.94	-1.01	0.32	Chain A, H48c Yeast Cu(II)Zn SUPEROXIDE DISMUTASE Structure
			dsYTX/MeOH	0.27	1.26	0.32	
			MeOH/untreated	0.24	-1.28	0.32	
150	BY4741 dsYTX	4-7	dsYTX/untreated	0.046	1.34	0.18	Chain G, Yeast Cytochrome Bc1 Complex With Bound Substrate Cytochrome C
			dsYTX/MeOH	0.29	1.18	0.18	
			MeOH/untreated	0.5	1.13	0.18	
151	BY4741 dsYTX	4-7	dsYTX/untreated	0.0025	-1.54	0.00081	N/A
			dsYTX/MeOH	0.0057	-1.55	0.00081	
			MeOH/untreated	0.88	1	0.00081	
154	BY4741 dsYTX	4-7	dsYTX/untreated	0.026	-2.08	0.018	<i>Chain O, Structure Of The Ribosomal 80s-Eef2-Sordarin Complex (Protein identification score was not significant)</i>
			dsYTX/MeOH	0.038	-1.63	0.018	
			MeOH/untreated	0.2	-1.27	0.018	
155	BY4741 dsYTX	6-11	dsYTX/untreated	0.06	-1.66	0.032	N/A
			dsYTX/MeOH	0.042	-1.8	0.032	
			MeOH/untreated	0.53	1.09	0.032	
156	BY4741 dsYTX	6-11	YTX/untreated	0.0061	-2.13	0.024	N/A
			YTX/MeOH	0.046	-2.33	0.024	
			MeOH/untreated	0.74	1.09	0.024	
157	BY4741 dsYTX	6-11	dsYTX/untreated	0.051	-1.87	0.024	N/A
			dsYTX/MeOH	0.044	-2.28	0.024	
			MeOH/untreated	0.23	1.22	0.024	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
158	ADA 12 μ M YTX (log)	4-7	YTX/untreated	0.034	-1.58	0.009	N/A
			YTX/MeOH	0.018	-1.78	0.009	
			MeOH/untreated	0.15	1.13	0.009	
159	ADA 12 μ M YTX (log)	4-7	YTX/untreated	0.085	-1.38	0.028	N/A
			YTX/MeOH	0.036	-1.53	0.028	
			MeOH/untreated	0.19	1.11	0.028	
160	ADA 12 μ M YTX (log)	4-7	YTX/untreated	0.013	-1.69	0.0024	N/A
			YTX/MeOH	0.0018	-1.74	0.0024	
			MeOH/untreated	0.59	1.03	0.0024	
161	ADA 12 μ M YTX (log)	4-7	YTX/untreated	0.1	-1.9	0.045	N/A
			YTX/MeOH	0.018	-2.18	0.045	
			MeOH/untreated	0.53	1.15	0.045	
162	ADA 12 μ M YTX (log)	4-7	YTX/untreated	0.07	-1.67	0.041	N/A
			YTX/MeOH	0.036	-1.79	0.041	
			MeOH/untreated	0.68	1.07	0.041	
163	ADA 12 μ M YTX (log)	4-7	YTX/untreated	0.02	-2.17	0.02	N/A
			YTX/MeOH	0.042	-1.99	0.02	
			MeOH/untreated	0.62	-1.09	0.02	
164	ADA dsYTX	4-7	dsYTX/untreated	0.095	1.75	0.066	<i>Phosphatidylinositol transfer protein (PITP); Pdr16p</i> (Protein identification score was not significant)
			dsYTX/MeOH	0.011	1.68	0.066	
			MeOH/untreated	0.82	1.04	0.066	
165	ADA dsYTX	4-7	dsYTX/untreated	0.021	2.41	0.0059	<i>YKR101Wp-like protein AWRI1631</i> (Protein identification score was not significant)
			dsYTX/MeOH	0.0099	2.5	0.0059	
			MeOH/untreated	0.79	-1.04	0.0059	
166	ADA dsYTX	4-7	dsYTX/untreated	0.0063	2.04	0.02	<i>Hypothetical protein identified by homology; Pau9p</i> (Protein identification score was not significant)
			dsYTX/MeOH	0.043	2.06	0.02	
			MeOH/untreated	0.91	-1.01	0.02	
168	ADA dsYTX	4-7	dsYTX/untreated	0.065	1.61	0.045	Chain A, Crystal Structure Of An Hsp90-Sba1 Closed Chaperone Complex
			dsYTX/MeOH	0.045	1.66	0.045	
			MeOH/untreated	0.85	-1.03	0.045	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
170	ADA dsYTX	4-7	dsYTX/untreated	0.027	1.78	0.014	<i>Chain A, Crystal Structure Analysis Of Sse1, A Yeast Hsp110 (Protein identification score was not significant)</i>
			dsYTX/MeOH	0.026	1.89	0.014	
			MeOH/untreated	0.61	-1.06	0.014	
172	ADA dsYTX	4-7	dsYTX/untreated	0.14	1.42	0.035	N/A
			dsYTX/MeOH	0.013	1.85	0.035	
			MeOH/untreated	0.2	-1.31	0.035	
173	ADA dsYTX	4-7	dsYTX/untreated	0.1	1.85	0.049	YLL024Cp-like protein AWRI1631
			dsYTX/MeOH	0.036	2.04	0.049	
			MeOH/untreated	0.67	-1.1	0.049	
174	ADA dsYTX	4-7	dsYTX/untreated	0.087	1.62	0.052	ATPase involved in protein folding and nuclear localisation signal (NLS)- directed nuclear transport; Ssa1p
			dsYTX/MeOH	0.023	1.58	0.052	
			MeOH/untreated	0.81	1.03	0.052	
175	ADA dsYTX	4-7	dsYTX/untreated	0.074	1.32	0.034	N/A
			dsYTX/MeOH	0.042	1.66	0.034	
			MeOH/untreated	0.18	-1.26	0.034	
176	ADA dsYTX	4-7	dsYTX/untreated	0.054	1.56	0.024	N/A
			dsYTX/MeOH	0.012	1.56	0.024	
			MeOH/untreated	0.98	-1	0.024	
177	ADA dsYTX	4-7	dsYTX/untreated	0.024	1.95	0.021	<i>Hypothetical protein identified by homology; Pau9p (Protein identification score was not significant)</i>
			dsYTX/MeOH	0.05	1.82	0.021	
			MeOH/untreated	0.64	1.07	0.021	
179	ADA dsYTX	4-7	dsYTX/untreated	0.06	1.51	0.036	N/A
			dsYTX/MeOH	0.025	1.61	0.036	
			MeOH/untreated	0.66	-1.07	0.036	
181	ADA dsYTX	4-7	dsYTX/untreated	0.012	1.5	0.013	<i>Hypothetical protein identified by homology; Pau9p (Protein identification score was not significant)</i>
			dsYTX/MeOH	0.03	1.66	0.013	
			MeOH/untreated	0.33	-1.1	0.013	
184	ADA dsYTX	4-7	dsYTX/untreated	0.074	1.6	0.029	<i>One of six ATPases of the 19S regulatory particle of the 26S proteasome; Rpt6p (Protein identification score was not significant)</i>
			dsYTX/MeOH	0.047	1.82	0.029	
			MeOH/untreated	0.22	-1.14	0.029	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
186	ADΔ dsYTX	4-7	dsYTX/untreated	0.19	-1.19	0.034	N/A
			dsYTX/MeOH	0.036	-1.5	0.034	
			MeOH/untreated	0.085	1.26	0.034	
188	ADΔ dsYTX	4-7	dsYTX/untreated	0.062	1.31	0.042	YLL024Cp-like protein AWRI1631
			dsYTX/MeOH	0.09	1.26	0.042	
			MeOH/untreated	0.38	1.04	0.042	
190	ADΔ dsYTX	4-7	dsYTX/untreated	0.13	1.27	0.072	actin
			dsYTX/MeOH	0.042	1.36	0.072	
			MeOH/untreated	0.52	-1.08	0.072	
191	ADΔ dsYTX	4-7	dsYTX/untreated	0.044	1.63	0.019	YGL071Wp-like protein AWRI1631 (Protein identification score was not significant)
			dsYTX/MeOH	0.015	1.65	0.019	
			MeOH/untreated	0.94	-1.01	0.019	
195	ADΔ dsYTX	4-7	dsYTX/untreated	0.022	-1.33	0.003	N/A
			dsYTX/MeOH	0.0078	-1.58	0.003	
			MeOH/untreated	0.031	1.19	0.003	
196	ADΔ dsYTX	4-7	dsYTX/untreated	0.08	-1.22	0.0063	N/A
			dsYTX/MeOH	0.011	-1.59	0.0063	
			MeOH/untreated	0.023	1.3	0.0063	
198	ADΔ dsYTX	4-7	dsYTX/untreated	0.053	-1.42	0.036	N/A
			dsYTX/MeOH	0.029	-1.5	0.036	
			MeOH/untreated	0.65	1.06	0.036	
199	ADΔ dsYTX	4-7	dsYTX/untreated	0.067	-1.44	0.019	N/A
			dsYTX/MeOH	0.035	-1.66	0.019	
			MeOH/untreated	0.065	1.15	0.019	
200	ADΔ dsYTX	4-7	dsYTX/untreated	0.0005	-2.26	0.00027	Mitochondrial translational activator YJM789 (Protein identification score was not significant)
			dsYTX/MeOH	0.0023	-2.36	0.00027	
			MeOH/untreated	0.43	1.04	0.00027	
201	ADΔ dsYTX	4-7	dsYTX/untreated	0.45	1.16	0.54	conserved protein YJM789
			dsYTX/MeOH	0.36	1.15	0.54	
			MeOH/untreated	0.94	1.01	0.54	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
204	ADΔ dsYTX	4-7	dsYTX/untreated	0.011	-1.34	0.0083	N/A
			dsYTX/MeOH	0.021	-1.51	0.0083	
			MeOH/untreated	0.18	1.12	0.0083	
205	ADΔ dsYTX	4-7	dsYTX/untreated	0.028	-1.7	0.02	<i>Putative protein of unknown function; Ylr146w-ap (Protein identification score was not significant)</i>
			dsYTX/MeOH	0.023	-1.81	0.02	
			MeOH/untreated	0.68	1.06	0.02	
206	ADΔ dsYTX	4-7	dsYTX/untreated	0.019	-1.37	0.005	<i>One of six subunits of the RNA polymerase III transcription initiation factor complex (TFIIIC); Tfc1p (Protein identification score was not significant)</i>
			dsYTX/MeOH	0.0056	-1.53	0.005	
			MeOH/untreated	0.19	1.11	0.005	
207	ADΔ dsYTX	4-7	dsYTX/untreated	0.69	1.06	0.52	Chain G, Yeast Cytochrome Bc1 Complex With Bound Substrate Cytochrome C
			dsYTX/MeOH	0.37	1.12	0.52	
			MeOH/untreated	0.29	-1.07	0.52	
208	ADΔ dsYTX	4-7	dsYTX/untreated	0.011	-1.28	0.027	YGR254Wp-like protein AWRI1631
			dsYTX/MeOH	0.046	-1.42	0.027	
			MeOH/untreated	0.33	1.11	0.027	
209	ADΔ dsYTX	4-7	dsYTX/untreated	0.002	-1.45	0.029	YGR254Wp-like protein AWRI1631
			dsYTX/MeOH	0.052	-1.65	0.029	
			MeOH/untreated	0.4	1.14	0.029	
210	ADΔ dsYTX	4-7	dsYTX/untreated		-1.72		Enolase II; Eno2p
			dsYTX/MeOH		-1.57		
			MeOH/untreated		-1.1		
211	ADΔ dsYTX	4-7	dsYTX/untreated	0.0025	-1.49	0.022	dehydrogenase isozyme I, alcohol
			dsYTX/MeOH	0.049	-1.54	0.022	
			MeOH/untreated	0.8	1.03	0.022	
213	ADΔ dsYTX	6-11	dsYTX/untreated	0.02	1.41	0.0036	N/A
			dsYTX/MeOH	0.0058	1.58	0.0036	
			MeOH/untreated	0.11	-1.13	0.0036	
214	ADΔ dsYTX	6-11	dsYTX/untreated	0.067	1.82	0.027	N/A
			dsYTX/MeOH	0.036	1.95	0.027	
			MeOH/untreated	0.57	-1.07	0.027	

Spot #	Experiment	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
215	ADΔ dsYTX	6-11	dsYTX/untreated	0.11	1.69	0.037	Translational elongation factor EF-1 alpha; Tef2p, TEF1
			dsYTX/MeOH	0.026	2.1	0.037	
			MeOH/untreated	0.3	-1.24	0.037	
217	ADΔ dsYTX	6-11	dsYTX/untreated	0.014	-2.09	0.02	<i>Unnamed protein product</i> (Protein identification score was not significant)
			dsYTX/MeOH	0.044	-2.14	0.02	
			MeOH/untreated	0.95	1.02	0.02	
218	ADΔ dsYTX	6-11	dsYTX/untreated	0.42	-1.11	0.43	pyruvate kinase
			dsYTX/MeOH	0.13	-1.14	0.43	
			MeOH/untreated	0.81	1.03	0.43	
219	ADΔ dsYTX	6-11	dsYTX/untreated	0.0016	-1.59	0.017	<i>Alcohol dehydrogenase isoenzyme V; Adh5p</i> (Protein identification score was not significant)
			dsYTX/MeOH	0.038	-1.82	0.017	
			MeOH/untreated	0.39	1.15	0.017	
220	ADΔ dsYTX	6-11	dsYTX/untreated	0.2	-1.33	0.093	pyruvate kinase
			dsYTX/MeOH	0.014	-1.49	0.093	
			MeOH/untreated	0.47	1.12	0.093	

3.2 HL-60 cells

HL60 cells were cultured until they were approximately 80% confluent. The cells were then divided into 10 mL cultures containing 9×10^6 cells. Samples were then treated with 1 μ M of YTX or dsYTX. Control samples were treated with methanol (0.02% v/v final concentration).

3.2.1 Morphological analysis

The morphology of all HL60 samples was observed at 0 h and 24 h after incubation with toxin or methanol control. No obvious morphological differences were observed in flasks of cells treated with YTX or control samples (Fig. 12). After 24 h of incubation flasks of cells treated with dsYTX had clumps of floating cells (Fig. 13). Staining of the cells with trypan blue indicated that cells in these clumps were dead.

Figure 12 HL60 cells in culture at 0 and 24 h after addition of toxin at 20x magnification. No morphological differences were observed in flasks of cells treated with toxin compared with control cells treated with methanol, with the exception of clumps forming in dsYTX treated cultures.

Figure 13 HL60 cells after 24 h incubation with dsYTX at 10x magnification. Arrows indicate aggregated cells.

Analysis of trypan blue staining gave a percentage cell viability of 86.1% at 0 h. After 24 h of incubation the percentage cell viability was 91.7% for control samples incubated with methanol, and 79.0% and 67.7% for samples incubated YTX and dsYTX respectively.

3.2.2 2D-DIGE analysis

The effects of YTX and dsYTX on HL60 cells were examined using 2D DIGE. The goal was to identify proteins that changed for comparison with known effects of YTX on HepG2 cells (Young *et al.*, 2009), and to the microtubule stabilising agent Pel A on HL60 cells (Wilmes *et al.*, 2012). The protein extracts treated with 1 μ M YTX or dsYTX and control samples (0.02% methanol) were analysed for changes in levels of protein expression by 2D DIGE. A change in protein abundance ≥ 2 -fold, $p \leq 0.01$ was again considered significant. MALDI mass fingerprinting was used to identify protein spots excised from 2D gels.

Table 6 Protein spots detected as changing by DeCyder analysis, in HL60 cells in response to YTX or dsYTX. The numbers in brackets are the number of protein spots which match the fold and p -value criteria when the FDR filter was not applied.

	Comparison of change						Total spots
	YTX/MeOH		dsYTX/MeOH		dsYTX/YTX		
	≥ 1.5-fold, p ≤ 0.05	≥ 2-fold, p ≤ 0.01	≥ 1.5-fold, p ≤ 0.05	≥ 2-fold, p ≤ 0.01	≥ 1.5-fold, p ≤ 0.05	≥ 2-fold, p ≤ 0.01	
pH 4-7	2(51)	1(16)	61(86)	0(26)	0(6)	0(0)	2413
pH 6-11	0(12)	0(0)	0(22)	0(6)	0(2)	0(0)	2439

2D-DIGE analysis detected a total of 119 protein spots undergoing a change in abundance by comparing all conditions (when the FDR filter was not applied). Thirty-eight protein spots were identified as changing significantly (≥ 2 -fold, $p \leq 0.01$) when comparing toxin treated cells to controls. Ten protein spots showed significant changes in abundance in response to both toxins, six changed only in YTX treated cells and 22 changed in cells treated with dsYTX. Of the six significant YTX specific changes, four were also possibly changing in response to dsYTX (≥ 1.5 -fold (but less than 2), $p \leq 0.05$). Of the 22 dsYTX specific changes, 13 were also possibly changing with YTX. Potential changes (≥ 1.5 -fold (but less than 2-fold), $p \leq 0.05$) were also detected in an additional 80 proteins. Eight were specific to YTX, 46 were specific to dsYTX and 26 were detected in both. Figure 14 shows the location of protein spots identified by DIGE analysis as undergoing a change in abundance, on pH 4-7 and 6-11 Coomassie stained polyacrylamide gels. Figures 15 and 16 summarise protein changes in response to toxin treatment (when the FDR filter was not or was applied, respectively).

Figure 14 Coomassie blue stained gel with protein spots of interest from HL60 cells treated with YTX or dSTYX. Black numbers are protein spots which did not undergo a change of abundance in response to toxin treatment but were extracted and successfully identified by MALDI analysis. Red represents increase in abundance of at least 2-fold, $p \leq 0.01$. **Pink** represents an increase in abundance of between 1.5 and 2-fold, $p \leq 0.05$. **Green** represents a decrease in abundance of at least 2-fold, $p \leq 0.01$. **Pale green** represents a decrease in abundance between 1.5 and 2-fold, $p \leq 0.05$.

Figure 15 Distribution of protein changes detected by 2D DIGE for toxin vs. methanol when the FDR was not applied. **Red** numbers are those that underwent an increase in abundance in response to toxin. **Green** numbers are those proteins whose abundance decreased in response to toxin. Proteins almost always responded in the same direction in response to either YTX or dsYTX. The one exception to this is spot number 58. * Denotes proteins which also showed a difference in abundance in dsYTX treated cells compared to YTX. Numbers 1-92 are proteins in the pH 4-7 range. Numbers 93-119 are proteins in the pH 6-11 range.

Figure 16 Distribution of protein changes detected by 2D DIGE for toxin vs. methanol when the FDR was applied. **Red** numbers are those that underwent an increase in abundance in response to toxin. **Green** numbers are those proteins whose abundance decreased in response to toxin. Numbers 1-92 are proteins in the pH 4-7 range. Numbers 93-119 are proteins in the pH 6-11 range.

Protein spots which changed in response to both toxins seemed to be affected similarly by YTX and dsYTX. In almost all such cases a protein which had an increased abundance in YTX treated cells also had an increased abundance in dsYTX treated cells and vice versa. Of the 53 proteins with at least a 1.5-fold change in abundance ($p \leq 0.05$), 36 showed similar fold changes with either toxin. Seventeen changed at least 2-fold ($p \leq 0.01$) with only one toxin, while changing between 1.5 and 2-fold with the other toxin.

The only exception to proteins being affected in the same way was protein spot 58. Although neither toxin caused a change in abundance meeting the required cut off criteria (≥ 1.5 -fold, $p \leq 0.05$), a possible decrease was detected in dsYTX treated cells compared to those treated with YTX (-1.65 fold, $t = 0.0016$). YTX caused an apparent increase of 1.49 fold ($t = 0.033$). DsYTX did not cause a change in abundance (-1.11 fold compared to methanol control, $t = 0.54$).

When the FDR filter was applied (Fig. 16) 62 protein spots were identified as potentially changing. The majority (60 spots) of these were at the $p \leq 0.05$ level only, and in response to dsYTX (59 spots). Protein spot 82 had a significant change in abundance (≥ 2 -fold, $p \leq 0.01$) in response to YTX treatment but only reached the $p \leq 0.05$ level for dsYTX treatment.

3.2.3 Protein identification by peptide mass fingerprinting

Sixty-four of the 119 protein spots identified as changing by 2D-DIGE analysis were extracted from gels for identification by MALDI-TOF/TOF for peptide mass fingerprinting. Thirteen of these were successfully identified with a significant score at the $p \leq 0.05$ level. An additional 13 protein spots which showed no change in abundance were also identified. Tables 7 and 8 list the results of the DIGE analysis and the identity of the protein spots.

Four identified proteins were similarly affected by YTX and dsYTX in HL60 cells. Stress response proteins GRP78 (BiP) precursor and HSP60 (chaperonin) showed significant increases in abundance in dsYTX treated cells (approximately 2 and 2.5 fold

respectively, $p \leq 0.01$, Table 7). However when the FDR filter was applied they only reached the $p \leq 0.05$ level. An apparent increase was seen in these proteins in YTX treated cells, but did not reach the 2-fold criterion for significance. The mitochondrial glycoprotein p32/tat-associated protein (chain A) and protein disulfide isomerase related protein 5 showed apparent changes in both YTX and dsYTX treated HL60, but these did not make the criteria for significance (≥ 2 -fold, $p \leq 0.01$). When the FDR filter was applied, both of the detected changes remained at the ≥ 1.5 -fold, $p \leq 0.05$ level.

Actin, hnRNP A, and BiP all appeared to have increased abundances in dsYTX treated HL60 cells, with actin having a significant increase (Table 7). These proteins all decreased in abundance in YTX treated HepG2 cells (Fig. 17, Young *et al.*, 2009). No identified proteins showed changes in abundance in both YTX treated HL60 and HepG2 cells.

Spots number 2 and 106 showed a possible increase in abundance when treated with dsYTX compared to controls. These were identified as tumour rejection antigen (gp96) 1 and nucleolin, respectively (Table 7). The levels of the gp96 precursor have been shown to be affected by YTX in HepG2 cells (Young *et al.*, 2009). Application of the FDR filter resulted in the change detected in nucleolin no longer reaching the p-value cut-off criteria. The gp96 precursor change still met the ≥ 1.5 -fold, $p \leq 0.05$ cut-off.

Spot 96 showed an apparent decrease in HL60 cells treated with YTX. This was matched by MASCOT to an unnamed protein, containing a K-homology domain (type 1). This domain is found in a range of proteins of the ribosome and in transcription factors. A BLAST search of the top ranked protein match indicated that this protein may be the far upstream element binding protein 1 (Supplementary Data CD). This protein is involved in c-myc expression. C-myc was one of the predicted hub molecules identified in the pathway affected by Pel A in HL60 cells detected by IPA.

No identified proteins were changing in common between HL60 cells treated with YTX, Pel A, or PTX (Fig. 18). Actin and possibly tumour rejection antigen 1 increased in abundance in HL60 cells treated with dsYTX or Pel A (Fig. 19). BiP appeared to increase in abundance in dsYTX and Pel A treated cells. In PTX treated HL60 cells and YTX treated HepG2 cells however it decreased (Wilmes *et al.*, 2012; Young *et al.*, 2009).

HnRNP A showed an apparent increase in response to dsYTX. It was also found to increase in Pel A or PTX treated HL60 cells (Wilmes *et al.*, 2011, 2012). In YTX treated HepG2 cells however it was shown to decrease (Young *et al.*, 2009).

Spot 82 still met the criteria for a significant change in abundance in response to YTX compared with controls, when the FDR filter was applied. The top-ranked match for this protein was the signal recognition particle; however the identification did not have a significant Mascot score.

A total of 288 spots were picked from HL60 gels. Twenty-four of these were identified with a significant Mascot score. Thirty-one had MS spectra of comparable quality to those that were identified, but did not provide a significant match.

Table 7 HL60 Protein abundance change (Av. Ratio) and associated statistics acquired from DeCyder and protein identification where applicable of proteins of interest and picked protein spots as numbered in Figs. 14-16. N/A for the protein name indicates that a protein spot was not selected for identification due to low abundance. An absence of DeCyder statistics is indicative that a protein spot was not present in all protein spot maps. Av. Ratio is the protein volume of the first condition compared to the second in the comparison. For example negative value when comparing dsYTX/untreated means that the protein has undergone a decrease in abundance in response to dsYTX respective to the untreated control. * Indicates protein identification is not the top-ranked match.

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
1	4-7	YTX/MeOH	9.50E-05	1.87	2.20E-05	<i>Immunoglobulin heavy chain variable region</i> (protein identification score was not significant)
		dsYTX/MeOH	0.00056	2.13	2.20E-05	
		dsYTX/YTX	0.11	1.14	2.20E-05	
2	4-7	YTX/MeOH	0.063	1.27	0.0014	tumour rejection antigen (gp96) 1
		dsYTX/MeOH	0.00047	1.63	0.0014	
		dsYTX/YTX	0.033	1.28	0.0014	
3	4-7	YTX/MeOH	0.0089	-1.74	0.0049	N/A
		dsYTX/MeOH	0.01	-1.89	0.0049	
		dsYTX/YTX	0.58	-1.08	0.0049	
4	4-7	YTX/MeOH	0.019	1.4	0.003	<i>opioid growth factor receptor and/or S100 calcium-binding protein A8</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0053	1.55	0.003	
		dsYTX/YTX	0.21	1.1	0.003	
5	4-7	YTX/MeOH	0.081	1.61	0.0089	<i>MHC class I antigen</i> (protein identification score was not significant)
		dsYTX/MeOH	0.00051	2.02	0.0089	
		dsYTX/YTX	0.21	1.26	0.0089	
6	4-7	YTX/MeOH	0.018	1.5	0.0046	N/A
		dsYTX/MeOH	0.013	1.65	0.0046	
		dsYTX/YTX	0.23	1.1	0.0046	
7	4-7	YTX/MeOH	0.001	2.37	8.30E-05	<i>hCG2015481</i> (protein identification score was not significant)
		dsYTX/MeOH	0.00017	2.9	8.30E-05	
		dsYTX/YTX	0.21	1.22	8.30E-05	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
8	4-7	YTX/MeOH	0.0023	1.88	0.00019	hCG2044129 (protein identification score was not significant)
		dsYTX/MeOH	0.00064	2.31	0.00019	
		dsYTX/YTX	0.12	1.23	0.00019	
9	4-7	YTX/MeOH	0.0044	1.65	0.00018	GRP78 precursor
		dsYTX/MeOH	0.00046	2.07	0.00018	
		dsYTX/YTX	0.045	1.25	0.00018	
10	4-7	YTX/MeOH	0.013	1.49	0.0006	
		dsYTX/MeOH	0.00061	1.82	0.0006	
		dsYTX/YTX	0.064	1.22	0.0006	
11	4-7	YTX/MeOH	0.043	1.44	0.0042	
		dsYTX/MeOH	0.002	1.7	0.0042	
		dsYTX/YTX	0.15	1.18	0.0042	
12	4-7	YTX/MeOH	0.054	1.54	0.0096	BiP protein
		dsYTX/MeOH	0.0033	1.8	0.0096	
		dsYTX/YTX	0.28	1.17	0.0096	
13	4-7	YTX/MeOH	0.022	1.99	0.0032	PRO1095 (protein identification score was not significant)
		dsYTX/MeOH	0.0038	2.57	0.0032	
		dsYTX/YTX	0.18	1.3	0.0032	
14	4-7	YTX/MeOH	0.11	1.58	0.021	N/A
		dsYTX/MeOH	0.0046	2.01	0.021	
		dsYTX/YTX	0.24	1.28	0.021	
15	4-7	YTX/MeOH	0.001	-1.76	0.001	N/A
		dsYTX/MeOH	0.0062	-1.71	0.001	
		dsYTX/YTX	0.87	1.03	0.001	
16	4-7	YTX/MeOH	0.18	-1.23	0.015	N/A
		dsYTX/MeOH	0.017	-1.61	0.015	
		dsYTX/YTX	0.043	-1.3	0.015	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
17	4-7	YTX/MeOH	0.044	-1.41	0.0078	N/A
		dsYTX/MeOH	0.0069	-1.66	0.0078	
		dsYTX/YTX	0.18	-1.18	0.0078	
18	4-7	YTX/MeOH	0.0038	-1.62	0.00071	<i>Unnamed protein product (highly similar to absent in melanoma 1 protein) (protein identification score was not significant)</i>
		dsYTX/MeOH	0.0024	-1.95	0.00071	
		dsYTX/YTX	0.11	-1.2	0.00071	
19	4-7	YTX/MeOH	0.034	-1.37	0.0029	<i>unnamed protein product {contains Ribosomal L3 superfamily domain; highly similar to mitochondrial 39s ribosomal protein L3} (protein identification score was not significant)</i>
		dsYTX/MeOH	0.006	-1.7	0.0029	
		dsYTX/YTX	0.034	-1.24	0.0029	
20	4-7	YTX/MeOH	0.0033	-1.63	0.00027	<i>hCG2040114, isoform CRA_b (protein identification score was not significant)</i>
		dsYTX/MeOH	0.0012	-2.04	0.00027	
		dsYTX/YTX	0.043	-1.25	0.00027	
21	4-7	YTX/MeOH	0.003	-1.59	0.00029	<i>hCG2042223 (protein identification score was not significant)</i>
		dsYTX/MeOH	0.00029	-1.63	0.00029	
		dsYTX/YTX	0.86	-1.02	0.00029	
22	4-7	YTX/MeOH	0.014	-2.81	0.0019	<i>translation initiation factor eIF-4gamma (protein identification score was not significant)</i>
		dsYTX/MeOH	0.0061	-2.58	0.0019	
		dsYTX/YTX	0.36	1.09	0.0019	
23	4-7	YTX/MeOH	0.0013	-2.68	0.00015	<i>cytotoxic T-lymphocyte-associated protein 4 (protein identification score was not significant)</i>
		dsYTX/MeOH	0.00037	-2.55	0.00015	
		dsYTX/YTX	0.63	1.05	0.00015	
24	4-7	YTX/MeOH	0.12	1.49	0.026	<i>ferritin light polypeptide (protein identification score was not significant)</i>
		dsYTX/MeOH	0.015	2.1	0.026	
		dsYTX/YTX	0.18	1.41	0.026	
25	4-7	YTX/MeOH	0.056	1.65	0.007	<i>immunoglobulin lambda chain variable region (protein identification score was not significant)</i>
		dsYTX/MeOH	0.0046	2.38	0.007	
		dsYTX/YTX	0.12	1.44	0.007	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
26	4-7	YTX/MeOH	0.0075	1.76	0.0059	N/A
		dsYTX/MeOH	0.0093	1.67	0.0059	
		dsYTX/YTX	0.74	-1.06	0.0059	
27	4-7	YTX/MeOH	0.012	-1.45	0.0083	N/A
		dsYTX/MeOH	0.014	-1.54	0.0083	
		dsYTX/YTX	0.53	-1.07	0.0083	
28	4-7	YTX/MeOH	0.00038	2.05	4.20E-05	<i>hCG2012006</i> (protein identification score was not significant)
		dsYTX/MeOH	0.00026	2.55	4.20E-05	
		dsYTX/YTX	0.11	1.24	4.20E-05	
29	4-7	YTX/MeOH	0.0012	2.12	6.20E-05	<i>heat shock 60kDa protein 1 (chaperonin), isoform CRA_c</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0004	2.89	6.20E-05	
		dsYTX/YTX	0.037	1.36	6.20E-05	
30	4-7	YTX/MeOH	0.0034	1.86	9.30E-05	heat shock 60kDa protein 1 (chaperonin), isoform CRA_c
		dsYTX/MeOH	0.00032	2.54	9.30E-05	
		dsYTX/YTX	0.021	1.37	9.30E-05	
31	4-7	YTX/MeOH	0.03	1.53	0.0014	<i>immunoglobulin heavy chain variable region</i> (protein identification score was not significant)
		dsYTX/MeOH	0.00093	2	0.0014	
		dsYTX/YTX	0.056	1.31	0.0014	
32	4-7	YTX/MeOH	0.3	1.22	0.026	<i>unnamed protein product {GroEL/chaperonin type I/II/chaperonin like domain.}</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0028	1.57	0.026	
		dsYTX/YTX	0.12	1.28	0.026	
33	4-7	YTX/MeOH	0.22	1.23	0.049	N/A
		dsYTX/MeOH	0.0092	1.51	0.049	
		dsYTX/YTX	0.23	1.23	0.049	
34	4-7	YTX/MeOH	0.0031	1.75	0.00021	<i>hCG2030305, isoform CRA_a</i> (protein identification score was not significant)
		dsYTX/MeOH	0.00074	2.1	0.00021	
		dsYTX/YTX	0.093	1.2	0.00021	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
35	4-7	YTX/MeOH	0.096	1.31	0.01	<i>calreticulin precursor variant</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0062	1.56	0.01	
		dsYTX/YTX	0.095	1.19	0.01	
36	4-7	YTX/MeOH	0.034	-1.72	0.037	N/A
		dsYTX/MeOH	0.031	-1.62	0.037	
		dsYTX/YTX	0.7	1.06	0.037	
37	4-7	YTX/MeOH	0.045	1.39	0.0068	N/A
		dsYTX/MeOH	0.0062	1.58	0.0068	
		dsYTX/YTX	0.18	1.14	0.0068	
38	4-7	YTX/MeOH	0.13	1.34	0.0083	N/A
		dsYTX/MeOH	0.00089	1.77	0.0083	
		dsYTX/YTX	0.1	1.32	0.0083	
39	4-7	YTX/MeOH	0.012	-1.76	0.0065	<i>NOS1 protein</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0017	-1.79	0.0065	
		dsYTX/YTX	0.98	-1.02	0.0065	
40	4-7	YTX/MeOH	0.051	1.56	0.0095	N/A
		dsYTX/MeOH	0.0009	1.8	0.0095	
		dsYTX/YTX	0.34	1.16	0.0095	
41	4-7	YTX/MeOH	0.029	-1.57	0.014	<i>unnamed protein product {non-specific BLAST hit to NAD-dependent glycerol-3-phosphate dehydrogenase C-terminus domain}</i> (protein identification score was not significant)
		dsYTX/MeOH	0.013	-1.77	0.014	
		dsYTX/YTX	0.45	-1.13	0.014	
42	4-7	YTX/MeOH	0.046	-1.5	0.0085	N/A
		dsYTX/MeOH	0.01	-1.8	0.0085	
		dsYTX/YTX	0.14	-1.2	0.0085	
43	4-7	YTX/MeOH	0.059	-1.57	0.042	N/A
		dsYTX/MeOH	0.034	-1.79	0.042	
		dsYTX/YTX	0.49	-1.14	0.042	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
44	4-7	YTX/MeOH	0.025	-2	0.018	N/A
		dsYTX/MeOH	0.02	-1.96	0.018	
		dsYTX/YTX	0.86	1.02	0.018	
45	4-7	YTX/MeOH	0.0018	2.02	3.20E-05	<i>KRT8 protein</i> (protein identification score was not significant)
		dsYTX/MeOH	5.70E-05	2.81	3.20E-05	
		dsYTX/YTX	0.026	1.39	3.20E-05	
46	4-7	YTX/MeOH	0.052	2.34	0.0095	<i>zinc finger protein 441</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0099	3.55	0.0095	
		dsYTX/YTX	0.16	1.51	0.0095	
47	4-7	YTX/MeOH	0.065	-1.38	0.011	N/A
		dsYTX/MeOH	0.004	-1.61	0.011	
		dsYTX/YTX	0.27	-1.17	0.011	
48	4-7	YTX/MeOH	0.04	-1.5	0.016	N/A
		dsYTX/MeOH	0.0094	-1.55	0.016	
		dsYTX/YTX	0.88	-1.04	0.016	
49	4-7	YTX/MeOH	0.0029	2.45	0.00074	N/A
		dsYTX/MeOH	0.0004	2.16	0.00074	
		dsYTX/YTX	0.62	-1.13	0.00074	
50	4-7	YTX/MeOH	0.00097	2.53	8.50E-05	<i>tyrosine hydroxylase</i> (protein identification score was not significant)
		dsYTX/MeOH	0.00055	2.42	8.50E-05	
		dsYTX/YTX	0.73	-1.05	8.50E-05	
51	4-7	YTX/MeOH	0.0039	2.03	0.00052	<i>unnamed protein product{nonspecific BLAST hit to BACK domain}</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0021	2.03	0.00052	
		dsYTX/YTX	0.94	-1	0.00052	
52	4-7	YTX/MeOH	0.024	1.62	0.0048	protein disulfide isomerase-related protein 5
		dsYTX/MeOH	0.0072	1.73	0.0048	
		dsYTX/YTX	0.45	1.07	0.0048	
53	4-7	YTX/MeOH	0.095	-1.28	0.0073	N/A
		dsYTX/MeOH	0.004	-1.64	0.0073	
		dsYTX/YTX	0.077	-1.28	0.0073	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
54	4-7	YTX/MeOH	0.19	1.71	0.061	unnamed protein product {specific BLAST hit to actin}
		dsYTX/MeOH	0.0092	2.27	0.061	
55		dsYTX/YTX	0.33	1.32	0.061	β -actin*
56	4-7	YTX/MeOH	0.21	2.12	0.049	<i>unnamed protein product{specific hit to actin domain} (protein identification score was not significant)</i>
		dsYTX/MeOH	0.0061	3.19	0.049	
		dsYTX/YTX	0.27	1.51	0.049	
57	4-7	YTX/MeOH	0.0064	-1.83	0.0053	N/A
		dsYTX/MeOH	0.046	-1.46	0.0053	
		dsYTX/YTX	0.082	1.26	0.0053	
58	4-7	YTX/MeOH	0.033	1.49	0.0074	N/A
		dsYTX/MeOH	0.54	-1.11	0.0074	
		dsYTX/YTX	0.0016	-1.65	0.0074	
59	4-7	YTX/MeOH	0.013	-1.57	0.0036	N/A
		dsYTX/MeOH	0.0059	-1.82	0.0036	
		dsYTX/YTX	0.25	-1.16	0.0036	
60	4-7	YTX/MeOH	0.03	1.54	0.012	N/A
		dsYTX/MeOH	0.0098	1.75	0.012	
		dsYTX/YTX	0.42	1.14	0.012	
61	4-7	YTX/MeOH	0.09	-1.4	0.044	N/A
		dsYTX/MeOH	0.034	-1.68	0.044	
		dsYTX/YTX	0.31	-1.19	0.044	
62	4-7	YTX/MeOH	0.022	-1.26	0.00018	N/A
		dsYTX/MeOH	0.00044	-1.56	0.00018	
		dsYTX/YTX	0.0017	-1.24	0.00018	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
63	4-7	YTX/MeOH	0.092	-1.32	0.033	zinc finger protein 441 (protein identification score was not significant)
64		dsYTX/MeOH	0.0042	-1.56	0.033	tyrosine hydroxylase (protein identification score was not significant)
		dsYTX/YTX	0.42	-1.18	0.033	
65	4-7	YTX/MeOH	0.0096	-1.41	0.002	immunoglobulin heavy chain variable region (protein identification score was not significant)
		dsYTX/MeOH	0.0017	-1.59	0.002	
		dsYTX/YTX	0.27	-1.13	0.002	
		YTX/MeOH	0.084	-1.24	0.02	
		dsYTX/MeOH	0.02	-1.38	0.02	
		dsYTX/YTX	0.15	-1.12	0.02	
66	4-7	YTX/MeOH	0.0038	1.76	0.002	N/A
		dsYTX/MeOH	0.0072	1.92	0.002	
		dsYTX/YTX	0.58	1.09	0.002	
67	4-7	YTX/MeOH	0.052	-1.41	0.021	N/A
		dsYTX/MeOH	0.008	-1.51	0.021	
		dsYTX/YTX	0.67	-1.07	0.021	
68	4-7	YTX/MeOH	0.027	1.61	0.0053	Chain A, Crystal Structure Of Human P32, A Doughnut-Shaped Acidic Mitochondrial Matrix Protein
		dsYTX/MeOH	0.0058	1.75	0.0053	
		dsYTX/YTX	0.39	1.09	0.0053	
69	4-7	YTX/MeOH	0.055	-1.3	0.0064	unnamed protein product {match to Zn finger 285A protein} (protein identification score was not significant)
		dsYTX/MeOH	0.0059	-1.52	0.0064	
		dsYTX/YTX	0.1	-1.16	0.0064	
70	4-7	YTX/MeOH	0.011	-1.44	0.0016	immunoglobulin heavy chain variable region (protein identification score was not significant)
		dsYTX/MeOH	0.0015	-1.52	0.0016	
		dsYTX/YTX	0.52	-1.06	0.0016	
71	4-7	YTX/MeOH	0.00061	2.35	0.00013	N/A
		dsYTX/MeOH	0.0069	1.64	0.00013	
		dsYTX/YTX	0.0041	-1.43	0.00013	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
72	4-7	YTX/MeOH	0.0016	-2.26	0.00025	<i>similar to RIKEN cDNA 1700016G05, isoform CRA_c (protein identification score was not significant)</i>
		dsYTX/MeOH	0.002	-1.88	0.00025	
		dsYTX/YTX	0.082	1.2	0.00025	
73	4-7	YTX/MeOH	0.019	1.32	0.0021	N/A
		dsYTX/MeOH	0.0035	1.57	0.0021	
		dsYTX/YTX	0.08	1.19	0.0021	
74	4-7	YTX/MeOH	0.13	-1.25	0.0081	<i>CALD1 protein (protein identification score was not significant)</i>
		dsYTX/MeOH	0.0078	-1.57	0.0081	
		dsYTX/YTX	0.027	-1.26	0.0081	
75	4-7	YTX/MeOH	0.01	1.73	0.0027	N/A
		dsYTX/MeOH	0.0029	1.7	0.0027	
		dsYTX/YTX	0.97	-1.02	0.0027	
76	4-7	YTX/MeOH	0.018	1.51	0.009	N/A
		dsYTX/MeOH	0.037	1.42	0.009	
		dsYTX/YTX	0.24	-1.06	0.009	
77	4-7	YTX/MeOH	0.0079	-1.26	2.30E-05	<i>STK35 protein (protein identification score was not significant)</i>
		dsYTX/MeOH	0.00011	-1.66	2.30E-05	
		dsYTX/YTX	0.00093	-1.32	2.30E-05	
78	4-7	YTX/MeOH	0.00044	2.91	5.50E-05	<i>complement receptor (protein identification score was not significant)</i>
		dsYTX/MeOH	0.012	1.63	5.50E-05	
		dsYTX/YTX	0.00019	-1.79	5.50E-05	
79	4-7	YTX/MeOH	0.0044	3.14	0.0021	<i>CDC28 protein kinase regulatory subunit 1B (protein identification score was not significant)</i>
		dsYTX/MeOH	0.037	1.91	0.0021	
		dsYTX/YTX	0.013	-1.64	0.0021	
80	4-7	YTX/MeOH	2.40E-05	2.12	3.90E-05	<i>T cell receptor V alpha 9.1=specific for mycobacterial heat shock protein 60-derived peptide P1 {clone 2.4, complementarity-determining region 3} [human, peripheral blood T cells, Peptide Partial, 29 aa] (protein identification score was not significant)</i>
		dsYTX/MeOH	0.12	1.22	3.90E-05	
		dsYTX/YTX	0.00096	-1.74	3.90E-05	
81	4-7	YTX/MeOH	0.012	-1.73	0.006	N/A
		dsYTX/MeOH	0.026	-1.53	0.006	
		dsYTX/YTX	0.19	1.13	0.006	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
82	4-7	YTX/MeOH	2.70E-06	3.26	2.60E-07	<i>signal recognition particle 9kDa, isoform CRA_c (protein identification score was not significant)</i>
		dsYTX/MeOH	0.00014	2.1	2.60E-07	
		dsYTX/YTX	0.0006	-1.55	2.60E-07	
83	4-7	YTX/MeOH	0.00019	1.98	3.90E-05	N/A
		dsYTX/MeOH	0.0098	1.34	3.90E-05	
		dsYTX/YTX	0.0015	-1.47	3.90E-05	
84	4-7	YTX/MeOH	0.00073	2.17	0.00022	<i>unnamed protein product (protein identification score was not significant)</i>
		dsYTX/MeOH	0.027	1.43	0.00022	
		dsYTX/YTX	0.0014	-1.51	0.00022	
85	4-7	YTX/MeOH	0.00014	1.81	3.90E-05	N/A
		dsYTX/MeOH	0.00073	1.52	3.90E-05	
		dsYTX/YTX	0.057	-1.19	3.90E-05	
86	4-7	YTX/MeOH	0.0033	1.66	0.0021	N/A
		dsYTX/MeOH	0.0026	1.57	0.0021	
		dsYTX/YTX	0.68	-1.06	0.0021	
87	4-7	YTX/MeOH	0.11	-1.28	0.0036	N/A
		dsYTX/MeOH	0.0032	-1.72	0.0036	
		dsYTX/YTX	0.02	-1.34	0.0036	
88	4-7	YTX/MeOH	0.0016	1.83	0.00035	N/A
		dsYTX/MeOH	0.0021	1.61	0.00035	
		dsYTX/YTX	0.19	-1.14	0.00035	
89	4-7	YTX/MeOH	0.13	-1.21	0.011	<i>Unknown {specific BLAST hit to α crystalline domain found in small HSPαB crystalline.} (protein identification score was not significant)</i>
		dsYTX/MeOH	0.012	-1.53	0.011	
		dsYTX/YTX	0.05	-1.26	0.011	
90	4-7	YTX/MeOH	0.24	-1.26	0.02	<i>Full=MIF4G domain-containing protein; AltName: Full=SLBP-interacting protein 1; Short=hSLIP1 (protein identification score was not significant)</i>
		dsYTX/MeOH	0.019	-1.66	0.02	
		dsYTX/YTX	0.015	-1.31	0.02	
91	4-7	YTX/MeOH	0.0059	-2.13	0.00055	N/A
		dsYTX/MeOH	0.0002	-2.69	0.00055	
		dsYTX/YTX	0.31	-1.26	0.00055	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
92	4-7	YTX/MeOH	0.0025	1.74	4.30E-05	<i>immunoglobulin heavy chain variable region</i> (protein identification score was not significant)
		dsYTX/MeOH	0.00012	2.17	4.30E-05	
		dsYTX/YTX	0.022	1.24	4.30E-05	
93	6-11	YTX/MeOH	0.031	1.65	0.024	N/A
		dsYTX/MeOH	0.086	1.32	0.024	
		dsYTX/YTX	0.088	-1.26	0.024	
94	6-11	YTX/MeOH	0.012	-1.57	0.014	N/A
		dsYTX/MeOH	0.066	-1.34	0.014	
		dsYTX/YTX	0.18	1.17	0.014	
95	6-11	YTX/MeOH	0.0015	-1.62	0.00046	N/A
		dsYTX/MeOH	0.018	-1.33	0.00046	
		dsYTX/YTX	0.0048	1.22	0.00046	
96	6-11	YTX/MeOH	0.012	-1.7	0.0076	unnamed protein product {specific BLAST hit K homology RNA-binding domain, type I. KH binds ss RNA/DNA}
		dsYTX/MeOH	0.071	-1.39	0.0076	
		dsYTX/YTX	0.027	1.22	0.0076	
97	6-11	YTX/MeOH	0.024	1.27	0.038	N/A
		dsYTX/MeOH	0.037	1.55	0.038	
		dsYTX/YTX	0.27	1.22	0.038	
98	6-11	YTX/MeOH	0.029	2.04	0.024	N/A
		dsYTX/MeOH	0.034	2.09	0.024	
		dsYTX/YTX	0.97	1.03	0.024	
99	6-11	YTX/MeOH	0.0046	1.63	0.0018	N/A
		dsYTX/MeOH	0.0032	1.7	0.0018	
		dsYTX/YTX	0.75	1.04	0.0018	
100	6-11	YTX/MeOH	0.039	2.23	0.016	<i>hCG1647242 {specific BLAST hit to Bel/Pao family of RNase HI in long-term repeat retroelements}</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0071	3.06	0.016	
		dsYTX/YTX	0.29	1.37	0.016	
101	6-11	YTX/MeOH	0.014	1.92	0.0018	<i>hCG2042223</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0012	2.47	0.0018	
		dsYTX/YTX	0.2	1.29	0.0018	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
102	6-11	YTX/MeOH	0.055	1.68	0.013	<i>tyrosine hydroxylase</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0028	2.13	0.013	
		dsYTX/YTX	0.28	1.27	0.013	
103	6-11	YTX/MeOH	0.28	1.19	0.047	N/A
		dsYTX/MeOH	0.027	1.5	0.047	
		dsYTX/YTX	0.12	1.26	0.047	
104	6-11	YTX/MeOH	0.082	-1.38	0.011	N/A
		dsYTX/MeOH	0.0027	-1.89	0.011	
		dsYTX/YTX	0.16	-1.36	0.011	
105	6-11	YTX/MeOH	0.15	-1.55	0.031	N/A
		dsYTX/MeOH	0.046	-2.42	0.031	
		dsYTX/YTX	0.046	-1.56	0.031	
106	6-11	YTX/MeOH	0.052	1.63	0.025	nucleolin, isoform CRA_c
		dsYTX/MeOH	0.02	1.72	0.025	
		dsYTX/YTX	0.69	1.05	0.025	
107	6-11	YTX/MeOH	0.074	1.41	0.0075	HNRPA1 protein
		dsYTX/MeOH	0.0011	1.7	0.0075	
		dsYTX/YTX	0.17	1.21	0.0075	
108	6-11	YTX/MeOH	0.003	1.62	0.00034	N/A
		dsYTX/MeOH	0.00025	1.77	0.00034	
		dsYTX/YTX	0.37	1.09	0.00034	
109	6-11	YTX/MeOH	0.01	1.79	0.00052	N/A
		dsYTX/MeOH	0.0015	2.22	0.00052	
		dsYTX/YTX	0.035	1.24	0.00052	
110	6-11	YTX/MeOH	0.02	2.32	0.024	N/A
		dsYTX/MeOH	0.072	1.84	0.024	
		dsYTX/YTX	0.23	-1.26	0.024	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
111	6-11	YTX/MeOH	0.38	1.11	0.01	N/A
		dsYTX/MeOH	0.013	1.57	0.01	
		dsYTX/YTX	0.017	1.41	0.01	
112	6-11	YTX/MeOH	0.0007	1.85	9.50E-05	N/A
		dsYTX/MeOH	0.00096	1.61	9.50E-05	
		dsYTX/YTX	0.1	-1.15	9.50E-05	
113	6-11	YTX/MeOH	0.1	-1.21	0.00022	N/A
		dsYTX/MeOH	0.00013	-1.76	0.00022	
		dsYTX/YTX	0.0031	-1.46	0.00022	
114	6-11	YTX/MeOH	0.009	-1.43	0.0024	<i>transgelin 2, isoform CRA_a</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0044	-1.71	0.0024	
		dsYTX/YTX	0.14	-1.19	0.0024	
115	6-11	YTX/MeOH	0.074	-1.38	0.047	<i>apolipoprotein M, isoform CRA_b</i> (protein identification score was not significant)
		dsYTX/MeOH	0.031	-1.54	0.047	
		dsYTX/YTX	0.5	-1.11	0.047	
116	6-11	YTX/MeOH	0.017	-1.41	0.0022	N/A
		dsYTX/MeOH	0.0031	-1.65	0.0022	
		dsYTX/YTX	0.13	-1.17	0.0022	
117	6-11	YTX/MeOH	0.037	-1.33	0.00075	N/A
		dsYTX/MeOH	0.0012	-1.75	0.00075	
		dsYTX/YTX	0.012	-1.32	0.00075	
118	6-11	YTX/MeOH	0.27	1.26	0.0046	<i>unnamed protein product {gene region Rossmann-fold NADP(+)-binding protein; highly similar to pyruvate dehydrogenase regulatory subunit, mRNA}</i> (protein identification score was not significant)
		dsYTX/MeOH	0.0072	2.23	0.0046	
		dsYTX/YTX	0.0026	1.77	0.0046	
119	6-11	YTX/MeOH	0.054	2.05	0.026	N/A
		dsYTX/MeOH	0.036	2.09	0.026	
		dsYTX/YTX	0.82	1.02	0.026	

Table 8 HL60 protein abundance changes (Av. Ratio) and associated statistics acquired from DeCyder and protein identification for picked protein spots which did not show a change in abundance, as numbered in Fig. 14. Av. Ratio is the protein volume of the first condition compared to the second in the comparison. For example negative value when comparing dsYTX/untreated means that the protein has undergone a decrease in abundance in response to dsYTX respective to the untreated control.

* Indicates protein identification is not the top-ranked match.

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
01	4-7	YTX/MeOH	0.53	1.23	0.22	MTHSP75*
		dsYTX/MeOH	0.0062	1.43	0.22	
		dsYTX/YTX	0.38	1.16	0.22	
		YTX/MeOH	0.93	1.06	0.61	
		dsYTX/MeOH	0.099	1.2	0.61	
		dsYTX/YTX	0.48	1.14	0.61	
02	4-7	YTX/MeOH	0.27	-1.24	0.38	heat shock 70kDa protein 8 isoform 1 variant*
		dsYTX/MeOH	0.21	-1.13	0.38	
		dsYTX/YTX	0.5	1.1	0.38	
03	4-7	YTX/MeOH	0.42	-1.12	0.36	unnamed protein product {BLAST matches to protein disulfide isomerase (PDI) a, a', b, b' families; ER}
		dsYTX/MeOH	0.49	1.09	0.36	
		dsYTX/YTX	0.22	1.22	0.36	
04	4-7	YTX/MeOH	0.85	-1	0.18	Chaperonin (HSP60)
		dsYTX/MeOH	0.022	1.26	0.18	
		dsYTX/YTX	0.16	1.26	0.18	
05	4-7	YTX/MeOH	0.67	1.05	0.2	Chain A, TapasinERP57 HETERODIMER
		dsYTX/MeOH	0.087	1.2	0.2	
		dsYTX/YTX	0.17	1.14	0.2	

Spot #	pH	Comparison	T-test	Av. Ratio	1-way ANOVA	Name
06	4-7	YTX/MeOH	0.64	-1.06	0.64	unnamed protein product {specific BLAST hit to β tubulin}
		dsYTX/MeOH	0.25	-1.16	0.64	
		dsYTX/YTX	0.71	-1.1	0.64	
07	4-7	YTX/MeOH	0.38	-1.14	0.48	unnamed protein product {specific BLAST hit to β tubulin}
		dsYTX/MeOH	0.22	-1.18	0.48	
		dsYTX/YTX	0.87	-1.04	0.48	
08	4-7	YTX/MeOH	0.49	1.23	0.45	unnamed protein product {specific BLAST hit to α tubulin}*
		dsYTX/MeOH	0.13	1.35	0.45	
		dsYTX/YTX	0.64	1.1	0.45	
09	4-7	YTX/MeOH	0.13	-1.32	0.14	unnamed protein product {specific BLAST hit to α tubulin}
		dsYTX/MeOH	0.041	-1.38	0.14	
		dsYTX/YTX	0.89	-1.05	0.14	
010	4-7	YTX/MeOH	0.53	-1.12	0.71	unnamed protein product {specific BLAST hit to actin}
		dsYTX/MeOH	0.4	-1.16	0.71	
		dsYTX/YTX	0.94	-1.04	0.71	
011	4-7	YTX/MeOH	0.28	-1.25	0.42	unnamed protein product {specific BLAST hit to actin}
		dsYTX/MeOH	0.17	-1.28	0.42	
		dsYTX/YTX	0.96	-1.02	0.42	
012	6-11	YTX/MeOH	0.16	1.32	0.13	Chain A, Crystal Structure Of Human Phosphoglycerate Kinase Bound To D-Adp/phosphoglycerate kinase 1, isoform CRA_b
		dsYTX/MeOH	0.89	-1.03	0.13	
		dsYTX/YTX	0.037	-1.36	0.13	
		YTX/MeOH	0.82	-1.03	0.93	
		dsYTX/MeOH	0.71	-1.06	0.93	
		dsYTX/YTX	0.92	-1.03	0.93	
013	6-11	YTX/MeOH	0.23	-1.09	0.16	17 kDa cyclophilin A (internal fragment)
		dsYTX/MeOH	0.11	-1.21	0.16	
		dsYTX/YTX	0.33	-1.1	0.16

Figure 17 Proteins identified as changing in HL60 cells treated with YTX or dsYTX with MeOH as control, compared to changes found in HepG2 cells following 24 h incubation with YTX (Young *et al.*, 2009). * denotes a 2 fold change, 0.01 (dsYTX or YTX only). Upwards pointing arrows indicate proteins which increased in abundance in response to treatment. Downwards pointing arrows indicate proteins which decreased in response to treatment.

Figure 18 Proteins identified as changing in HL60 cells treated with either YTX with MeOH as control, or peloruside A (Pel A) or paclitaxel (PTX) (Wilmes *et al.*, 2010, 2011). * denotes a 2 fold change, 0.01 (dsYTX or YTX only). Upwards pointing arrows indicate proteins which increased in abundance in response to treatment. Downwards pointing arrows indicate proteins which decreased in response to treatment.

Figure 19 Proteins identified as changing in HL60 cells treated with dsYTX with MeOH as control, or peloruside A (Pel A) or paclitaxel (PTX) (Wilmes *et al.*, 2011, 2012). * denotes a 2 fold change, 0.01 (dsYTX or YTX only). Upwards pointing arrows indicate proteins which increased in abundance in response to treatment. Downwards pointing arrows indicate proteins which decreased in response to treatment.

4 DISCUSSION

4.1 *S. cerevisiae*

In initial experiments wild type *S. cerevisiae* cells were exposed to 1.2 μ M YTX. This concentration was selected to be comparable to the amount used by Young *et al.*, in HepG2 cells.

The effects of YTX or dsYTX on yeast cell proteins were small and did not meet the criteria for significant change (Karp *et al.*, 2007). These small changes were only observed when the FDR filter was not applied. The changes are reported, however, for comparison with effects seen in mammalian cells.

DIGE analysis showed no significant changes, using the ≥ 2 -fold, $p \leq 0.01$ criteria, in response to 1.2 μ M YTX. Of four protein spots showing a possible change (≥ 1.5 fold, $p \leq 0.05$) in abundance, two were identified with significant Mascot scores. Cytosolic aldehyde dehydrogenase showed a possible 1.51-fold decrease in abundance (spot 10) in YTX treated cells compared to untreated controls. *S. cerevisiae* phosphoglycerate mutase showed a possible 1.75-fold decrease in YTX treated cells when compared with untreated cells, but not methanol treated (spot 32). Spot 5 and Spot 17 also possibly decreased in abundance when treated with YTX compared with untreated or methanol treated controls respectively.

Yeast cells often require a higher toxin dose to have an effect than is seen with mammalian cells due to the presence of the yeast cell wall and a number of export pumps. Therefore in order to test if the small number of changes was due to the concentration of YTX used being too low, the effect of a 10-fold higher concentration on wild type stationary phase yeast cells was tested. This resulted in an increase in the number of changes detected when proteins from YTX treated cells were compared with those from methanol treated cells. These differences in abundance did not reach the criteria for significance and were not observed when toxin treated proteins were compared to those from untreated cells.

Log phase wild type cells were then exposed to YTX, as they can be more susceptible than stationary phase cells to the chemical effects. No significant changes in

abundance were observed using the defined criteria. Seven of the 12 possible (≥ 1.5 -fold, $p \leq 0.05$) changes detected when YTX treated proteins were compared to untreated control samples also changed when the methanol treated and untreated controls were compared with each other. The fact that the changes were detected when controls were compared suggests that these differences in abundance were artefacts and not effects of the toxin.

The ABC transporter pump knock-out mutant, AD Δ , was used to examine whether the small number of changes observed was due to the toxin being removed from the cell before it had a chance to exert an effect. No changes were detected in stationary phase cells treated with 1.2 or 12 μ M YTX. Basic proteins from log phase cells treated with YTX also showed no differences from controls. While no significant protein changes were detected, three protein spots showed possible decreases in abundance (Spots 158, 160 and 163, Table 5) when compared with methanol treated or untreated controls. Three other protein spots showed apparent decreases in response to YTX when compared with methanol treated cells only. No changes were detected between the two control groups. Due to the small number of changes and the fact that the majority of the changing protein spots were low abundance, these protein spots were not excised from gels for identification.

YTX did not appear to have an effect on *S. cerevisiae* cells; however inhibition of yeast growth by YTX has been observed previously (Ogino *et al.*, 1997). However in that study, YTX was applied at a much higher concentration (50 μ M), than was used in this project. It was also applied on a disk to *Candida rugosa* plate cultures, rather than administered in liquid cultures.

Significant changes in protein abundance were only detected in yeast cells treated with dsYTX. In wild type cells, GRE1 was identified as undergoing a 2.37-fold decrease in abundance in response to dsYTX compared with untreated controls. GRE1 is a hydrophillin induced in response to stress, and is regulated by the (HOG) MAPK-pathway. Spot 156 showed a 2.13-fold decrease, but was too low in abundance to be extracted for MALDI TOF/TOF MSMS analysis. A possible, but not significant, change in abundance was also detected in mitochondrial aldehyde dehydrogenase. However

Mascot scores for these identifications were below the significance threshold (score > 56, $p \leq 0.05$). These proteins were not identified as undergoing a change in abundance in HepG2 cells treated with YTX. The greater effect seen with dsYTX is consistent with dsYTX being a more potent inhibitor of yeast growth than YTX (Ogino *et al.*, 1997).

DIGE analysis detected more changes in protein abundance in pump knock-out mutant cells exposed to dsYTX compared with wild type. Three protein spots showed significant changes in cells exposed to toxin. Spots 165 and 166 showed 2.5 and 2-fold increases in response to dsYTX treatment respectively. Spot 200 showed an approximately 2.3-fold decrease when exposed to dsYTX. Unfortunately mass spectrometry analysis of these protein spots did not yield significant matches. The top-ranked match for each spot in order was YKR101Wp-like protein, Pau9 hypothetical protein product, and mitochondrial translational activator. Possible (≥ 1.5 -fold, $p \leq 0.05$) increases in abundance were detected in protein spots identified as HSP90-Sba1 closed chaperone complex (chain A, spot 168, Table 5), YLL024Cp-like protein (spot 173), ATPase (involved in nuclear localisation signal directed nuclear transport), and translational elongation factor EF-1 α (spot 215). A possible decrease was detected in alcohol dehydrogenase isozyme I (spot 211). HSP90B1 and elongation factor 1 δ were shown to decrease and increase respectively, in HepG2 cells exposed to YTX.

There did not seem to be any common pattern to the protein changes seen in *S. cerevisiae*. The exception was spots 38 and 159 (Figs. 7 and 10) which migrated to similar positions on their respective gels so may be the same protein. Both spots, in stationary wild type cells or log phase pump mutant cells respectively, showed a possible decrease when exposed to 12 μ M YTX. They are also possibly the same as spot 26 (Fig. 6), which was identified as triosephosphate isomerase, chain A. Spot 26 did not have a change in abundance in stationary wild type cells exposed to 1.2 μ M YTX.

Twenty-three proteins, which showed no change in protein abundance, were identified with significant Mascot scores (Appendix H). These included fructose-1,6-aldoase, pyruvate kinase, actin, and SSE1 (ATPase component of the HSP90 co-

chaperone complex) and the HSP90 co-chaperone protein. Changes in abundance were seen in proteins similar to these in HepG2 cells treated with YTX (Young *et al.*, 2009). These were aldolase A, pyruvate kinase 3 (isoform 1), β -actin, and HSP90B1.

Possible reasons for the lack of a strong effect seen with YTX or dsYTX on *S. cerevisiae* cells include poor penetration of the toxins through the yeast cell wall, removal of the toxin by efflux pumps other than the ABC transporters, or lack of proteins in *S. cerevisiae* structurally homologous to targets in mammalian cells. For example, although there are proteins functionally homologous to hnRNPs in yeast, they are not structurally identical to those in mammalian cells (Matunis *et al.*, 1994, Denisenko *et al.*, 2001, Jimeno *et al.*, 2006).

4.1.1 Summary

Few changes were seen in *S. cerevisiae* cells treated with YTX. Significant changes in protein abundance were only observed in cells treated with dsYTX, with a possibly greater effect seen in the pump knock-out mutant strain. However, these changes were non-significant when the FDR filter was applied. A slight increase in the number of affected proteins was also seen in log phase cells from the mutant strain when treated with YTX. A significant decrease was seen in the abundance of GRE1 in YTX treated cells compared to untreated controls. Apparent increases were detected in the HSP90-Sba1 closed chaperone complex (chain A) and translational elongation factor EF-1 α , when cells were exposed to dsYTX, compared to methanol treated controls. These proteins were also affected by YTX in HepG2 cells (Young *et al.*, 2009).

No change in abundance was detected in fructose-1,6-aldolase, pyruvate kinase, actin, and SSE1 (ATPase component of the HSP90 co-chaperone complex) and the HSP90 co-chaperone protein. However in HepG2 cells treated with YTX, changes in abundance were detected in protein similar to these. These were: aldolase A, pyruvate kinase 3 (isoform 1), β -actin, and HSP90B1 (Young *et al.*, 2009).

The greater effect seen with dsYTX is consistent with previous research which showed dsYTX was a more potent inhibitor of yeast growth than YTX (Ogino *et al.*, 1997).

The slightly higher number of protein changes detected in the pump knock-out mutant implies that the ABC transporters may be involved in export of the toxin.

As only a small number of changes in protein abundance were observed in *S. cerevisiae* cells exposed to YTXs, the planned chemical genetics experiments could not be carried out.

4.2 HL60 cells

4.2.1 Morphological changes

Following 24 h exposure to YTX, no differences in morphology were observed in HL60 cells compared to controls. However trypan blue staining showed a 12.7% decrease in the number of live cells. Exposure to dsYTX, caused cells to aggregate into clumps of various sizes. Trypan blue staining showed that the cells in these clumps were dead. A 24% decrease in the number of live cells was also seen.

4.2.2 Protein changes

4.2.2.1 DeCyder Analysis

A greater number of protein changes was observed in HL60 cells treated with YTX, than was seen in yeast. Sixteen protein spots were identified as undergoing a significant change in response to YTX. As was seen in yeast, dsYTX seemed to have a greater effect (Table 6, Figs. 15 and 16), with 32 proteins showing a significant change (Table 6). Of the 119 spots identified as changing, 33 showed a significant increase in abundance. Five showed a significant decrease in abundance.

When protein abundances from dsYTX treated cells were compared to those from YTX treated cells no significant differences were detected. DeCyder analysis showed that protein abundances seemed to be affected in a similar way by either toxin. Eight protein spots did, however, show a possible difference (between 1.5 and 2-fold

change, $p \leq 0.05$) between the two toxins, but no identification was attained for these proteins.

4.2.2.2 Protein identifications and comparison to HepG2, Pel A, and PTX

4.2.2.2.1 YTX vs. dsYTX in HL60 cells

Four identified proteins changed in both YTX and dsYTX treated HL60 cells. Two of these were stress response proteins (HSP60 (chaperonin), GRP78 (BiP) precursor), one was a mitochondrial glycoprotein (chain A, p32/tat associated protein), the fourth was protein disulfide isomerase-related protein 5. In HepG2 cells treated with YTX the protein disulfide isomerase precursor was found to be affected in HepG2 cells (Young *et al.*, 2009). The two stress response proteins both showed a significant increase (approximately 2 and 2.5-fold for GRP78 precursor and HSP60, respectively, $p \leq 0.01$) in abundance in response to dsYTX. Apparent increases were also observed in response to YTX. Protein disulfide isomerase-related protein 5 and p32 (chain A) both showed possible increases of approximately 1.6 and 1.7-fold in response to YTX and dsYTX respectively. When the FDR filter was applied all four of these proteins no longer met the $p \leq 0.01$ cut-off but were significant at $p \leq 0.05$.

An unnamed protein, containing a K homology RNA binding, type 1 domain was identified as possibly decreasing (1.7-fold) in response to YTX. This domain is found in wide range of proteins, including proteins of the ribosome and transcription factors, including hnRNP K, and is involved in binding single stranded RNA and DNA. A BLAST search of the top ranked protein identification suggests that it may be far upstream element-binding protein 1, which is involved in c-myc expression. Myc was identified as a hub protein in the IPA network analysis of proteins affected by Pel A (Wilmes *et al.*, 2011).

Nucleolin and tumour rejection antigen 1 (gp96) showed increases in abundance, or 1.72 and 1.63 fold respectively, in HL60 cells treated with dsYTX compared to controls. The gp96 precursor underwent an increase in abundance in HepG2 cells following 24 h incubation with YTX (Young *et al.*, 2009). When the FDR filter was applied the gp96

precursor change still met the ≥ 1.5 -fold, $p \leq 0.05$ cut-off. Nucleolin did not meet this criteria.

4.2.2.2.2 YTX and dsYTX in HL60 cells vs. HepG2 cells treated with YTX

Identified proteins changing in HL60 cells when incubated with YTX or dsYTX for 24 h were compared with those previously found to be changing in HepG2 treated with YTX after 24 h of incubation (Fig. 17).

Actin (β), hnRNP A, and BiP were found to be affected by dsYTX in HL60 cells and YTX in HepG2 cells. HnRNP A and BiP showed increases of 1.7 and 1.8-fold respectively. Actin (β) showed a significant 2.27 fold increase. In HepG2 cells treated with YTX these proteins all decreased in abundance (Fig. 17, Young *et al.*, 2009).

4.2.2.2.3 Comparison of identified protein changes in HL60 cells

No identified proteins were found to be changing in common between HL60 cells treated with PTX or Pel A and YTX. (Fig. 18)

BiP and hnRNP A were found to be changing in HL60 cells treated with PTX, Pel A or dsYTX. In almost all cases these proteins increased in abundance. The only exception was in PTX treated cells, where BiP underwent a decrease in abundance (Fig. 19).

Two identified proteins were affected by both Pel A and dsYTX. These were actin and tumour rejection antigen 1. Both proteins had an increase (actin: ≥ 2 -fold, 0.01 in dsYTX treated) in abundance compared to controls.

No identified proteins were found to be affected by both PTX and dsYTX, but not Pel A in HL60 cells.

4.2.2.2.4 Other proteins

No changes in abundance were detected in response to either toxin for mitochondrial HSP75, HSP 70 kDa protein 8 isoform 1 variant, tapasin/ERP57 heterodimer (chain A), phosphoglycerate kinase (chain A), cyclophilin A (17 kDa fragment), or unnamed products with specific BLAST search hits to α - or β -tubulin. An unnamed protein product had a BLAST search match to protein disulfide isomerase a, a', b and b'

families. This contrasts to the results obtained for HL60 cells treated with PTX or Pel A (Wilmes *et al.*, 2011, 2012) and HepG2 cells treated with YTX (Young *et al.*, 2009). Tubulin decrease in abundance in HepG2 cells treated with YTX and increased in HL60 cells treated with Pel A. α -tubulin abundance increased in HL60 cells when treated with PTX (α -tubulin 1A), or when treated with PTX or Pel A (α -tubulin 1C).

4.2.3 Summary

YTX and dsYTX were more potent against mammalian HL60 cells than yeast. No change in morphology was observed in cells exposed to YTX for 24 h, however trypan blue staining showed a 12.7% decrease in the number of live cells present. DsYTX may be affecting cell-cell adhesion in some way as when cells were exposed to it for 24 h, they aggregated into clumps of dead cells. A 24% decrease in the number of live cells was also observed. It is not clear whether cell death occurred prior to or after cluster formation. Only a small number of the detected protein changes were able to be successfully identified. Of these hnRNP A and BiP (GRP78, HSP70) were identified as changing in response to treatment with dsYTX, Pel A or PTX in HL60 cells, and in response to YTX in HepG2 cells. Actin (β) was affected by dsYTX and Pel A in HL60 cells, and YTX in HepG2 cells.

HSP60, GRP78 precursor, p32/tat associated protein, and protein disulfide isomerase-related protein 5, underwent a change in abundance in response to YTX treatment and to dsYTX treatment. These proteins are mostly related to stress response.

Unexpectedly, none of the identified proteins which changed with response to YTX in HL60 cells were also changing in YTX treated HepG2 cells or in Pel A or PTX treated HL60 cells. However as the number of proteins identified was limited, this does not mean that there are no similar protein changes. This means that we were unable to distinguish toxin specific protein changes from those which are cell line specific.

4.3 Conclusions

The small number of protein identifications limits the formation of a comprehensive conclusion. No significant changes in protein abundance or cell morphology were observed in *S. cerevisiae* cells treated with YTX. This suggests that YTX does not exert any significant effect on *S. cerevisiae* cells at the concentration used in either stationary or log phase. Significant changes in abundance were detected when stationary phase cells were exposed to dsYTX. This suggests that dsYTX is potentially more toxic to yeast than YTX. This disulfated compound may have greater penetration through the cell wall or a different mechanism of action or protein target than YTX which makes it more toxic to yeast.

A greater effect of both toxins was seen in mammalian cells compared with yeast. HL60 cells showed no morphological differences from controls when treated with YTX, however dsYTX treated cells aggregated into clumps of dead cells. Actin (β), hnRNP A, and BiP were found to be affected by both YTX and dsYTX in HL60 cells. These proteins were also affected in YTX treated HepG2 cells (Young *et al.*, 2009). These proteins increased in abundance in HL60 cells but were reported to decrease in HepG2 cells (Young *et al.*, 2009). BiP and hnRNP A also showed an increase in HL60 cells treated with Pel A or PTX (Wilmes *et al.*, 2011, 2012). This suggests that these proteins are a general response of HL60 cells to stress. No identified proteins were detected as changing in response to YTX in both HL60 and HepG2 cells. However, due to the small number of significant protein identifications it cannot be concluded that there were no similarities.

A greater number of changes in protein levels appeared to be produced by dsYTX compared with YTX in HL60 cells. However when the DIGE analyses of the two toxins were compared, there were few differences in abundance detected, even for protein spots which showed a change in abundance response to only one of the toxins. Proteins which underwent a change in abundance in response to treatment with either toxin were changed similarly. For example a protein spot which underwent an increase in abundance in response to YTX would also show an increase in response to dsYTX, although the fold-change in abundance could be different.

4.4 Future directions

Further proteomic analysis of effects in HL60 cells could use either larger gels, which allow the application of greater protein loads than 7cm gels, or gel-free shotgun proteomics methods. The advantage of the gel-based approach is that it allows the ready detection of protein isoforms formed from different post-translational modifications. Protein identifications should be validated by Western Blot analysis. Once a larger set of protein identifications is obtained IPA should then be used to identify the possible target pathways of YTX and dsYTX. These should then be compared to each other, and to those obtained by Young *et al.* (2009), in YTX treated HepG2 cells, and Wilmes *et al.* (2011) in HL60 cells treated with Pel A.

Observation of the cells over the course of exposure to the toxin could provide insight into how the toxic effects of each compound progresses. In particular it would be useful to learn more about the development of the cell aggregation seen with dsYTX treatment. Comparison of protein profiles from different time points would also be interesting to carry out, to observe any time dependent effects on protein expression. Annexin V/PI staining should also be carried out to determine if the cell death observed is apoptosis or necrosis.

If larger amounts of toxin are available, the effect of higher toxin concentrations on *S. cerevisiae* and HL60 cells could be investigated. Determination of the LC₅₀ and IC₅₀ of YTX and dsYTX in these cell lines could help to determine the optimal concentration to use to observe toxin specific effects, with the least amount of general stress and cell death responses.

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APPENDICES

Appendix A

Symbol key for IPA networks

Function

Circle	Other
Circle (double)	Group/complex
Diamond	Enzyme
Hexagon	Translation regulator
Oval	Transcription regulator
Rectangle (dotted)	Ion channel
Rectangle (solid)	G-protein nuclear receptor
Square	Cytokine
Trapezium	Transporter
Triangle (downward pointing)	Kinase
Triangle (upward pointing)	Phosphatase

Interaction

Arrow, solid line	Direct action (protein acts on another to influence expression)
Arrow, dotted line	Indirect association
Dotted line	Indirect action
Solid line	Binding

Gene abbreviations

HepG2 cells and YTX

ACTB	β -actin
AHCY	S-adenosylhomocysteine hydrolase
ALDOA	aldolase A
BAG2	BCL2-associated anthanogene 2
CCN2	calponin 2
DNAJC3	DNA J (Hsp40) homologue, subfamily C, member 3
HNRNPA2B1	hnRNP A2/B1
HNRNPL	hnRNP L
HNRNPK	hnRNP K
HSP90B1	heat shock protein 90 kDa β (Grp94), member 1
HSPA8	heat shock 70 kDa protein 8
HSPA9	heat shock 70 kDa protein 9 (mortalin)
PCBP1	hnRNP E1
PKM2	pyruvate kinase, muscle
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5

HL60 cells and Pel A

ACAT2	acetyl-coenzyme A acetyltransferase 2
BIK	BCL2-interacting killer
CAPZB	F-actin-capping subunit β
CLIC4	chloride intracellular channel 4
COTL1	coactosin-like 1
DSTN	destrin
EEF1D	eukaryotic translation elongation factor 1 δ
EIF3D	eukaryotic translation initiation factor 3, subunit D
EIF3F	eukaryotic translation initiation factor 3, subunit F
EIF4E	eukaryotic translation initiation factor 4E
ERN1	ER to nucleus signalling 1 protein
GARS	glycyl-tRNA synthetase
HNRNPC	heterogeneous nuclear ribonucleoprotein C
HSP90AA2	heat shock protein 90 kDa α , class A member 2
HSPA1B	heat shock protein 1B
IFI6	interferon, alpha-inducible protein 6
IGK@	immunoglobulin kappa locus
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B cells, kinase epsilon
Immunoproteasome Pa28/20s	proteasome activator subunit 1 (PA28 alpha)
KHSRP	KH-type splicing regulatory protein
LMNB2	lamin B2
MSR1	macrophage scavenger receptor 1
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)

MYH9	myosin
PDGFB	platelet derived growth factor, B polypeptide
PRDX3	peroxiredoxin 3
Proteasome PA700/20s	proteasome activator subunit 1 (PA28 alpha)
PSMA1	proteasome subunit, alpha type 1
PSMB4	proteasome subunit, beta type, 4
PSMC4	proteasome 26s subunit
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein
SRXN1	sulfiredoxin 1 homolog (S. cerevisiae)
STMN1	stathmin 1/oncoprotein 18
TNF	tumour necrosis factor (TNF superfamily, member 2)

Appendix B

LC-MS analysis of the compounds was carried out by Andy Selwood of the Cawthron Institute, Nelson, New Zealand. The analysis determined that the stock solutions of each compound were pure. The YTX stock contained no detectable traces of other YTX analogues. The dsYTX stock contained only trace (ca 1%) amounts of YTX.

The YTX and dsYTX samples sent for analysis were supposed to contain 100 µg and 25 µg of toxin respectively. LC-MS-MS analysis determined the amount of YTX to be 50 µg. UV comparison of the dsYTX sample to the Cawthron Institute's dsYTX standard determined the quantity of toxin present to be 19 µg. This means there is potentially some difference between the intended concentration of toxin used to treat samples, and the actual. However since only small samples (10 µL and 5 µL for YTX and dsYTX respectively), it is also possible that some may have evaporated in transit between Wellington and Nelson.

UV spectrum of YTX

UV spectrum of dsYTX

Positive ion MS spectrum of dsYTX

LC-MS negative ions MS spectrum of dsYTX

Appendix C

Example template for a DIGE experiment (n=4, 3 treatment conditions)

PROTEIN LOAD: 20 µg/dye/strip

DYE/PROTEIN: 80 pmols/20 µg

WORKING DYE SOL'N.: 1.2 µL dye + 4.8 (5) µL DMF (1 pH range)
2.4 µL dye + 9,6 (10) µL DMF (2 pH ranges)

STRIPS: 7cm; 4-7/6-11

CHECK pH SAMPLES (>8)

LABELLING						
Cy3	SAMPLE	µg prot.	µL lysate	µL Dye	µL lysine	Tot volume
tube 1	MeOH 1	20	2.46	1	1	4.46
tube 2	MeOH 4	20	2.06	1	1	4.06
tube3	YTX 6	20	2.99	1	1	4.99
tube 4	YTX 7	20	2.45	1	1	4.45
tube 5	dsYTX 10	20	3.02	1	1	5.02
tube 6	dsYTX 11	20	2.66	1	1	4.66
TOTAL Cy3 480 pmol/ 120 µg protein						
Cy5	SAMPLE	µg prot.	µL lysate	µL Dye	µL lysine	Tot volume
tube 7	MeOH 2	20	2.04	1	1	4.04
tube 8	MeOH 3	20	2.70	1	1	4.70
tube 9	YTX 5	20	2.27	1	1	4.27
tube 10	YTX 8	20	2.41	1	1	4.41
tube 11	dsYTX 9	20	2.18	1	1	4.18
tube 12	dsYTX 12	20	2.46	1	1	4.46
TOTAL Cy5 480 pmol/ 120 µg protein						
INTERNAL ST.		µg prot.	µL lysate	µL Dye	µL lysine	Tot volume
		120	14.85			
tube 13		10/sample		6	6	26.85
TOTAL Cy2 480 pmol/120 µg prot.						
SAMPLE MIX						
1	tube 1 + tube 9 + 4.47 µL i.s					
2	tube 2 + tube 12 + 4.47 µL i.s					
3	tube 3 + tube 7 + 4.47 µL i.s					
4	tube 4 + tube 11 + 4.47 µL i.s					
5	tube 5 + tube 8 + 4.47 µL i.s					
6	tube 6 + tube 10 + 4.47 µL i.s					

EXPERIMENT DESIGN			$\mu\text{L R.B.}$	
			pH 4-7	pH 6-11
1	strip# 11347	Cy3 MeOH 1	125	100
		Cy5 YTX 5		
		i.s.Cy2		
2	strip# 11349	Cy3 MeOH 4	125	100
		Cy5 dsYTX 12		
		i.s.Cy2		
3	strip# 11350	Cy3 YTX 6	125	100
		Cy5 MeOH 2		
		i.s.Cy2		
4	strip# 11350	Cy3 YTX 7	125	100
		Cy5 dsYTX 9		
		i.s.Cy2		
5	strip# 11350	Cy3 dsYTX 10	125	100
		Cy5 MeOH 3		
		i.s.Cy2		
6	strip# 11350	Cy3 dsYTX 11	125	100
		Cy5 YTX 8		
		i.s.Cy2		

Appendix D

Example of “noise” 3D view

Example of dust 3D view

Example of a protein spot 3D view

Appendix E

Reference masses for MS internal reflector positive procedural method

m/z monoisotopic	NAME	TYPE	CHARGE
904.4680	des-Arg1-bradykinin	r	+1
1296.6850	Angiotensin 1	r	+1
1570.6770	Glu1-fibrinopeptide	r	+1
2093.0870	ACTH (1-17)	r	+1
2465.1990	ACTH (18-39)	r	+1
3657.9294	ACTH (7-38)	r	+1

Reference masses for MS/MS internal reflector positive procedural method

m/z monoisotopic	NAME	TYPE	CHARGE
255.1090	B2-17(-NH ₃) fragment ion	r	+1
354.1780	B3-17(-NH ₃) fragment ion	r	+1
784.4110	B6 fragment ion	r	+1
1181.6580	Angiotensin Y9 fragment ion	r	+1
1183.6010	Angiotensin fragment ion	r	+1

Appendix F

Exclusion masses for MALDI TOF/TOF MS/MS

m/z monoisotopic	NAME	TOLERANCE (m/z)
659.384	Trypsin	0.03
805.417	Trypsin	0.03
861.060	CHCA	0.10
877.000	Polyethylene glycol	0.10
906.505	Trypsin	0.03
1020.503	Trypsin	0.03
1153.574	Trypsin	0.03
1175.523	Trypsin	0.03
1296.680	Angiotensin 1	0.03
1433.721	Trypsin	0.03
1493.599	Trypsin	0.03
1676.777	Trypsin	0.03
1774.851	Trypsin	0.03
2093.080	ACTH (clip 1-17)	0.03
2163.057	Trypsin	0.03
2193.003	Trypsin	0.03
2193.995	Trypsin	0.03
2273.160	Trypsin	0.03
2289.155	Trypsin	0.03
2305.150	Trypsin	0.03
2465.190	ACTH (clip 18-39)	0.03
2514.339	Trypsin	0.03
2550.233	Trypsin	0.03
2612.181	Trypsin	0.03
2613.350	Trypsin	0.03
3211.475	Trypsin	0.03
3227.40	Trypsin	0.03

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Appendix G

The top ranked protein identification for each picked protein spot from *S. cerevisiae* experiments. Match scores greater than 56 are significant, $p = 0.05$ and are in **bold**. Fold changes in **bold** are significant (≥ 2 -fold, $p \leq 0.01$). Identifications without a score were identified using Data Explorer and searched through Profound. Profound scores were not determined (n.d.). Protein identifications acquired through Profound were considered significant if there were at least 5 matched peptides, sequence coverage $\geq 20\%$ and a difference between first and second ranked identifications of at least 1×10^{-3} .

Spot #	Expt	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										YTX/un	YTX/MeOH	MeOH/un
1	BY4741 1.2 μ M, 4-7	Formylglycinamide-ribonucleotide (FGAM)-synthetase, catalyses a step in the 'de novo' purine nucleotide biosynthetic pathway; Ade6p	gi 6321498	150.14	5.1	n.d.	8.9×10^{-7}	26	27/131	-1.3	-1.1	-1.18
2	BY4741 1.2 μ M, 4-7	SSE1 protein	gi 533365	77.68	5.1	n.d.	3.4×10^{-5}	36	21/93	-1.19	-1.39	1.16
5	BY4741 1.2 μ M, 4-7	Cytoplasmic tryptophanyl-tRNA synthetase, aminoacylates tryptophanyl-tRNA; Wrs1p	gi 6324475	49.661	6.35	28	31	12	5/45	-1.57	-1.59	1.01
9	BY4741 1.2 μ M, 4-7	Enolase II, catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis; Eno2p	gi 6321968	46.942	5.67	198	3.4×10^{-16}	19	10/75	-1.32	-1.33	1.01
10	BY4741 1.2 μ M, 4-7	Cytosolic aldehyde dehydrogenase, activated by Mg^{2+} and utilizes NADP ⁺ as the preferred coenzyme; required for conversion of acetaldehyde to acetate; constitutively expressed; locates to the mitochondrial outer surface upon oxidative stress; Ald6p	gi 6325196	54.80	5.3	n.d.	6.7×10^{-4}	33	15/132	-1.51	-1.44	-1.05

Spot #	Expt	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										YTX/un	YTX/MeOH	MeOH/un
11	BY4741 1.2 μ M, 4-7	cytosolic aldehyde dehydrogenase	gi 1336076	54.96	5.4	n.d.	8.9 $\times$ 10 ⁻⁷	49	23/99	-1.21	-1.6	1.32
12	BY4741 1.2 μ M, 4-7	cytosolic aldehyde dehydrogenase	gi 1336076	54.96	5.4	n.d.	5.7 $\times$ 10 ⁻⁷	49	23/117	-1.24	-1.29	1.05
15	BY4741 1.2 μ M, 4-7	Threonine synthase, conserved protein, catalyses formation of threonine from O-phosphohomoserine; Thr4p	gi 6319901	57.553	5.46	97	4.4e-006	17	10/64	-1.18	-1.24	1.05
16	BY4741 1.2 μ M, 4-7	Aromatic aminotransferase I; Aro8p	gi 6321236	56.371	5.68	143	1.1e-010	15	11/75	-1.03	-1.15	1.12
18	BY4741 1.2 μ M, 4-7	Enolase II, catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis; Eno2p	gi 6321968	46.942	5.67	194	8.5e-016	18	11/75	-1.07	-1.19	1.11
19	BY4741 1.2 μ M, 4-7	Enolase II, catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis; Eno2p	gi 6321968	46.942	5.67	433	1.1e-039	30	15/75	-1.12	-1.21	1.08
						429	2.7e-039	34	18/75	-1.1	-1.18	1.07
20	BY4741 1.2 μ M, 4-7	Enolase II, catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis; Eno2p	gi 6321968	46.942	5.67	315	6.7e-028	29	15/75	-1.47	-1.31	-1.12

Spot #	Expt	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										YTX/ un	YTX/ MeOH	MeOH/ un
23	BY4741 1.2 μ M, 4-7	Fructose 1,6-bisphosphate aldolase, required for glycolysis and gluconeogenesis; catalyses conversion of fructose 1,6 bisphosphate to glyceraldehyde-3-P and dihydroxyacetone-P; locates to mitochondrial outer surface upon oxidative stress; Fba1p	gi 6322790	39.881	5.51	123	1.1e-008	14	8/75	-1.31	-1.2	-1.1
25	BY4741 1.2 μ M, 4-7	Subunit d of the five-subunit V0 integral membrane domain of vacuolar H ⁺ -ATPase (V-ATPase), an electrogenic proton pump found in the endomembrane system; stabilizes VO subunits; required for V1 domain assembly on the vacuolar membrane; Vma6p	gi 6323480	40.12	4.5	n.d.	6.2 $\times$ 10 ⁻³	32	8/90	-1.16	-1.2	1.04
26	BY4741 1.2 μ M, 4-7	Chain A, Electrophilic Catalysis In Triosephosphate Isomerase: The Role Of Histidine-95	gi 349951	26.75	5.6	n.d.	3.2 $\times$ 10 ⁻⁴	47	11/87	1.03	-1.07	1.11
28	BY4741 1.2 μ M, 6-11	Chain A, Circularly Permuted Phosphoglycerate Kinase; Pgk P72	gi 157876436	44.595	7.71	401	1.7e-036	40	18/75	-1.03	-1.07	1.04
29	BY4741 1.2 μ M, 6-11	Chain A, Circularly Permuted Phosphoglycerate Kinase; Pgk P72	gi 157876436	44.595	7.71	369	2.7e-033	44	18/75			
32	BY4741 1.2 μ M, 6-11	Chain E, Saccharomyces Cerevisiae Phosphoglycerate Mutase	gi 157884466	26.687	8.27	335	6.7e-030	44	15/75	-1.75	-1.64	-1.06
33	BY4741 1.2 μ M, 6-11	Protein component of the small (40S) ribosomal subunit, the least basic of the non-acidic ribosomal proteins; phosphorylated in vivo; essential for viability; Rps5p	gi 6322583	25.080	8.63	345	6.7e-031	35	16/75	-1.03	1.07	-1.1

Spot #	Expt	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										(ds)YTX /un	(ds)YTX /MeOH	MeOH/un
41	BY4741 12 μ M, 4-7, log	unknown	gi 825555	22.362	5.10	37	4.6	17	5/23	1.58	1.02	1.55
43	BY4741 12 μ M, 4-7, log	YLL024Cp-like protein, AWRI1631	gi 207343245	59.805	7.00	494	8.5e-046	28	21/75	1.54	-1.1	1.7
45	BY4741 12 μ M, 4-7, log	unknown	gi 825555	22.362	5.10	22	1.4e+002	17	6/56	1.63	1.02	1.59
49	BY4741 12 μ M, 4-7, log	Chain A, The Structure Of Tap42 Alpha4 Subunit	gi 152149535	27.360	5.58	20	2.3e+002	9	4/69	1.75	-1.06	1.86
58	BY4741 12 μ M, 4-7, log	<i>No hit</i>								1.51	1	1.5
66	BY4741 12 μ M, 4-7, log	<i>No hit</i>								-1.5	1.06	-1.6
72	BY4741 12 μ M, 6-11, log	hypothetical protein identified by homology. See FEBS Letters [2000] 487:31-36.; Pau9p	gi 13129151	4.521	9.52	23	1.1e+002	52	2/75	1.63	-1.02	1.66
88	BY4741 12 μ M, 6-11, log	Ics2p	gi 30267748	3.320	10.29	32	12	90	3/59	-1.56	-1.1	-1.42
92	BY4741 12 μ M, 6-11, log	YNL022Cp-like protein AWRI1631	gi 207341631	56.825	9.02	27	40	17	7/70	-1.52	-1.01	-1.51
98	BY4741 12 μ M, 6-11, log	Thymidylate synthase, required for de novo biosynthesis of pyrimidine deoxyribonucleotides; expression is induced at G1/S; Cdc21p	gi 83578104	35.310	6.67	26	52	19	7/67	-1.75	1.05	-1.83
113	BY4741 12 μ M, 6-11, log	Ydr381wp	gi 849202	11.932	10.72	26	56	42	6/75	-1.57	-1.02	-1.54
123	BY4741 dsYTX, 4-7	Hsp90 cochaperone, interacts with the Ssa group of the cytosolic Hsp70 chaperones; activates the ATPase activity of Ssa1p; homolog of mammalian Hop protein; Sti1p	gi 6324601	66.395	5.45	93	1.1e-005	19	12/61	-1.01	-1.02	1.01

Spot #	Expt	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										dsYTX/un	dsYTX/MeOH	MeOH/un
127	BY4741 dsYTX, 4-7	Thymidylate synthase, required for de novo biosynthesis of pyrimidine deoxyribonucleotides; expression is induced at G1/S; Cdc21p	gi 83578104	35.310	6.67	30	20	22	8/75	1.51	1.39	1.09
136	BY4741 dsYTX, 4-7	Phosphomannomutase, involved in synthesis of GDP-mannose and dolichol-phosphate-mannose; required for folding and glycosylation of secretory proteins in the ER lumen; Sec53p	gi 14318474	29.216	5.14	124	8.5e-009	32	14/75	-1.02	-1	-1.01
138	BY4741 dsYTX, 4-7	protein GRE1 RM11-1a	gi 190407740	18.926	4.60	113	1.1e-007	42	8/75	-2.37	-1.84	-1.28
142	BY4741 dsYTX, 4-7	hypothetical protein identified by homology. See FEBS Letters [2000] 487:31-36.; Pau9p	gi 13129151	4.521	9.52	24	77	52	2/61	-1.52	-1.39	-1.09
147	BY4741 dsYTX, 4-7	Chain A, H48c Yeast Cu(ii)ZN superoxide dismutase room temperature (298k) Structure	gi 6730103	15.851	5.48	555	6.7e-052	60	13/75	-1.01	1.26	-1.28
150	BY4741 dsYTX, 4-7	Chain G, Yeast Cytochrome Bc1 Complex With Bound Substrate Cytochrome C	gi 20151124	14.482	5.63	68	0.0035	28	6/75	1.34	1.18	1.13
164	ADA dsYTX, 4-7	Phosphatidylinositol transfer protein (PITP) controlled by the multiple drug resistance regulator Pdr1p, localizes to lipid particles and microsomes, controls levels of various lipids, may regulate lipid synthesis, homologous to Pdr17p; Pdr16p	gi 6324098	40.802	7.72	21	1.9e+002	13	5/67	1.75	1.68	1.04
165	ADA dsYTX, 4-7	YKR101Wp-like protein AWRI1631	gi 207343311	69.792	9.04	19	2.4e+002	12	6/69	2.41	2.5	-1.04

Spot #	Expt	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										dsYTX/un	dsYTX/MeOH	MeOH/un
166	ADΔ dsYTX, 4-7	hypothetical protein identified by homology. See FEBS Letters [2000] 487:31-36.; Pau9p	gi 13129151	4.521	9.52	24	85	52	2/64	2.04	2.06	-1.01
168	ADΔ dsYTX, 4-7	Chain A, Crystal Structure Of An Hsp90-Sba1 Closed Chaperone Complex	gi 99031945	77.927	5.06	95	6.6e-006	8	8/75	1.61	1.66	-1.03
170	ADΔ dsYTX, 4-7	Chain A, Crystal Structure Analysis Of Sse1, A Yeast Hsp110	gi 159795398	73.710	5.17	24	89	5	4/70	1.78	1.89	-1.06
173	ADΔ dsYTX, 4-7	YLL024Cp-like protein AWRI1631	gi 207343245	59.805	7.00	280	2.1e-024	23	16/75	1.85	2.04	-1.1
177	ADΔ dsYTX, 4-7	hypothetical protein identified by homology. See FEBS Letters [2000] 487:31-36.; Pau9p	gi 13129151	4.521	9.52	23	1.1e+002	52	2/75	1.95	1.82	1.07
181	ADΔ dsYTX, 4-7	hypothetical protein identified by homology. See FEBS Letters [2000] 487:31-36.; Pau9p	gi 13129151	4.521	9.52	23	1.1e+002	52	2/75	1.5	1.66	-1.1
184	ADΔ dsYTX, 4-7	One of six ATPases of the 19S regulatory particle of the 26S proteasome involved in the degradation of ubiquitinated substrates; bound by ubiquitin-protein ligases Ubr1p and Ufd4p; localized mainly to the nucleus throughout the cell cycle; Rpt6p	gi 6321390	45.471	9.09	19	2.5e+002	14	4/72	1.6	1.82	-1.14
188	ADΔ dsYTX, 4-7	YLL024Cp-like protein AWRI1631	gi 207343245	59.805	7.00	92	1.3e-005	13	11/74	1.31	1.26	1.04
190	ADΔ dsYTX, 4-7	actin	gi 3328	41.907	5.53	98	3.3e-006	14	9/52	1.27	1.36	-1.08
191	ADΔ dsYTX, 4-7	YGL071Wp-like protein AWRI1631	gi 207345331	51.776	5.74	31	16	14	9/75	1.63	1.65	-1.01
200	ADΔ dsYTX, 4-7	mitochondrial translational activator [Saccharomyces cerevisiae YJM789]	gi 151944573	57707	9.18	25	67	15%	7/75	-2.26	-2.36	1.04
201	ADΔ dsYTX, 4-7	conserved protein YJM789	gi 151942578	18.896	4.60	168	3.4e-013	34	8/63	1.16	1.15	1.01
205	ADΔ dsYTX, 4-7	Putative protein of unknown function; Ylr146w-ap	gi 82795259	7.130	4.75	24	87	37	4/75	-1.7	-1.81	1.06

Spot #	Expt	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										dsYTX/un	dsYTX/MeOH	MeOH/un
206	ADΔ dsYTX, 4-7	One of six subunits of the RNA polymerase III transcription initiation factor complex (TFIIIC); part of the TauA globular domain of TFIIIC that binds DNA at the BoxA promoter sites of tRNA and similar genes; human homolog is TFIIIC-63; Tfc1p	gi 6319600	73.678	5.23	24	85	14	8/71	-1.37	-1.53	1.11
207	ADΔ dsYTX, 4-7	Chain G, Yeast Cytochrome Bc1 Complex With Bound Substrate Cytochrome C	gi 20151124	14.482	5.63	79	0.00026	28	6/75	1.06	1.12	-1.07
208	ADΔ dsYTX, 4-7	YGR254Wp-like protein AWRI1631	gi 207344913	41.740	6.23	94	8.1e-006	8	3/75	-1.28	-1.42	1.11
209	ADΔ dsYTX, 4-7	YGR254Wp-like protein AWRI1631	gi 207344913	41.740	6.23	120	2.1e-008	4	2/31	-1.45	-1.65	1.14
211	ADΔ dsYTX, 4-7	dehydrogenase isozyme I, alcohol	gi 223142	31.954	6.38	84	8.1e-005	12	6/75	-1.49	-1.54	1.03
215	ADΔ dsYTX, 6-11	Translational elongation factor EF-1 alpha; also encoded by TEF1; functions in the binding reaction of aminoacyl-tRNA (AA-tRNA) to ribosomes; Tef2p	gi 6319594	50.400	9.14	112	1.3e-007	13	10/75	1.69	2.1	-1.24
217	ADΔ dsYTX, 6-11	unnamed protein product	gi 395245	12.862	10.33	20	2.3e+002	21	3/75			
218	ADΔ dsYTX, 6-11	pyruvate kinase	gi 4180	54.964	8.00	197	4.3e-016	14	14/75	-1.11	-1.14	1.03
219	ADΔ dsYTX, 6-11	Alcohol dehydrogenase isoenzyme V; involved in ethanol production; Adh5p	gi 6319621	38.194	5.94	31	15	5	3/72	-1.59	-1.82	1.15
220	ADΔ dsYTX, 6-11	pyruvate kinase	gi 4180	54.964	8.00	341	1.7e-030	17	19/75	-1.33	-1.49	1.12

Appendix H

Proteins identified as not having a significant change of abundance by 2D-DIGE analysis of YTX in *S. cerevisiae*.

Experiment	Protein name	Spot number(s)
BY4741, 1.2 μ M YTX	FGAM-synthetase	1
BY4741, 1.2 μ M YTX	SSE1 protein (ATPase component of HSP90 cochaperone complex)	2
BY4741, 1.2 μ M YTX	Enolase II	9, 18-20
BY4741, 1.2 μ M YTX	Cytosolic aldehyde dehydrogenase	11, 12
BY4741, 1.2 μ M YTX	Threonine synthase	15
BY4741, 1.2 μ M YTX	Aromatic aminotransferase I	16
BY4741, 1.2 μ M YTX	Fructose 1,6-biphosphate aldolase	22
BY4741, 1.2 μ M YTX	V0 integral membrane domain of vacuolar H ⁺ -ATPase, subunit d	25
BY4741, 1.2 μ M YTX	Electrophilic catalysis in triosephosphate isomerase, chain A	26
BY4741, 1.2 μ M YTX; ADA dsYTX	Pyruvate kinase	27, 218, 220
BY4741, 1.2 μ M YTX	Circularly permuted phosphoglycerate kinase, chain A	28, 29
BY4741, 1.2 μ M YTX	Protein component of small ribosomal subunit	33
BY4741, 12 μ M YTX, log phase; ADA dsYTX	YYL024Cp-like protein	43, 62, 188
BY4741, 12 μ M YTX, log phase	ATPase	44
BY4741, 12 μ M YTX, log phase	Nucleoside diphosphate kinase	96
BY4741 dsYTX	HSP90 cochaperone	123
BY4741 dsYTX	Phosphomannomutase	136
BY4741 dsYTX	Egd2p	137
BY4741 dsYTX	Yeast Cu(Li)Zn superoxide dismutase	147
BY4741 dsYTX; ADA dsYTX	Cytochrome Bcl complex	150, 207
ADA dsYTX	Actin	190
ADA dsYTX	Conserved protein, YJM789	201
ADA dsYTX	YGR254Wp-like protein	208, 209

Appendix I

The top ranked protein identification for each picked protein spots from HL60 experiment which showed at least a 1.5-fold change in abundance, p-value = 0.05. Match scores greater than 66 are significant, p = 0.05 and are in **bold**. *Indicates a protein identification which is the best match overall (including Mw, pI), but is not the top-ranked identification. Fold changes in **bold** are significant (≥ 2 -fold, $p \leq 0.01$).

Spot #	pH	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										YTX/MeOH	dsYTX/MeOH	dsYTX/YTX
1	4-7	Immunoglobulin heavy chain variable region	gi 112699019	10.962	7.88	34	95	40%	3/65	1.87	2.13	1.14
2	4-7	tumour rejection antigen (gp96) 1	gi 61656607	92.567	4.77	71	0.018	10%	14/75	1.27	1.63	1.28
		heat shock protein 90kDa beta, member 1	gi 4507677	92.696	4.76	71	0.018	10%	14/75			
4	4-7	opioid growth factor receptor and/or S100 calcium-binding protein A8	gi 66347254	11.600	5.54	44	9.3	32%	5/75	1.4	1.55	1.1
			gi 21614544	10.885	6.51	36	62	43%	6/75			
5	4-7	MHC class I antigen	gi 85815622	10.392	5.30	35	76	46%	4/64	1.61	2.02	1.26
7	4-7	hCG2015481	gi 119618893	7.512	9.04	24	7.9e+002	43%	3/75	2.37	2.9	1.22
8	4-7	hCG2044129 {region: DUF132, contains SGF29 tudor-like domain; pfam07039; poss RNA binding}	gi 119572656	6.273	6.31	36	50	24%	2/7	1.88	2.31	1.23
9, 10, 11	4-7	GRP78 precursor	gi 386758	72.185	5.03	446	5.5e-040	30%	23/75	1.65	2.07	1.25
										1.49	1.82	1.22
										1.44	1.7	1.18
12	4-7	BiP protein	gi 6470150	71.002	5.23	145	6.9e-010	16%	12/75	1.54	1.8	1.17
13	4-7	PRO1095 {predicted protein product of HQ1095}	gi 11493413	4.854	9.59	29	2.8e+002	57%	4/42	1.99	2.57	1.3
18	4-7	unnamed protein product	gi 193786969	99.132	7.32	39	26	4%	5/12	-1.62	1.95	-1.2
19	4-7	unnamed protein product {contains Ribosomal L3 superfamily domain; highly similar to mitochondrial 39s ribosomal protein L3}	gi 194376318	27.262	9.64	48	3.8	16%	4/14	-1.37	-1.7	-1.24

Spot #	pH	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										YTX/MeOH	dsYTX/MeOH	dsYTX/YTX
20	4-7	hCG2040114, isoform CRA_b {ch/some 2}	gi 119620565	15.179	8.56	31	1.8e+002	29%	3/34	-1.63	-2.04	-1.25
21	4-7	hCG2042223 {ch/some 1}	gi 119615331	8.095	11.92	50	2	38%	3/11	-1.59	-1.63	-1.02
22	4-7	translation initiation factor eIF-4gamma	gi 510307	79.884	5.44	35	6	5%	4/11	-2.81	-2.58	1.09
23	4-7	cytotoxic T-lymphocyte-associated protein 4	gi 553245	4.397	9.61	39	29	50%	4/44	-2.68	-2.55	1.05
24	4-7	ferritin light polypeptide	gi 171702799	5.863	5.74	31	1.9e+002	44%	4/75	1.49	2.1	1.41
25	4-7	immunoglobulin lambda chain variable region	gi 3153364	11.031	9.07	44	8.9	43%	3/41	1.65	2.38	1.44
28	4-7	hCG2012006 {ch/some 2}	gi 119590542	3.398	9.39	34	85	51%	3/62	2.05	2.55	1.24
29	4-7	heat shock 60kDa protein 1 (chaperonin), isoform CRA_c	gi 119590557	41.067	5.09	47	4.8	16%	7/75	2.12	2.89	1.36
30	4-7	heat shock 60kDa protein 1 (chaperonin), isoform CRA_c	gi 119590557	41.067	5.09	67	0.04	21%	11/75	1.86	2.54	1.37
31	4-7	immunoglobulin heavy chain variable region	gi 11137459	11.216	10.01	34	81	58%	5/66	1.53	2	1.31
32	4-7	unnamed protein product {GroEL/chaperonin type I/II/chaperonin like domain. Chaperonins are involved in productive folding of proteins}	gi 221041730	58.705	5.82	52	1.2	7%	10/75	1.22	1.57	1.28
34	4-7	hCG2030305, isoform CRA_a {ch/some 6}	gi 119568344	6.997	9.77	29	3e+002	24%	2/15	1.75	2.1	1.2
35	4-7	calreticulin precursor variant	gi 62897681	47.061	4.30	31	1.8e+002	11%	9/75	1.31	1.56	1.19
39	4-7	NOS1 protein	gi 118600892	10.454	5.08	40	25	45%	4/41	-1.76	-1.79	-1.02
41	4-7	unnamed protein product {non-specific hit to NAD-dependent glycerol-3-phosphate dehydrogenase C-terminus domain}	gi 193785041	13.475	7.63	35	66	18%	4/42	-1.57	-1.77	-1.13

Spot #	pH	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										YTX/MeOH	dsYTX/MeOH	dsYTX/YTX
45	4-7	KRT8 protein	gi 14198278	30.802	5.02	26	5.5e+002	21%	4/31	2.02	2.81	1.39
46	4-7	zinc finger protein 441	gi 119604646	78.130	8.95	38	32	9%	6/20	2.34	3.55	1.51
50	4-7	tyrosine hydroxylase	gi 548153	3.717	8.31	36	55	73%	3/15	2.53	2.42	-1.05
51	4-7	unnamed protein product {nonspecific hit to BACK domain}	gi 221044806	23.358	6.88	34	91	11%	3/14	2.03	2.03	-1
52	4-7	protein disulfide isomerase-related protein 5	gi 1710248	46.512	4.95	161	1.7e-011	14%	8/75	1.62	1.73	1.07
54	4-7	unnamed protein product {specific hit to actin}	gi 194376310	38.950	5.19	116	5.5e-007	18%	8/75	1.71	2.27	1.32
55		ACTB protein*	gi 15277503	40.536	5.55	84	0.00081	10	5/75			
56	4-7	unnamed protein product {specific hit to actin domain}	gi 194376310	38.950	5.19	65	0.069	15%	6/48	2.12	3.19	1.51
63	4-7	zinc finger protein 441	gi 119604646	78.130	8.95	52	1.3	9%	6/17	-1.32	-1.56	-1.18
64		tyrosine hydroxylase	gi 548153	3.717	8.31	35	63	73%	3/16			
65	4-7	immunoglobulin heavy chain variable region	gi 27650504	8.847	8.07	30	2.4e+002	23%	4/75	-1.41	-1.59	-1.13
68	4-7	Chain A, Crystal Structure Of Human P32, A Doughnut-Shaped Acidic Mitochondrial Matrix Protein	gi 4930073	23.844	4.32	141	1.7e-009	32%	6/75	1.61	1.75	1.09
69	4-7	unnamed protein product {match to Zn finger 285A protein}	gi 16550013	51.777	8.85	35	68	8%	4/16	-1.3	-1.52	-1.16
70	4-7	immunoglobulin heavy chain variable region	gi 21999882	6.980	5.04	37	41	56%	4/75	-1.44	-1.52	-1.06
72	4-7	similar to RIKEN cDNA 1700016G05, isoform CRA_c	gi 119604393	25.383	9.19	34	89	15%	3/15	-2.26	-1.88	1.2
74	4-7	CALD1 protein	gi 116283250	17.756	9.69	39	30	20%	4/15	-1.25	-1.57	-1.26
77	4-7	STK35 protein	gi 84798766	28.027	7.78	33	1.1e+002	16%	8/74	-1.26	-1.66	-1.32
78	4-7	complement receptor	gi 395334	3.082	9.22	37	47	44%	2/14	2.91	1.63	-1.79

Spot #	pH	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										YTX/ MeOH	dsYTX/ MeOH	dsYTX/YTX
79	4-7	CDC28 protein kinase regulatory subunit 1B	gi 55960117	8.079	5.88	47	4	38%	3/16	3.14	1.91	-1.64
80	4-7	T cell receptor V alpha 9.1=specific for mycobacterial heat shock protein 60-derived peptide P1 {clone 2.4, complementarity-determining region 3} [human, peripheral blood T cells, Peptide Partial, 29 aa]	gi 1086967	3.287	10.05	33	1.1e+002	75%	3/75	2.12	1.22	-1.74
82	4-7	signal recognition particle 9kDa, isoform CRA_c	gi 119590159	5.570	6.54	31	1.8e+002	34%	2/21	3.26	2.1	-1.55
84	4-7	unnamed protein product	gi 194380768	19.682	11.21	32	1.5e+002	17%	5/75	2.17	1.43	-1.51
89	4-7	Unknown {specific hit to α crystalline domain found in small HSP α B crystalline. sHSPs are molecular chaperones, protect against cell stress}	gi 2852648	22.435	7.18	36	62	15%	3/14	-1.21	-1.53	-1.26
90	4-7	Full=MIF4G domain-containing protein; AltName: Full=SLBP-interacting protein 1; Short=hSLIP1	gi 189081938	25.749	5.23	30	2.4e+002	9%	2/5	-1.26	-1.66	-1.31
92	4-7	immunoglobulin heavy chain variable region	gi 112698573	11.110	9.10	42	13	32%	3/19	1.74	2.17	1.24
96	6-11	unnamed protein product {specific hit K homology RNA-binding domain, type I. KH binds ss RNA/DNA}	gi 194377024	70.150	9.47	67	0.043	8%	8/12	-1.7	-1.39	1.22
100	6-11	hCG1647242 {specific hit to Bel/Pao family of RNase HI in long-term repeat retroelements}	gi 119612025	58.863	9.16	26	5.9e+002	17%	4/50	2.23	3.06	1.37
101	6-11	hCG2042223 {ch/some 1}	gi 119615331	8.095	11.92	45	6.8	38%	3/16	1.92	2.47	1.29
102	6-11	tyrosine hydroxylase	gi 548153	3.717	8.31	32	1.3e+002	73%	2/21	1.68	2.13	1.27

Spot #	pH	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										YTX/MeOH	dsYTX/MeOH	dsYTX/YTX
106	6-11	nucleolin, isoform CRA_c	gi 119591368	58.576	4.57	208	3.5e-016	18%	14/75	1.63	1.72	1.05
107	6-11	HNRNPA1 protein	gi 75517570	29.482	9.19	131	1.7e-008	26%	9/75	1.41	1.7	1.21
114	6-11	transgelin 2, isoform CRA_a	gi 119573144	19.458	8.87	49	2.9	35%	7/75	-1.43	-1.71	-1.19
115	6-11	apolipoprotein M, isoform CRA_b	gi 119623867	18.021	8.52	43	10	22%	7/75	-1.38	-1.54	-1.11
118	6-11	unnamed protein product {gene region Rossmann-fold NADP(+)-binding protein; highly similar to pyruvate dehydrogenase regulatory subunit, mRNA}	gi 193788467	32.249	9.61	30	2e+002	15%	2/7	1.26	2.23	1.77

Appendix J

The top ranked protein identification for each picked protein spots from HL60 experiment which had a significant match score, but were not identified as changing by 2D-DIGE analysis. *Indicates a protein identification which is the best match (including Mw, pI), but is not the top-ranked identification.

Spot #	pH	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										YTX/MeOH	dsYTX/MeOH	dsYTX/YTX
01	4-7	unnamed protein product {hits to DNA K molecular chaperone, highly similar to HSP70 mitochondrial protein}	gi 221039760	47504	6.20	253	1.1e-020	32%	19/75	1.23	1.43	1.16
		MTHSP75*	gi 292059	74019	5.97	229	2.8e-018	21%	19/75	1.06	1.2	1.14
02	4-7	Chain A, Crystal Structure Of Hsc70BAG1 IN COMPLEX WITH ATP	gi 225698069	42120	6.38	154	8.7e-011	21%	11/75	-1.24	-1.13	1.1
		heat shock 70kDa protein 8 isoform 1 variant*	gi 62897129	71083	5.28	151	1.7e-010	15%	13/75			
03	4-7	unnamed protein product {matches to protein disulfide isomerase (PDI) a, a', b, b' families; ER}	gi 194373909	51620	4.77	186	5.5e-014	21%	14/75	-1.12	1.09	1.22
04	4-7	Chaperonin (HSP60)	gi 31542947	61187	5.70	220	2.2e-017	31%	20/75	-1	1.26	1.26
05	4-7	Chain A, TapasinERP57 heterodimer (nb: ERP is part of PDI family)	gi 220702506	54541	5.61	301	1.7e-025	24%	19/75	1.05	1.2	1.14

Spot #	pH	Name	gi #	Calc. Mw (kDa)	Calc. pI	Score	Expect.	Seq. cov. (%)	Matched /total	Fold change		
										YTX/MeOH	dsYTX/MeOH	dsYTX/YTX
06	4-7	unnamed protein product {specific hit to β tubulin}	gi 221045918	46936	4.83	118	3.5e-007	30%	21/75	-1.06	-1.16	-1.1
07	4-7	unnamed protein product {specific hit to β tubulin}	gi 221045918	46936	4.83	99	2.6e-005	27%	19/75	-1.14	-1.18	-1.04
08	4-7	unnamed protein product {from tubulin Ftsz region}	gi 194387512	20259	4.39	74	0.0095	19%	3/75	1.23	1.35	1.1
		unnamed protein product {specific hit to α tubulin}	gi 221039556	58636	4.94	71	0.017	9%	3/75			
09	4-7	unnamed protein product^ {specific hit to α tubulin}	gi 221039556	58636	4.94	91	0.00018	5%	2/75	-1.32	-1.38	-1.05
010	4-7	unnamed protein product {specific hit to actin}	gi 194375299	37667	5.49	239	2.8e-019	19%	5/20	-1.12	-1.16	-1.04
011	4-7	unnamed protein product {specific hit to actin}	gi 194375299	37667	5.49	226	5.5e-018	33%	14/75	-1.25	-1.28	-1.02
012	6-11	Chain A, Crystal Structure Of Human Phosphoglycerate Kinase Bound To D-Adp/phosphoglycerate kinase 1, isoform CRA_b	gi 193506632	45266	8.32	288	3.5e-024	24%	13/75	1.32	-1.03	-1.36
										-1.03	-1.06	-1.03
013	6-11	17 kDa cyclophilin A {internal fragment} [human, first trimester decidual and placental tissue, Peptide Partial, 29 aa]	gi 1041969	3190	4.10	127	4.4e-008	62%	2/75	-1.09	-1.21	-1.1