Investigation of chiralities of propranolol and atenolol by comparative proteomic analysis of vascular smooth muscle cells

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Investigation of Chiralities of Propranolol and Atenolol by Comparative Proteomic Analysis of Vascular Smooth Muscle Cells

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School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2009

Dedicated to my family, especially my mother and my father

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TABLE OF CONTENTS

ACKNOWLEDGMENTS	i
TABLE OF CONTENTS	iii
SUMMARY	vi
LIST OF TABLES	viii
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xiv
PUBLICATIONS	xvii
1. Introduction	1
1.1 Chiral Drug	1
1.2 β -Adrenoceptors	4
1.3 β -Blockers	8
1.4 High Performance Liquid Chromatography	9
1.5 Proteomics	12
1.6 Quantitative Proteomics	16
1.7 Application of Mass Spectrometry-based Proteomics	22
1.7.1 Functional Studies of Protein Profiles with Proteomics	23
1.7.2 Protein Interactions with Proteomics	25
1.7.3 Analysis of Protein Modifications with Proteomics	27
1.8 Metabolomics	28
1.9 Objectives of Research Project	30
2. Materials and Methods	32
2.1 Cell Cultures	32
2.2 Cell Incubation with Drugs	32
2.3 MTT Assay	32
2.4 Cell Lysis	33
2.5 Conditioned Medium	34
2.6 Protein Quantification	34
2.7 Protein Precipitation	35
2.8 Protein Digestion, and Labeling with iTRAQ Reagents	35
2.9 On-Line 2D Nano LC-MS/MS Analysis	37
2.10 Data Analysis and Interpretation	40
2.11 NAD ⁺ /NADH Assay	42
2.12 ROS Detection	43
2.13 Real-time Reverse Transcription-Polymerase Chain Reaction	43
2.13.1 RNA Extraction	43
2.13.2 RNA Quantitation	44
2.13.3 One-Step Reverse Transcription – Polymerase Chain Reaction	44
2.13.4 Real-time RT-PCR	45
2.14 Intracellular Ca ²⁺ Concentration Measurements	47
2.15 Immunostaining and Fluorescence Microscopy	48
2.16 Western Blot Analysis	48
2.16.1 Preparation of water-saturated butanol	48

2.16.2 Preparation of SDS-PAGE.....	49
2.16.3 Western Transfer	50
2.16.4 Western Analysis	50
2.17 Data Analysis and Statistics	52
3. Protein Profile in A7r5 Cells Incubated.....	53
with <i>S</i> - and <i>R</i> -enantiomers of Propranolol.....	53
3.1 Introduction.....	53
3.2 Results.....	55
3.2.1 MTT Assay and Concentration of Propranolol	55
3.2.2 iTRAQ Analysis of BSA	56
3.2.3 iTRAQ Analysis of Differentially Expressed Proteins.....	59
3.2.4 Analysis of Cytoskeletal Proteins	66
3.2.5 Analysis of Signaling Proteins	67
3.2.6 Up-Regulation of Metabolic Enzymes	68
3.3 Discussion	73
3.3.1 Enzymes Involved in Anabolism and Actin Polymerization.....	73
3.3.2 Enzymes Involved in Antioxidant Activity	75
4. Comparative Proteomics Analysis of A7r5.....	77
Cells Incubated with <i>S</i> - and <i>R</i> -Enantiomers of Atenolol.....	77
4.1 Introduction.....	77
4.2 Results.....	79
4.2.1 MTT Assay and Concentration of Atenolol.....	79
4.2.2 iTRAQ Analysis and Differentially Expressed Proteins	81
4.2.3 Up-regulation of Metabolic Enzymes	87
4.2.4 Down-regulation of Calcium-binding Proteins	88
4.3 Discussion	95
5. Secretion of T-Kininogen from A7r5	100
Cells in Response to <i>S</i> -Enantiomer of Propranolol	100
5.1 Introduction.....	100
5.2 Results.....	100
5.3 Discussion	102
6. Secretion of T-Kininogen from A7r5	108
Cells in Response to <i>S</i> -Enantiomer of Atenolol	108
6.1 Introduction.....	108
6.2 Results and Discussion	108
7. Conclusions.....	112
8. Future Directions.....	121
LIST OF REFERENCES	123
APPENDIX	135
Appendix I: Supplementary table 1 for the identified 361 proteins (score >2) in A7r5 cells incubated with propranolol	136
Appendix II: Supplementary table 2 for the identified 323 proteins (score>2) in	

A7r5 cells incubated with atenolol 149

Appendix III: MATLAB program for plot of cumulative distribution..... 161

SUMMARY

Propranolol is a nonselective β -blocker exerting blocking effect on the β -adrenergic receptors, with the *S*-enantiomer being more active than the *R*-enantiomer. Utilization of iTRAQ coupled with two-dimensional LC-MS/MS system, we report here the first study of protein profiles in vascular smooth muscle cells (A7r5) in response to two individual enantiomers of propranolol, respectively. In this study four categories of cellular proteins including cytoskeletal proteins, signaling molecules, metabolic enzymes and those associated with DNA synthesis/protein translation showed differentially expressed protein levels. The higher protein levels of several enzymes involved in cellular anabolism and antioxidant activity in *S*-enantiomer of propranolol-incubated A7r5 cells, as revealed by LC MS/ MS, was further validated by real-time PCR. Significantly, the increase in the anabolic activity associated with the higher level of metabolic enzymes was also supported by the higher intracellular concentration of the metabolic cofactor NAD^+ , which was the product resulting from oxidation of NADH. The higher expression level of thioredoxin might account for increased antioxidant activity, as indicated by the lower ROS level in *S*-enantiomer of propranolol-incubated cells than that for *R*-enantiomer of propranolol-incubated cells and control ones.

Atenolol is a β_1 -selective drug, exhibiting greater blocking activity on β_1 -adrenoreceptors than on β_2 ones, with the *S*-enantiomer being more active than *R*-enantiomer. It was found that a number of metabolic enzymes such as glutathione *S*-transferase *P*, aspartate aminotransferase, NADH-cytochrome *b*₅ reductase, and alpha-N-acetylgalactosaminidase precursor were up-regulated in the A7r5 cells incubated with *S*-enantiomer of atenolol compared with *R*-enantiomer of atenolol

treatment and normal state. More importantly several calcium-binding proteins including calmodulin, protein S100-A4, protein S100-A11 and annexin A6 were down-regulated and showed relatively lower protein levels in the *S*-enantiomer of atenolol-incubated A7r5 cells, with the lower intracellular Ca^{2+} concentration involved. Ca^{2+} signals transduced by calcium-binding proteins acted on cytoskeletal proteins such as nestin and β -tropomyosin, which can play a complex role in phenotypic modulation and regulation of the cytoskeletal modeling. Similarly the involvement of NADH-cytochrome *b*₅ reductase in the intracellular anabolic activity was validated by NAD^+/NADH assay with a higher ratio of NAD^+/NADH , indicating a higher proportion of NAD^+ .

Our results also indicated that secretion of T-kininogen 1 by *S*-enantiomer of propranolol-incubated cells was greatly enhanced as compared with that for *R*-enantiomer incubated-cells or control cells. It can be inferred that the *S*-enantiomer of propranolol will induce more T-kinin, the vasoactive peptides. Interestingly the similar result for *S*-enantiomer of atenolol-incubated cells was obtained that the secretion of T-kininogen 1 was stimulated compared with *R*-enantiomer of atenolol incubated cells or control cells.

Our results therefore provide molecular evidence on metabolic effects involved with propranolol or atenolol treatment. The differentially expressed metabolic enzymes may serve as useful targets for future pharmaceutical interventions to reduce clinical side effects. More importantly, our findings may be used for explaining the differences in the potential mechanism of the actions of individual enantiomers of propranolol or atenolol, as well as providing possible link of treatment of cardiovascular diseases associated with propranolol or atenolol treatment.

LIST OF TABLES

Table 2.1: Sequence of DNA oligonucleotides for real time PCR.....	47
Table 2.2: Sequence of DNA oligonucleotides for real time PCR.....	47
Table 3.1: List of differential expressed proteins in A7r5 cells incubated with propranolol ^a	62
^a S:C is the ratio of different protein expression level in the S-enantiomer incubated cells relative to the control cells; R:C is the ratio of different protein expression level in the R-enantiomer incubated cells relative to the control cells. The <i>p</i> values are listed if predefined criteria for statistical testing were met.....	
	62
Table 3.2: Ratios of intracellular NAD ⁺ /NADH in control cells, S-enantiomer of propranolol-incubated cells and R-enantiomer of propranolol-incubated cells.....	70
Table 4.1: List of differential expressed proteins in A7r5 cells incubated with atenolol ^a	86
^a S:C is the ratio of different protein expression level in the S-enantiomer incubated cells relative to the control cells; R:C is the ratio of different protein expression level in the R-enantiomer incubated cells relative to the control cells. The sequence coverage was calculated from one typical experiment. The <i>p</i> values are calculated for those meeting the predefined criteria for statistical testing.	
	86
Table 4.2: Ratios of intracellular NAD ⁺ /NADH in control cells, S-enantiomer of atenolol-incubated cells and R-enantiomer of atenolol-incubated cells.	88
Table 4.3: Quantification of the ratio of intracellular Ca ²⁺ concentration in S-enantiomer- and R-enantiomer-incubated cells as compared with that of control cells. Ratio between S-enantiomer-incubated cells and control cells (S:C) as well as between R-enantiomer-incubated cells and control cells (R:C) is shown.	93
Table 5.1: List of secreted proteins in conditioned culture medium of A7r5 cells treated with single enantiomers of propranolol respectively ^a	104
^a S:C is the ratio of the level of each protein secreted by S-enantiomer of propranolol incubated cells relative to that by the control cells; R:C is the ratio of the level of each protein secreted by R-enantiomer of propranolol incubated cells relative to that by control cells. “no. of experiments” indicates the number of times the protein was detected in the three independent experiments.	
	104
Table 5.2: Differentially expressed protein, T-Kininogen 1 in conditioned culture medium of A7r5 cells treated with single enantiomer of propranolol respectively ^a	105
^a S:C is the ratio of the level of each protein secreted by S-enantiomer of propranolol incubated cells relative to that by the control cells; R:C is the ratio of the level of each protein secreted by R-enantiomer of propranolol incubated cells relative to that by control cells. “no. of experiments” indicates the times the protein was detected in the three independent experiments. The <i>p</i> values indicated statistical significance of the observed differences.	
	105

Table 6.1: List of secreted proteins in conditioned culture medium of A7r5 cells treated with single enantiomer of atenolol respectively ^a 110

^a S:C is the ratio of the level of each protein secreted by S-enantiomer of atenolol incubated cells relative to that by the control cells; R:C is the ratio of the level of each protein secreted by R-enantiomer of atenolol incubated cells relative to that by control cells. “no. of experiments” indicates the number of times the protein was detected in the three independent experiments..... 110

Table 6.2: Differentially expressed protein, T-Kininogen 1 in conditioned culture medium of A7r5 cells treated with single enantiomer of atenolol respectively ^a 111

^a S:C is the ratio of the level of each protein secreted by S-enantiomer of atenolol incubated cells relative to that by the control cells; R:C is the ratio of the level of each protein secreted by R-enantiomer of atenolol incubated cells relative to that by control cells. “no. of experiments” indicates the times the protein was detected in the three independent experiments. The *p* values indicated statistical significance of the observed differences. 111

LIST OF FIGURES

Figure 1.1: Functional domains of the β_2 -adrenoceptor. Large-diameter segments represent the transmembrane-spanning regions (1-7), the upper portion of the structure represents the extracellular and the lower portion the intracellular domains. GRKs, G protein-coupled receptor kinases. (Adapted from: Summers, R. J., and McMartin, L. R. (1993) Adrenoceptors and their second messenger systems. *J Neurochem* 60, 10-23). 6

Figure 1.2: Mass spectrometers used in proteome research. The left and right upper panels illustrate the sample introduction and ionization process in ESI and MALDI. The different instrumental designs (a-f) are featured with their typical ion source. a, The reflector time-of-flight instrument; b, The TOF-TOF instrument; c, The quadrupole instrument or linear ion trap; d, The quadrupole TOF instrument; e, The (three-dimensional) ion trap; f, The FT-MS instrument. (Adapted from: Aebersold, R., Mann, M., (2003), Mass spectrometry-based proteomics. *Nature* 422, 198-207). 17

Figure 1.3: Cutaway view of the orbitrap mass analyzer. Ions are injected into the orbitrap at a point (arrow) offset from its equator ($z=0$) and perpendicular to the z -axis, where they begin coherent axial oscillations without the need for any further excitation. (Adapted from: Hu et al., (2005), The Orbitrap: a new mass spectrometer. *J Mass Spectrom*, 40, 430-443). 18

Figure 1.4: A schematic diagram illustrating the components and chemistry of the multiplexed isobaric tagging. (Adapted from: Ross, P., Huang, Y., Marchese, J., Williamson, B., Parker, K., Hattan, S., Khainovski, S., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlett-Jones, M., He, F., Jacobson, A., Pappin, D. (2004) Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents. *Mol. Cell. Proteomics* 3, 1154-1169.) 21

Figure 1.5: A representative MS/MS spectrum of peptide TPHPALTEAK derived from a mixture of protein digests by labeling four individual cleaved peptides with each member of the four isobaric reagent tags and then pooling the reaction mixtures in a ratio of 1:1:1:1. (Adapted from: Ross, P., Huang, Y., Marchese, J., Williamson, B., Parker, K., Hattan, S., Khainovski, S., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlett-Jones, M., He, F., Jacobson, A., Pappin, D. (2004) Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents. *Mol. Cell. Proteomics* 3, 1154-1169.) 22

Figure 2.1: A typical schematic of workflow for the preparation of the samples labeled with iTRAQ reagents. 37

Figure 2.2: Flow diagram for online nano 2D LC. (A) The 10-port valve was in position 1: bypassing SCX column, washing enrichment column 1, and analyzing peptides from enrichment column 2. (B) The 10-port valve was in position 2: bypassing SCX column, washing enrichment column 2, and analyzing peptides from enrichment column 1. 39

Figure 2.3: A typical schematic diagram of the setup of Bio-RAD semi-dry system. (Reproduced from: Tan T. L., Ph.D. dissertation, Role of Hepatitis B virus X protein (HBx) in cell adhesion and cytoskeletal reorganization-implications on virus

replication, (2007)). _____ 52

Figure 3.1: Cell viability of vascular smooth muscle cells after 24 h of exposure to R- and S-enantiomer of propranolol, respectively. Three independent experiments were carried out for each enantiomer of propranolol. _____ 57

Figure 3.2: Tandem mass spectra of BSA tryptic peptides prepared by labeling with each of the four isobaric iTRAQ tags and pooling the reaction mixtures in a 1:1:0.75:1.5 ratio. The enlarged low-mass region shows the report ions m/z 114, 115, 116, and 117 an approximate 1:1:0.75:1.5 ratio. 'J' is the iTRAQ-modified lysine residue. _____ 58

Figure 3.3: Normally distributed "data" from the calculated values of S:C ratios with a mean of 0.99 and a variance of 0.12. _____ 64

Figure 3.4: Normally distributed "data" from the calculated values of R:C ratio with a mean of 0.99 and a variance of 0.11. _____ 65

Figure 3.5: ROS levels in A7r5 cells incubated with R-, S-enantiomer of propranolol, respectively. The pictures on the left-handed panels were taken under fluorescent filter. ROS in untreated and treated cells incubated with carboxy-H2DCFDA can cause the green fluorescence; the cells incubated with S-enantiomer of propranolol (D) emitted the lowest fluorescence intensity as compared with control cells or with R-enantiomers of propranolol-treated cells. The pictures on the right-handed panels were taken at phase-contrast setting and overlaid with the corresponding images on the left. The control cells without treatment were used as the negative control (B), the cells with TBHP treatment as positive control (A). The cells incubated with the R-enantiomer of propranolol are shown in (C). _____ 71

Figure 3.6: Real-time PCR analysis of mRNA levels of genes coding for metabolic enzymes in S-enantiomer of propranolol- and R-enantiomer of propranolol incubated cells. Ratio between the S-enantiomer incubated and control cells (S:C), as well as between the R-enantiomer incubated and control cells (R:C) was shown. _____ 72

Figure 4.1: Cell viability of A7r5 cells after 24 h of exposure to R- and S-enantiomer of atenolol, respectively. _____ 79

Figure 4.2: Tandem mass spectra of BSA tryptic peptides prepared by labeling with each member of the four isobaric iTRAQ reagent tags and pooling the reaction mixtures in a ratio of 1:1:0.75:1.5. The enlarged low-mass region shows the report ions m/z 114, 115, 116, and 117 an approximate 1:1:0.75:1.5 ratio. _____ 80

Figure 4.3: A MS/MS spectrum representing a peptide, ADQLTEEQIAEFK, from calmodulin. The ion assignments are as follows: control A7r5 = iTRAQ 114; A7r5 incubated with the S-enantiomer of atenolol = iTRAQ 115; A7r5 incubated with the R-enantiomer of atenolol = iTRAQ 117. _____ 83

Figure 4.4: A MS/MS spectrum representing a peptide, DLESDIIGDTSGHFQK, from annexin A6. The ion assignments are as follows: control A7r5 = iTRAQ 114; A7r5 incubated with the S-enantiomer of atenolol = iTRAQ 115; A7r5 incubated with the R-enantiomer of atenolol = iTRAQ 117. _____ 84

Figure 4.5: Distribution of all proteins identified after iTRAQ labeling and tandem mass spectrometry into different functional categories. _____ 85

Figure 4.6: Western blot analysis of protein levels of calmodulin and annexin A6 in S-enantiomer- and R-enantiomer-incubated cells. (A) Lane Control, cells in the absence of atenolol; Lane S, cells treated with the S-enantiomer of atenolol; Lane R, cells incubated with the R-enantiomer of atenolol. (B), quantification of the protein level based on Western blot analysis. The ratio between S-enantiomer-incubated cells and control cells (S:C) as well as between R-enantiomer-incubated cells and control cells (R:C) is shown. The p values indicated statistical significance of the observed differences with $p < 0.05$ considered as statistically significant and $p > 0.05$ considered as not statistically significant. _____ 91

Figure 4.7: Real time PCR analysis of mRNA levels of genes coding for calcium-binding proteins in S-enantiomer and R-enantiomer of atenolol-incubated cells. The ratio between the S-enantiomer-incubated and control cells (S:C) as well as between the R-enantiomer-incubated and control cells (R:C) is shown. The p values indicated statistical significance of the observed differences with $p < 0.05$ considered as statistically significant and $p > 0.05$ considered as not statistically significant. ____ 92

Figure 4.8: The immunofluorescence images of control A7r5 cell (A), S-enantiomer of atenolol-incubated A7r5 cells (B), and R-enantiomer of atenolol-incubated A7r5 cells (C). The scale bar represents 20 μm . _____ 94

Figure 5.1: A MS/MS spectrum representing a peptide, RPPGFSPFR, from T-kininogen 1. The ion assignments are as follows: control A7r5 = iTRAQ 114; A7r5 incubated with the S-enantiomer of propranolol = iTRAQ 115; A7r5 incubated with the R-enantiomer of propranolol = iTRAQ 117. _____ 106

Figure 5.2: Western blot analysis of T-kininogen 1 in the conditioned medium of A7r5 cells incubated with individual enantiomers of propranolol. Lane C, control, cells in the absence of propranolol. Lane S, cells incubated with the S-enantiomer of propranolol. Lane R, cells incubated with the R-enantiomer of propranolol. The quantification of the protein level based on Western blot analysis was displayed in the table below the Western blot results. Ratio between S-enantiomer incubated cells and control cells (S:C), as well as between R-enantiomer incubated cells and control cells (R:C) was shown. _____ 107

Figure 7.1: A schematic diagram depicting the possible role of metabolic enzymes associated with cellular anabolism and antioxidant activity and the stimulated secretion of T-kininogen in A7r5 cells incubated with S-enantiomer of propranolol. The oval represents the cell membrane, where the seven transmembrane β -adrenoceptors are located. The curved up arrows indicate the changes in increased direction, whereas the curved down arrows indicate the changes in decreased direction. _____ 119

Figure 7.2: A schematic diagram illustrating the possible role of metabolic enzymes involved in cellular anabolism, the possible role of Ca^{2+} binding proteins and the enhanced secretion of T-kininogen in A7r5 cells incubated with S-enantiomer of atenolol. The oval represents the cell membrane, where the seven transmembrane

β -adrenoceptors are located. The curved up arrows indicate the changes in increased direction, whereas the curved down arrows indicate the changes in decreased direction.

LIST OF ABBREVIATIONS

iTRAQ	isobaric tag for relative and absolute quantitation
2D	two-dimensional
2-DE	two-dimensional gel electrophoresis
ADF	actin-depolymerizing factor
B1R	bradykinin receptor B1
BK	bradykinin
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
carboxy-H2DCFDA	5-(and-6)-carboxy-20, 70-dichlorodihydrofluorescein diacetate
CID	collision-induced dissociation
DBK	des-Arg ⁹ -BK
DMEM	Dulbecco's modified Eagle's medium
DRF	diaphanous-related formins
ECL	enhanced chemiluminescence
ECs	endothelial cells
EF	error factor
ESI	electrospray ionization
FT-MS	Fourier transform ion cyclotron
GAPs	GTPase-activating proteins
GDP	guanosine diphosphate
GPRs	G-protein-coupled receptors
GRKs	G protein-coupled receptor kinases
GTP	guanosine triphosphate

HBSS	Hank's Buffered Salt Solution
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HUVECs	human umbilical vein endothelial cells
I/R	ischemia/reperfusion
KCL	potassium chloride
LC	liquid chromatography
MALDI	matrix-assisted laser desorption/ionization
MAPK	mitogen activated protein kinases
MS	mass spectrometry
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NAD ⁺	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide plus hydrogen
NADt	total NAD ⁺ and NADH
PCR	polymerase chain reaction
PTMs	post-translational modifications
RGS	regulator of G protein signaling
ROCKs	Rho kinases
ROS	reactive oxygen species
RP	reversed-phase
SCX	strong cation exchange
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SILAC	amino acids in cell culture
TBHP	tert-Butyl hydroperoxide

T-kinin	Ile-Ser-bradykinin
TOF	time-of-flight
TrkA	tyrosine kinase receptor
VSMCs	vascular smooth muscle cells

PUBLICATIONS

1. **Jianjun Sui**, Jianhua Zhang, Chi Bun Ching, Wei Ning Chen.(2009) Expanding proteomics into chiralities of chiral drugs. *Mol. BioSyst*, 5(6), 603-608.
2. **Jianjun Sui**, Jianhua Zhang, Chi Bun Ching, Wei Ning Chen. (2008) Comparative proteomic analysis of extracellular proteins revealed secretion of T-kininogen from vascular smooth muscle cells in response to incubation with S-enantiomer of propranolol. *Mol. Pharm*, 5(5), 885-890.
3. **Jianjun Sui**, Jianhua Zhang, Tuan Lin Tan, Chi Bun Ching, Wei Ning Chen. (2008) Comparative proteomic analysis of vascular smooth muscle cells incubated with S- and R-enantiomers of atenolol using iTRAQ-coupled 2D LC-MS/MS. *Mol. Cell. Proteomics*, 7(6), 1007-1018.
4. **Jianjun Sui**, Tuan Lin Tan, Jianhua Zhang, Chi Bun Ching, Wei Ning Chen. (2007) iTRAQ-coupled 2D LC-MS/MS analysis on protein profile in vascular smooth muscle cells treated with S- and R-enantiomers of propranolol: possible role of metabolic enzymes involved in cellular anabolism and antioxidant activity. *J. Proteome Res*, 6(5), 1643-1651.
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1. Introduction

1.1 Chiral Drug

Chirality originated from Greek, referring to handedness, is a property of asymmetry important in the living world. In pharmacology, there is increasing awareness of the importance of drug chirality and the role it plays in explaining the oftentimes dramatic differences in biological activities of individual enantiomers of therapeutic agents (Triggle 1997). The number of the enantiomers is dependent on the number of chiral centers in the molecule and each of them cannot superimpose on the other as the orientation of the atoms or groups in each form is different in space, though with the same structure formula (Wsol *et al.* 2004). An enantiomer can be named by the direction that it rotates the plane of the polarized plane light, as designated as (+) or (−). For simple systems with only a single asymmetric carbon atom, an enantiomer can be named by relating the molecule to the smallest commonly used chiral molecule, glyceraldehyde, with the nomenclature system referred as the D/L. The most important nomenclature for denoting enantiomers is *R/S* system, in which each of the four groups of an enantiomer is assigned a priority based on atomic number and the chiral center is oriented in the way the lowest priority group is pointed away from the viewer, then there are two possibilities: the enantiomer is labeled *R* if the remaining three priorities decrease in clockwise direction and conversely, the counterclockwise direction gives rise to *S*-enantiomer.

Since the beginning of the 20th the investigation of the role of chirality in drug action and disposition has begun with the emphasis placing on enantioselectivity. For example, in Easson's study on adrenaline to determine which of the four groups

linking to the asymmetric carbon atom are involved in the attachment of the drug molecule to its specific receptor, it was found that L-adrenaline is more potent than D-adrenaline (Easson & Stedman 1933). By the 1970s a lot of research work has been done on the role of chirality in drug action and metabolism, and the achievement in this field has been summarized in several reviews (Portoghese 1970, Sastry 1973).

During 1980s, new powerful methods for the preparation of an enantiomerically pure drug and the enantioselective separation of an existing racemic drug into its enantiomers in a pure form have been developed. It was documented that single-enantiomer drugs accounts for an increasingly growing proportion of new drugs that are commercially available in the market, rising from approximately 20% of new drugs ten years ago to almost 75% today (Agranat *et al.* 2002). To date, chiral switch from existing racemic mixtures to single enantiomers (Tucker 2000, Agranat & Caner 1999) is now a major theme in the drug development portfolios. The potential advantages residing in the chiral switching are that single enantiomer is able to enhance therapy due to stereoselectivity and strong potency in pharmacokinetic properties as well as decreased side-effects and diminution of drug-drug interactions. Furthermore the stimulus for chiral switching to further profitability has driven the extension of the racemates with patents that are expiring in an effective way the racemic drug is substituted with single isomers — for example, omeprazole and fluoxetine (Tucker 2000).

The chiral drugs can be subdivided into three groups according to the degree of bioactivities of single enantiomers as follows: racemic drugs with one major bioactive enantiomer, whereas others are inactive or less active or even toxic; racemic drugs with equally bioactive enantiomers and racemic drugs with chiral inversion (Drayer

1986, Waldeck 2003).

A majority of chiral drugs belong to the first group such as β -blockers, angiotensin-converting enzyme inhibitors, calcium channel antagonists and many antiarrhythmic drugs. A large amount of studies have demonstrated that single enantiomers of these chiral drugs exhibited different behaviors in their distribution, metabolic and pharmacokinetic profiles both quantitatively and qualitatively (Lin & Lu 1997). However there are only a few racemic drugs with enantiomers being equally bioactive, such as cyclophosphamide, flecainide and fluoxetine. For the third group where generally one enantiomer of the racemic drug can perform chiral inversion into another enantiomer, it is comprised of a number of nonsteroidal anti-inflammatory drugs such as ibuprofen, ketoprofen, fenpropfen and benoxaprofen. For example as an inhibitor of cyclo-oxygenase I, *S*-ibuprofen is approximately 100 times as active as *R*-ibuprofen. It was reported that only inactive *R*-enantiomer can undertake chiral inversion into the *S*-enantiomer in vivo and not vice versa (Landoni & Soraci 2001, Marzo & Heftmann 2002).

Examining and characterizing interactions between chiral drug and biomolecules are increasingly playing an instrumental role particularly, chiral recognition in various biological processes, for example, the enzyme–substrate interactions. Kawamura and Hindi reported the possibility of attaining high protein-binding selectivity by the use of simple chemical probes containing benzophenone, where the chirality center as well as the neighboring substituent in the vicinity of benzophenone played the major role in the protein-binding selectivity. In doing so they created a method that chiral recognition processes can be examined in complex proteomes with a simple chemical strategy using photoactive probes (Kawamura & Hindi 2005). In another example of

detailed investigation of enantioselective interaction of alprenolol and propranolol enantiomers with the protein Cel7a, a surface plasmon resonance and HPLC perturbation method were used. It was found that the interactions with the enantioselective and nonselective binding sites are highly pH dependent. The resulted underlining chiral mechanism is the large affinity differences between the two enantiomers, whereas the binding capacity difference accounts for a smaller influence (Arnell *et al.* 2006).

A number of chiral drugs act on cell receptors. Studying the chiral drug/receptor interaction will assist in elucidating the molecular mechanism of chiral drug therapy. Li and co-workers examined the effects of the natural and unnatural forms of neuroactive steroids in response to $\rho 1$ receptors and confirmed that the enantiomers of the natural steroids are much less effective at either potentiating ($3\alpha 5\alpha P$) or inhibiting ($3\alpha 5\beta P$) the activation of the $\rho 1$ receptor, whereas neurosteroids, and probably other steroids, act on the $\rho 1$ receptor by interacting with a chiral site with the receptor protein (Li *et al.* 2006).

1.2 β -Adrenoceptors

The endogenous catecholamines (noradrenaline and adrenaline) are the transmitters of the sympathetic nervous system, which can evoke their biological effects through stimulation of specific membrane-bound receptors, the adrenoceptors. The adrenoceptors can be categorized into the subtypes α and β to explain the differences in action of noradrenaline and adrenaline. With the development of molecular biology techniques into pharmacology, it rapidly became recognized that both α - and β -adrenoceptors can be further subdivided into the following subtypes: $\alpha 1A$, $\alpha 1B$ and $\alpha 1D$; $\alpha 2A$, $\alpha 2B$ and $\alpha 2C$; β_1 , β_2 and β_3 adrenoceptors (Bylund *et al.* 1994).

Among the three known types of β -adrenoceptors, particularly, β_1 - and β_2 -adrenoceptors are well investigated for their pharmacology. β_1 -adrenoceptors are located mainly in the heart and in the kidneys while β_2 -adrenoceptors located mainly in the lungs, gastrointestinal tract, vasculature, and uterus; however, it should be noted that the organ-specific subclassification of β -adrenoceptors into cardiac β_1 - and vascular and bronchial smooth muscle β_2 -adrenoceptors is inappropriate: it is now generally accepted that in a variety of organs and tissues, β_1 - and β_2 -adrenoceptors appear to coexist, whereby normally one subtype predominates (Stiles *et al.* 1983, Minneman *et al.* 1979). The most important β_1 -adrenoceptor-mediated effects of catecholamines are their effects on the heart, lipolysis and regulation of renin release (and by this activation of the renin-angiotensin-aldosterone system); the most important β_2 -adrenoceptor-mediated effects of the catecholamines are bronchodilation, relaxation of uterine and vascular smooth muscles and glycogenolysis (Brodde 2007).

The β -adrenoceptors belong to the family of G-protein-coupled receptors (Johnson 2006) (GPRs) characterized by seven transmembrane spanning domains, forming a pocket in which the agonists and competitive antagonists find their binding sites (Strader *et al.* 1989). For example, the functional domains of β_2 -adrenoceptor have been determined (Dixon *et al.* 1988, Dohlman *et al.* 1991), as illustrated in Figure 1.1. It was found that the ligand binding site is located in the transmembrane-spanning regions (Tota *et al.* 1991), whereas the glycosylation sites at Asn⁶ and Asn¹⁵ are lying outside the cell (Dixon *et al.* 1987b, Dixon *et al.* 1987a). Five (Cys^{106, 184, 190, 192, 341}) and Asp¹¹³ are believed to be essential for both agonist and antagonist binding (Raymond *et al.* 1990). Asp⁷⁹, Ser^{204, 207, 309}, Tyr³²⁶, and Phe^{289, 290} are in more affinity for agonists than antagonist.

Figure 1.1: Functional domains of the β_2 -adrenoceptor. Large-diameter segments represent the transmembrane-spanning regions (1-7), the upper portion of the structure represents the extracellular and the lower portion the intracellular domains. GRKs, G protein-coupled receptor kinases. (Adapted from: Summers, R. J., and McMartin, L. R. (1993) Adrenoceptors and their second messenger systems. *J Neurochem* 60, 10-23).

It is well accepted that β -adrenoceptor are coupled to their effector systems by heterotrimeric G proteins, which is composed with three subunits, the tightly associated β and γ subunits and α subunits. In the basal state, guanosine diphosphate (GDP) was bound to the catalytic site of GTPase on the α subunit, and was replaced by guanosine triphosphate (GTP) following interaction of agonists with a coupled receptor, resulting in dissociation of GTP- α subunit complex from the $G_{\beta\gamma}$ subunits(Hamm 1998). The GTP- α subunit complex interacts with the effector proteins including adenylyl cyclase, phospholipase C, ion channels or mitogen activated protein kinases (MAPK), resulting in a variety of cellular functions. One of the well documented signal transduction is that the GTP- α subunit complex will stimulate adenylyl cyclase activity, and modulate levels of intracellular second messenger cyclic adenosine monophosphate (cAMP)(Birnbaumer 1990). cAMP activates protein kinase A and thus leads to phosphorylate L-type calcium channels and myosin light-chain kinase (Nishikawa *et al.* 1984, Somlyo & Somlyo 1994), with Ca^{2+} entry facilitated and positive inotropic effects in ventricles and atria generated. Apart from the underlying mechanisms that explain the alterations in ion transport indirectly, β -adrenoceptor activation is more linked to ion channels directly (Brown 1990). Upon ligand binding, receptors also undergo adaptive changes such as homologous desensitization, i.e., GPRs rapidly lose their ability to respond to agonist. Homologous desensitization involves phosphorylation of ligand-occupied receptors by members of G protein-coupled receptor kinases (GRKs) (Summers & McMartin 1993, Huttenrauch *et al.* 2005). It was also documented that the Ser and Thr residues in the carboxy-terminus region shown in Figure 1.1 are involved in receptor phosphorylation by GRKs(Summers & McMartin 1993). Following receptor phosphorylation by GRKs specifically binding to the agonist-occupied receptor, arrestin is binding to preclude

receptor/G protein interaction, resulting in functional desensitization (Palczewski 1997, Krupnick & Benovic 1998). The activated G_α can in turn be inhibited by regulator of G protein signaling proteins (RGS), which are GTPase-activating proteins (GAPs) that reduce the signal transmitted by the receptor-activated (GTP bound) G_α subunit by rapidly returning it to the inactive state (GDP bound) (De Vries & Gist Farquhar 1999). It was reported that the three β -adrenoceptors share roughly 60% sequence homology (Summers & McMartin 1993).

1.3 β -Blockers

In contrast to β -adrenergic agonists, β -blockers (β -adrenoceptor antagonists) are compounds that bind to β -adrenoceptor but compete with β -adrenergic agonists and thus inhibit their physiological effects. The benefit of β -adrenergic blockade is that it is able to decrease excessive or inappropriate sympathetic activity in some clinical conditions to the degree that the blocking effect does not impair necessary sympathetic activity and thus preserve the normal sympathetic stimulation. Principally, β -blockers can be classified into three classes/generations (Bristow 2000). “First class/generation” β -blockers are nonselective that can have an effect on both β_1 -adrenoceptors and β_2 ones, with propranolol being the first β -blocker introduced for the treatment of angina (Black *et al.* 1964). “Second class/generation” β -blockers are drugs that have a higher affinity and thus exert greater blocking activity on β_1 -adrenoceptors than β_2 ones. “Third class/generation” β -blockers are drugs with additional vasodilating properties such as carvedilol, bucindolol and nebivolol.

As mentioned above, β -receptor is involved in mediating the effects of the sympathetic nervous system; in addition, an increased activity of the sympathetic nervous system can have an important role in various cardiovascular diseases such as

hypertension, coronary artery disease, ventricular and supraventricular arrhythmias (Brodde 2007). Therefore it is quite understandable that the blocking effects exerted by β -blockers can play an important therapeutic role in treatment of these diseases.

Despite the well documented blocking action of catecholamines on β -adrenoceptors, concerns regarding β -blockers' potential adverse effects such as β -blockers at usual doses bringing an undesirable risk of provoking type 2 diabetes (Taylor *et al.* 2006) and in particular, the underlying molecular mechanism remains to be addressed. Although to date many β -blockers are administered as the racemic mixture, things are changed as the development of separation methods have been applied to enhance the production of individual enantiomers and market them economically available especially for those with only one enantiomer being active at the receptor and accounting for most of the β -blocker effect (Stoschitzky *et al.* 1995, Vermeulen *et al.* 1990). It was generally accepted that biochemical responses to external forces such as disease or drugs are likely to be reflected in the patterns of protein expression and turnover in affected cells, tissues. The mechanism of action of β -blockers has been studied at gene expression level (Herndon *et al.* 2003). However, little is known about their action at the protein level. It will be of great interest to detect changes in quantitative protein profiles and to infer biological function from the observed patterns.

1.4 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) has greatly developed to meet the increasing demands of pharmaceutical industry to separate a mixture of molecules into individual ones and thus obtain molecule structural information coupled with other instruments such as mass spectrometry (MS) and nuclear magnetic resonance.

The instrumental component of HPLC system is the column where the actual separation occurs. Generally these columns can be subdivided into five groups of (i) normal-phase column which is normally packed in non-hydrated form with nonpolar solvents such as hexane being mobile phases, (ii) other bonded-phase silica columns specially used for separation of racemic mixtures with C8 side chains bonded on silica through Si–O–Si linkages to enable nonpolar elements of the mobile phase to move closer to the polar support, (iii) reverse-phase column (RP), featured with a nonpolar bound phase with a polar mobile phase to allow the most polar sample components to elute first, whereas the nonpolar compounds being retained for a longer time on the nonpolar column packing, (iv) ion-exchange column which requires salt plugs with a high concentration of salt or salt gradients to substitute the retained ionized material and thus elute them from a column, and (v) size-separation columns with different sizes being their separating mode. As discussed above each type of column is characterized with its typical configuration and utilized for separation of a specific range of molecules. In some cases where a mixture of complexity is needed to be separated and one type of column cannot achieve it, generally several types of columns with different separation mechanisms are combined to accomplish the task, for example, ion-exchange column coupled with reverse-phase column to resolve the digested peptides of more complexities.

Accompanying the development of specific columns, the advances in HPLC technique have further enhanced the application in molecules separation, such as the nano-HPLC with the nanoscale flow rate of mobile phase and the ultra-high pressure liquid chromatography, which possess the advantages of reducing residence time on the column with minimum sacrifice in resolution. For example, in the scenario that a very small amount of material needs to be analyzed the nano-HPLC is able to perform the

task successfully rather than the normal HPLC where a great volume of solvent is required. The small flow rate HPLC is more suitable to couple with other instrument such as the sensitive MS to obtain useful information of the extremely small amounts of material. However it is generally accepted that HPLC at higher flow rates is less prone to problems and this is the reason why it is widely used in industry.

Another important component of HPLC system is pump, specially designed for pumping mobile phase from the solvent source through the system to the waste tank. For a simple separation of materials of less complexity an isocratic pump system suffices to accomplish it by pumping an unchanged mobile phase with fixed composition. However for separation of a complex mixture of compounds, utilization of a simple mobile phase is not recommended. As a result, gradient solvent is used to achieve the separation characterized by changed compositions in mobile phase with the progress of time. For example, some Agilent 1200 Series nanoflow LC systems consist of two pumps, i.e., the isocratic pump and nanoflow LC pump, specialized for two-dimensional LC and providing better separation of a mixture of samples of complexity.

In addition to the advances in equipment configuration, the optimization of mobile phase compositions as well as the addition of additives in the mobile phase has been extensively investigated for HPLC separation. For example, it is generally accepted that addition of a small amount of formic acid in mobile phase to adjust the acidification had the added benefit of keeping the analyte to reside on the column longer and obtaining better separation(Wu *et al.* 2004). Another example demonstrated that selection of appropriate mobile phase for HPLC separation is very important. It was reported that a response for the compound in the water/methanol

mobile phase was approximately 25 times as high as that in the water/acetonitrile mobile phase (Jemal & Hawthorne 1999).

Last but not least the development of automation and program has the beneficial effect on molecules separation, enabling a complex mixture of compounds to be transformed into a number of separate fractions and facilitating the analysis with high sensitivity. A sophisticated gradient HPLC system equipped with an autosampler can be used to make a series of injections of the same sample exposed to several predefined gradient conditions to determine the optimum separation condition.

1.5 Proteomics

In 1996 the term 'proteome' was first introduced by Wilkins et al. (Wilkins *et al.* 1996) and refers to the entire complement of proteins expressed in a given cell, tissue or organism, the study of which is proteomics. Proteomics is often considered complementary with genomics in the study of biological systems, and in some cases proteomics provides more useful information about an organism than genomics as it directly addresses the level of genome products and their interactions.

In the rapidly expanding field of proteomics mass spectrometry has become an instrumental technology to address the increasing bioanalytical demands encountered in proteomic studies. Owing to its wide dynamic signal range and the facility to couple with chromatographic separation methods, mass spectrometry interfaced with HPLC has proved to be one of the most powerful tools in proteomics study. The LC/MS interface facilitates in ionizing samples that in turn are introduced into the high-vacuum mass spectrometer for analysis. Generally LC/MS interface can be classified into the following groups based on their individual mechanisms:

electrospray ionization (ESI) interface, atmospheric-pressure chemical ionization spray interface, matrix-assisted laser desorption/ionization (MALDI), and ion spray (IS) interface. As for proteomics study, ESI and MALDI are widely used. In ESI-MS, the analyte dissolved in polar solvent is infused into the ionization source where a high electrical potential is applied, highly charged droplets were formed and then vaporized with the aid of a neutral gas. In MALDI, a dry crystalline matrix is used to minimize the sample damage by direct laser beam and also serves to facilitate vaporization and ionization process by absorbing the laser radiation. Compared with ESI, MALDI interfaced with HPLC are, to some degree, more tolerant of the modest presence of salts and/or detergents (Breux *et al.* 2000) owing to the fact that analytes can break away from impurities during ionization in the same way that they escape the matrix, whilst when using ESI, impurities can compete with the analytes and are likely to influence the results. ESI, theoretically, has unlimited mass range because it tends to produce multiply charged species for proteins or peptides, and proteins with large molecular weights can display at lower m/z values. In contrast, MALDI, tends to generate singly charged species and this feature is greatly important, particularly for identifying the molecular ion of proteins, carbohydrates and lipids (El-Aneed *et al.* 2009). In addition, ESI enables to generate ions directly out of a solution and is therefore more readily coupled to liquid-based separation tools.

The development of proteomics has necessitated the need to separate the complex mixture of proteins or perform the fractionations of digested peptides before identified by mass spectrometry. One of the approaches is two-dimensional gel electrophoresis (2-DE). Briefly, a mixture of proteins is first separated on an immobilized pH gradient strip, further separated into individual spots (proteins) by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) slab gel and then stained

by silver or Coomassie Brilliant Blue. Following comparison of the staining patterns of those individual proteins from two samples the differentially expressed proteins of interest are defined, excised and cleaved into peptides by using proteolytic enzyme such as trypsin, which predominantly cleaves proteins at the C-terminal side of the lysine and arginine, with the exception that either of the two amino acids is followed by proline. The peptide digests are then identified by MS in particular, MALDI. 2-DE possesses the advantage of enabling a rapid comparison of differences in proteins between individual samples and is relatively economical as compared with LC-based proteomics. However, several practical limitations remain, including difficulty in focusing highly basic and acidic proteins, low resolution against hydrophobic and membrane proteins, inadequate sensitivity (Timperman & Aebersold 2000, Lilley *et al.* 2002). Due to these limitations, an alternative approach involves the use of liquid-based techniques including capillary electrophoresis and HPLC have gradually substituted the gel-based separation to meet the increasing demand for resolving of the high degree of complexity of cellular proteomes and detect low abundance of many of the proteins. In the LC-based technique the mixture of the cleaved peptides are bonded and then separated sequentially on the multi-dimensional columns based on charge or hydrophobicity of the ionized analytes with solvents, eluted into the MS for identification.

To date the rapid development of LC-based separation coupled with ESI-MS has further enhanced the technology in systematic analysis of more complexity of cellular proteome, primary sequence, post-translational modifications (PTMs) and protein-protein interactions. In addition to the two types of ion sources described above, a mass analyzer that measures gas phase ions with respect to the mass-to-charge ratio (m/z) and a detector which generates a current signal correlated

with the abundances of respective ion fragments are essential components of MS as well, with emphasis especially on the mass analyzer.

Several basic types of mass analyzer are used in proteomics research including quadrupole, reflector time-of-flight (TOF), quadrupole ion trap, Fourier transform ion cyclotron (FT-ICR) and orbitrap mass analyzers, illustrated in detail in Figure 1.2 (Aebersold & Mann 2003) and Figure 1.3 (Hu *et al.* 2005). In order to perform tandem mass analysis with quadrupole instruments it is necessary to place two or more quadrupoles in series. In the first quadrupole (Q_1), the time-varying electric fields between four rods are applied to scan across ions of a preset m/z range or select an ion of a particular m/z . The second quadrupole, known as the collision cell (q_2), introduces the collision gas and fragmented the selected ions, and the third quadrupole (Q_3) serves to read out the fragmented ions. For ion trap analyzer the physics behind it is similar as that for quadrupole analyzer except that the fragmented ions are trapped in a radio frequency field rather than passing through a quadrupole. FT-ICR is used to trap the ions with the introduction of strong magnetic fields. In TOF analyzer the ions are accelerated with high kinetic energy, separated along a flight tube based on their different velocities, returned from a reflector to make up for slight differences in kinetic energy, and finally reach on a detector that amplifies and registers arriving ions. The tandem mass spectrometry, quadrupole/time-of-flight, regarded as a triple quadrupole with the third quadrupole replaced by an orthogonal TOF, has found applications in rapid *de novo* peptide sequencing (Morris *et al.* 1996, Shevchenko *et al.* 1997). The orbitrap mass analyzer consists of an outer barrel-like electrode and an inner spindle-like central electrode that form an electrostatic field. Tangentially injected ions rotate around the central electrode and trapped when an appropriate

voltage between the outer and central electrodes is applied. The frequencies of axial oscillations of ions of specific mass-to-charge ratio are detected for mass analysis.

Advantages of the quadrupole ion trap are fast analysis, relatively inexpensive, simple vacuum requirements and simplicity of mechanical construction. Commercial quadrupole ion trap typically has a significantly lower resolution of about 2000 and mass range of 4000-6000 m/z (Payne & Glish 2005). TOF mass analyzer is particularly susceptible to variations in ion intensity due to its employed fast acquisition systems with inherently modest dynamic range (Wu & McAllister 2003, Blom 2001). It was reported that TOF cannot achieve the mass accuracy of 5 ppm over a signal range larger than a few hundred even with the aid of advance algorithms for intensity correction (Blom 2001, Colombo *et al.* 2004). Unlike TOFs, FT-ICR uses much slower acquisition systems with much higher dynamic range and showed an intra scan dynamic range of a few thousand with mass accuracy of a few ppm (Syka *et al.* 2004). In addition, FT-ICR is not a high-throughput method in the traditional sense (Zhang *et al.* 2005) and therefore is not particularly suitable with modern high-speed HPLC. Finally, the novel orbitrap mass analyzers have a high mass accuracy (1-2 ppm), a high resolving power (up to 200,000) and a high dynamic range (around 5000) (Makarov *et al.* 2006b, Makarov *et al.* 2006a).

1.6 Quantitative Proteomics

As is well established, 2 or more than 2 unique peptides suffice to recognize a protein. However, it is sometimes occurred that the protein-specific peptides cannot be identified or detected as they fall beyond the mass range analyzed or they are too strongly attached to the chromatographic column during analysis to be above the threshold of detection limited by the sensitivity of MS, which will inevitably lead to

the false conclusion that the protein is absent. Therefore, a very limited knowledge of protein abundance in a sample is provided by the MS protein identification schemes if no quantitative measures are taken along with it. More importantly, most changes in proteins induced by perturbation of a biological system can only be detected if the quantitative information is provided. In addition, quantitative data for modeling is increasingly required in system biology.

Figure 1.1: Mass spectrometers used in proteome research. The left and right upper panels illustrate the sample introduction and ionization process in ESI and MALDI. The different instrumental designs (a–f) are featured with their typical ion source. a, The reflector time-of-flight instrument; b, The TOF-TOF instrument; c, The quadrupole instrument or linear ion trap; d, The quadrupole TOF instrument; e, The (three-dimensional) ion trap; f, The FT-MS instrument. (Adapted from: Aebersold, R., Mann, M., (2003), Mass spectrometry-based proteomics. Nature 422, 198-207).

Figure 1.3: Cutaway view of the orbitrap mass analyzer. Ions are injected into the orbitrap at a point (arrow) offset from its equator ($z=0$) and perpendicular to the z -axis, where they begin coherent axial oscillations without the need for any further excitation. (Adapted from: Hu *et al.*, (2005), The Orbitrap: a new mass spectrometer. *J Mass Spectrom*, 40, 430-443).

Currently the well established technique of stable-isotope dilution has been widely adopted. The underlying principles are that a multiplex set of identical chemical analytes of different stable-isotope composition are capable of producing the same MS response signal and being detected on the detector of the mass spectrometer due to the same ionization efficiencies, but only differentiated by the mass difference resulted from the introduction by the stable isotopes, and the abundance ratio for the analytes can be directly determined from the ratio of signal intensities for those analytes. A number of methods have been developed and applied to stable isotope-based quantification for proteomics and further classified based on the way stable isotope labels are introduced onto the peptide or protein into the following 4 groups of (i) Spiking in an isotopically labeled peptide which was chemically synthesized as

internal standards with known quantities (Kusmierz *et al.* 1990), (ii) incorporation through an enzyme via transfer of ^{18}O from water to peptides during protein digestion (Mirgorodskaya *et al.* 2000, Yao *et al.* 2001), (iii) introducing isotope-tagging chemistries onto peptides or proteins and (iv) metabolic incorporation of the stable isotope to the cells in particular, stable isotope labeling by amino acids in cell culture (SILAC), in which one cell population grow in a medium that contains a 'light' (normal) amino acid and the other in a medium containing a 'heavy' amino acid. In so doing one cell state is labeled metabolically by, for example, ^{13}C -labelled arginine (Ong *et al.* 2002).

SILAC is experimentally straightforward: it allows mixing of labeled and unlabelled cell population and subsequent fractionation and purification of the pooled sample can be simply performed, introducing fewer errors in quantitation. However SILAC labeling limits its use in living cells whereas chemical labeling can be applied in other proteome including body fluids and secretory proteome. Of all the developed stable isotope-based quantification methods, isobaric tag for relative and absolute quantitation (iTRAQ) has recently gained popularity as it allows up to four samples to be examined within one experiment, increasing analytical throughput and resulting in great reduction of work and costs. However, more scan time is needed because the tandem MS is acquired for the quantitation with iTRAQ. In addition, mass analysis of iTRAQ-labeled samples cannot be performed on mass spectrometers with a low mass cut-off during MS/MS (Putz *et al.* 2005).

The complete iTRAQ molecule consists of a reporter group, a mass balance group and a peptide-reactive group as shown in Figure 1.4A (Ross *et al.* 2004). The overall mass of reporter group ion (ranging in m/z from 114.1 to 117.1) and mass balance group of

the molecule are kept constant by introducing the differential isotopic enrichment by ^{18}O , ^{15}N and ^{13}C atoms. When a tag reacted with a peptide, an amide linkage was formed to any peptide amine (N-terminal or ϵ amino group of lysine) shown in Figure 1.4B. A mixture of four identical peptides each reacted with one member of the multiplex set is shown as a singly unresolved precursor ion in MS (indistinguishable m/z). Following collision-induced dissociation (CID) MS/MS analysis of the precursor ion, the four reporter group ions separate as distinct masses (114–117 Da) displayed in Figure 1.4C. All other sequence-instructive fragment ions (b-, y-, etc.) are still isobaric and the relative concentration of the peptides is derived from the relative intensities of the reporter ions.

A MS/MS spectrum representative of peptide TPHPALTEAK from a mixture of labeled peptides by labeling four separate digests through reacting with each member of the four isobaric tags and combining the reaction mixtures in a ratio of 1:1:1:1 was shown in Figure 1.5 (Ross et al. 2004). Briefly, isotopic distribution of the precursor with a m/z of 1352.84 ($[\text{M}+\text{H}]^+$) was displayed in (i), the reporter ions in the low mass region shown in (ii) were used for quantitation, isotopic distribution of the b_6 fragment ion shown in (iii), and isotopic distribution of the y_7 fragment ion shown in (iv). It is clear that the precursor ion and as well as all the internal fragment ions denoted as b $^-$ and y $^-$ contain the four members of the tag set, but stay isobaric.

Figure 1.4: A schematic diagram illustrating the components and chemistry of the multiplexed isobaric tagging. (Adapted from: Ross, P., Huang, Y., Marchese, J., Williamson, B., Parker, K., Hattan, S., Khainovski, ., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlett-Jones, M., He, F., Jacobson, A., Pappin, D. (2004) Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents. *Mol. Cell. Proteomics* 3, 1154-1169.)

Figure 1.5: A representative MS/MS spectrum of peptide TPHPALTEAK derived from a mixture of protein digests by labeling four individual cleaved peptides with each member of the four isobaric reagent tags and then pooling the reaction mixtures in a ratio of 1:1:1:1. (Adapted from: Ross, P., Huang, Y., Marchese, J., Williamson, B., Parker, K., Hattan, S., Khainovski, ., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlett-Jones, M., He, F., Jacobson, A., Pappin, D. (2004) Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents. *Mol. Cell. Proteomics* 3, 1154-1169.)

1.7 Application of Mass Spectrometry-based Proteomics

With the developments of HPLC separation techniques, MS technologies and informatics/bioinformatics applied to analysis of large scale data, MS-based proteomics have become a crucial research tool with the potential benefits to a variety of protein samples and drug biomarker discovery. As discussed above, 2-DE coupled with MS, LC-MS- or MS/MS-based proteomics technologies have provided highly sensitive analytical capabilities (Qian *et al.* 2006). For example, the multidimensional liquid chromatography combined with a linear ion trap-orbitrap hybrid mass

spectrometer demonstrated the suitability of the comparative analysis of complex bio-analytical samples(Venable *et al.* 2007).

Substantial progress has been achieved in protein and peptide separation schemes, and as a result, increasingly robust and productive platforms have been established. Among them are the widely used two-dimensional (strong cation exchange/reversed phase)(Link *et al.* 1999, Wolters *et al.* 2001) or three-dimensional chromatographic (strong cation exchange/avidin/reversed phase) (Han *et al.* 2001) separations of peptide mixtures generated by tryptic digestion of protein samples, by which to date the number of proteins identified can reach into several hundreds and are capable of detecting proteins of very low abundance. Moreover MS-based proteomics has been widely applied in addressing a number of biological or clinical issues, including the generation of protein profiling, mapping protein-protein interactions and the determining protein modifications.

1.7.1 Functional Studies of Protein Profiles with Proteomics

The more common and versatile use of large-scale MS-based proteomics has been in investigating protein expression profiles as a function of cellular state as an aid to infer cellular function. Systematically analyzing the proteins expressed in an organism has been illustrated recently by the creation large-scale proteome maps of microorganisms such as yeast (Washburn *et al.* 2001) or the bacterium *Deinococcus radiodurans* (Lipton *et al.* 2002). Increasingly, stable-isotope dilution and LC-MS/MS are used to identify potentially clinically useful molecular patterns in cancer (Hanash 2004), heart disease (Stanley *et al.* 2004), and other common ailments, establish cellular signaling pathways in response to various external stimuli, including comparing normal and diseased conditions (Griffin *et al.* 2002, Katz-Jaffe *et al.* 2006,

Shiio *et al.* 2002, Tan & Chen 2005) and offer a nontargeted way to identify protein profiles or drug-activity markers since there is often no priori knowledge of the particular proteins that are likely to change (Tyers & Mann 2003). Goufman *et al.* well documented that the thermostable fractions of serum samples taken from patients diagnosed with uterus, ovarian, and breast cancers, as well as those samples from benign ovarian tumor, were investigated by mass spectrometry system (Goufman *et al.* 2006). Of them, the expression of R-1 acid glycoprotein and clusterin has been found to be down-regulated in breast cancer, whereas that of transthyretin decreased specifically in ovarian cancer (Goufman *et al.* 2006). It was reported that ATP synthase showed higher expression level in tumor tissues and existed on the plasma membrane of breast cancer cells. When treated with ATP synthase inhibitor, aurovertin B, breast cancer cells exhibited a significant decrease in cell density, indicating that aurovertin B can be served as an antitumorigenic agent and is likely to be exploited in cancer chemotherapy (Huang *et al.* 2008).

Currently vascular proteomics is still in its early stage compared with other areas in proteomic research. As the barrier to perform the proteomic analysis of vessels lies in their heterogeneous cellular composition (Mayr *et al.* 2006). The alternative approach to study blood vessels is the use of cultured cells, e.g., mature endothelial cells (ECs) obtained from large arteries and veins, human umbilical vein endothelial cells (HUVECs) drawn from transient fetal vessels and as well as smooth muscle cell (SMC) which is located within the vasculature and includes any connective tissue cell around the endothelial tube (Mahoney & Schwartz 2005). It was documented that a variety of studies have been carried out in which specific enzymes or proteins associated with hypertension are regulated and differentially expressed at the molecular level for cell culture models (Mayr *et al.* 2006). For example, using 2-DE in conjunction with

MALDI-MS techniques Fuchs *et al.* reported that HUVEC treated with the pro-atherosclerotic stressors homocysteine showed decreased protein expression levels in annexin V and lamin A. The mutations in nuclear lamin A can lead to perturbations of plasma lipids associated with hypertension (Fuchs *et al.* 2005). Interestingly, genistein, an abundant compound in soy reversed the stressor-induced decrease of these proteins, indicating the potential prevention of coronary heart disease by soy (Fuchs *et al.* 2005). As a result of proteomic analysis, elements of unknown signaling cascades, members of critical pathways and the potential molecular mechanisms underlying human hypertension might be found to serve as new targets for guidance of developing novel antihypertensive agents.

1.7.2 Protein Interactions with Proteomics

Most proteins exert their function by binding to other proteins and these partner proteins may be directly implicated in a cellular process. To study this question by MS, the protein itself is used as an affinity reagent. Compared with chip based and two-hybrid approaches, MS-based proteomics strategy possesses the advantages that the modified protein and fully processed can act as the affinity reagent to isolate its binding partners, the interactions occur in the cellular location and native environment, and that multi-component complexes can be separated and examined in a single operation (Ashman *et al.* 2001). For example, to explore and understand the critical regulatory role of Nedd8 in cell proliferation and development, Jones *et al.* have affinity purified the proteins modified and associated with Nedd8 from HEK293 cells stably expressing GST-Nedd8 and performed subsequent protein identification employing LC-MS/MS. A total of 496 proteins modified and associated with GST-Nedd8 have been identified, providing clues on the biological role of Nedd8 and thus laying the foundation for an in-depth analysis of the regulation of the Nedd8

pathway (Jones *et al.* 2008). Recently the MS-based approach to identify partners involved in tyrosine kinase receptor (TrkA) signaling in breast cancer cells has been reported. Wild type and modified TrkA chimeric constructs with green fluorescent protein were transfected in human breast adenocarcinoma cells, and co-immunoprecipitated proteins were resolved by SDS-PAGE before identification with nano LC-MS/MS. Several TrkA putative signaling partners were detected, one of which was the DNA repair protein Ku70, arousing increasing interest for its particular role in cell survival and carcinogenesis (Com *et al.* 2007).

Although such MS-based proteomics techniques proved to be successful, some challenging remains, as interacting partners could be expressed endogenously with low abundance in cells, protein interactions may be transient and the large amount of coprecipitated proteins may be present. To remove the coprecipitated proteins by biochemical purification, one always has to compromise with the tradeoff between specificity and sensitivity. Increasing the washing steps aimed to increase specificity in pull-down experiments will inevitably result in a diminishing number of purified proteins and simultaneously potentially leave behind true interacting partners along the way. However quantitative proteomics can provide an appropriate platform to resolve it (Blagoev *et al.* 2003, Ranish *et al.* 2003). Assuming the background proteins bind equally to control and bait, proteins interacting with control and bait are thus differentially isotopically labeled and combined. Consequently specific interactions with the bait will result in differential ratios for the proteins of interest, whereas background proteins lead to an approximate ratio of 1.

This method discussed above can be utilized to any class of affinity purification, including protein-drug and protein-oligonucleotide (Oda *et al.* 2003) interactions. One

example of applying quantitative proteomics to discover novel ILK-interacting proteins is examined by Dobrev et al., in which SILAC was used. Of 752 proteins identified in ILK immunoprecipitates, 24 proteins had SILAC ratios higher than those of previously identified as direct ILK-binding partner. Some of the newly identified proteins specifically enriched in ILK immunoprecipitates, with potentially interesting roles in ILK biology (Dobrev et al. 2008).

1.7.3 Analysis of Protein Modifications with Proteomics

Proteins are modified to their mature form through PTMs, which regulates biological function through a multitude of mechanisms. Specific mass spectrometric methods have been developed that can identify the peptides cleaved from a protein for the presence of a particular modification. Protein modifications are then determined by examining the measured mass and fragmentation spectra comparing with the original ones via manual or computer-assisted interpretation. Although great efforts have been put to define modifications on a whole proteome scale, at present it is obvious that scanning for proteome-wide modifications is not complete for the fact that identifying all modifications is difficult even in a single protein.

Current research focuses on identifying one type of modification made on all the proteins present in a sample, in particular, phosphorylation. A number of techniques have been developed with affinity selection that is specific for the phosphorylation modification of interest and thus purify the sub-proteome bearing this modification. For example, McNulty et al. made full use of the phosphate group possessing strong hydrophilicity to selectively trap and make fractionation of phosphopeptides based on their increased binding force under hydrophilic interaction chromatography conditions. The phosphopeptides from those fractions were subsequently enriched on

immobilized metal ion affinity chromatography, showing better than 99% selectivity. In a 300- μ g equivalent amount of HeLa cell lysate over 1000 unique phosphorylation sites were identified and more than 700 novel sites were included in the HeLa phosphoproteome (McNulty & Annan 2008). Another study was carried out by Kruger and coworkers to generate the tyrosine-phosphoproteome of the insulin signaling pathway on the platform of high resolution mass spectrometry in conjunction with phosphotyrosine immunoprecipitation and stable isotope labeling (SILAC) in differentiated brown adipocytes, providing a useful strategy to explain the insulin signaling pathways in intact cells (Kruger *et al.* 2008).

Proteomics, in particular quantitative proteomics, has been proved to probe most, if not all, of the proteins in a sample, enabling us to discern and distinguish pertinent changes from background proteins, providing a better biological understanding of the follow-up of functional experiments. However specific challenges for quantitative proteomics still remain, in particular, the issues of quantitating putative biomarkers with relatively low levels in human body fluids and characterizing whole proteomes comprehensively need to be addressed. The issues to be addressed will require the advances achieved through development of MS instrumentation and more intelligent algorithms and software.

1.8 Metabolomics

Metabolomics is the systematic analysis of the unique chemical molecules that are left behind along specific cellular processes, mainly in a purpose to study the resulting small molecule metabolite profiles (Daviss 2005). The metabolome is the large scale collection of all the small molecule metabolites including hormones, metabolic

intermediates, other signaling molecules and secondary metabolites as well, which existed within a biological sample, e.g. a single organism (Oliver *et al.* 1998).

Although greatly improved and comprehensive profiling of metabolites in an organism is not feasible, large scale quantitative and qualitative measurements of sizeable numbers of cellular metabolites by employing multiple technologies (Dettmer *et al.* 2007, Hollywood *et al.* 2006, Lenz & Wilson 2007, Seger & Sturm 2007, Villas-Boas *et al.* 2005) still can provide unique insight into the biochemical phenotype of an organism, which can be used to monitor and to assess the response of the biological system and give a complementary picture with mRNA gene expression data and proteomic analyses in a cell.

It is generally accepted that the metabolic profile is a reflection of enzymatic activity to monitor both normal biochemical events and perturbations that lead to disease. For example, a measurement of the change in the level of a particular metabolite can reflect a defective enzyme with impaired or even absent activity. It has been well documented that nearly 200 inherited enzymatic disorders can be characterized by perturbations in their associated metabolic profiles (Chace *et al.* 2002, Chace *et al.* 2003, Chace *et al.* 1993). Additionally, external forces, such as drugs or infection can induce the change in enzymatic activities. The widely used noninvasive source for metabolite studies has been focused on biofluids such as serum, plasma, and urine. Recently, increasing interests has been aroused in metabolite profiling derived from cells and tissues for a better understanding the biochemical events and thus identifying both disease and pharmaceutical biomarkers potentially.

Mass spectrometry has been proven a powerful system for endogenous metabolite identification in drug-metabolite analysis and pharmacokinetic studies (Dear *et al.*

1999, Deng *et al.* 2004, Plumb *et al.* 2003, Shockcor & Holmes 2002, Staack *et al.* 2005, Tiller & Romanynshyn 2002, Zhang *et al.* 2000). Mass spectrometry interfaced with chromatographic separation such as gas chromatography (GC), high-performance liquid chromatography, ultra-high-performance liquid chromatography or capillary electrophoresis offers a central system to most metabolomics methods combining sensitivity and selectivity currently.

The area of metabolite profiling, though expanding rapidly, still faces challenges. The primary challenge in metabolite profiling is in the generation of complete metabolite databases for characterizing metabolites of interest, an issue that is currently being addressed by metabolite researchers. A second challenge is in resolving thousands of components in samples; here the development of multi-separation technology will ultimately drive the process. A third challenge is data analysis, and the development of new algorithms and bioinformatics software packages will make the discovery process.

1.9 Objectives of Research Project

Proteomics directly addresses gene products quantitatively or qualitatively present in a given cell state and thus provides a complementary picture with genomics. It is capable of characterizing protein interactions, investigating protein activities and determining subcellular distributions.

It will be of great interest to detect changes in quantitative protein profiles and to infer biological function from the observed patterns to elucidate the underlying pharmaceutical mechanisms for drug development. Furthermore, the same technology is used to investigate the effects of candidate drugs in a purpose of reversing a disease

process.

Propranolol and atenolol are two β -blockers with two enantiomers, i.e., *S*-enantiomer and *R*-enantiomer, which exerts blocking activity on adrenoceptors. Developments in industrial chemical processes have made it possible and economical for pharmaceutical manufacturers to separate drugs that were originally sold in racemic form and register the individual enantiomers in market, each of which displaying unique properties. The objective of the project is to study the differentially expressed proteins within the proteome of vascular smooth muscle cells (VSMCs) in response to individual enantiomers of propranolol (atenolol) using quantitative proteomics techniques and thus infer the possible mechanism of the action of individual enantiomers of propranolol (atenolol). By overall comparison of the differentially expressed proteins in A7r5 cells incubated with propranolol or atenolol, the similarities and differences in the blocking effects of the two β -blockers are able to be generalized. Additionally, the secreted proteins with differential protein expression levels in culture medium of A7r5 cells treated separately with individual enantiomers of propranolol (atenolol) were also examined and can serve as complemented results to account for the extracellular effects of the drugs.

Experimentally, mass spectrometry interfaced with two-dimensional (2D) (strong cation exchange/reversed phase) chromatographic separations coupled with the widely used iTRAQ reagents were applied in this project. Working on this sophisticated approach, we hope our results can shed new lights and thereby provide a deep understanding of these two β -blockers' biological effect and as well as potential adverse effects.

2. Materials and Methods

2.1 Cell Cultures

A7r5 cells, purchased from the American Type Culture Collection (ATCC), were grown in Dulbecco's modified Eagle's medium (DMEM, supplemented with penicillin (100 U/mL), streptomycin (100 U/mL) and 10% fetal bovine serum). Cultured A7r5 cells were maintained at 37 °C in an atmosphere of 5% CO₂. All the culture media and supplements were purchased from Life Technologies.

2.2 Cell Incubation with Drugs

After reaching 80% confluence, the A7r5 cells were incubated respectively with the *S*-enantiomer, the *R*-enantiomer of propranolol (atenolol) of different concentrations up to 200 µM for 24 h in the absence of serum.

2.3 MTT Assay

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay initially proposed by Mosmann in 1983 (Mosmann 1983), has been used to test cytotoxicity of reagents and cell viability. The principle underlying the assay is that the mitochondrial dehydrogenase enzyme from viable cells are able to cleave the tetrazolium rings of the light yellow MTT and thus develop a dark blue formazan crystals which are accumulated within healthy cells. The cells are lysed by adding a detergent and the liberated crystals are solubilized. As the number of surviving cells is proportional to the level of the generated formazan product, a simple colorimetric assay can be used to measure the corresponding formazan quantity. Briefly the treated and untreated cells were cultured in a 96-well plate. After chemical exposure, the medium was

removed, and cells were incubated for 3 h with 5 mg/ml MTT dissolved in PBS. MTT was cleared out, and the formazan salts were solubilized in 100 μ L of dimethyl sulfoxide for each well. Plates were then read at 570 nm against a 660 nm reference wavelength on a microplate reader (Benchmark Plus). The cell viability was expressed as a percentage of the corresponding control value.

2.4 Cell Lysis

After performing MTT assay, the appropriate molar concentration of propranolol (atenolol) was determined as 20 μ M for incubation of the A7r5 cells. The cells were cultured as described in section 2.2.1. After reaching 80% confluence the cells were incubated with the *S*-enantiomer and *R*-enantiomer of propranolol (atenolol) at a concentration of 20 μ M respectively, for 24 h in the absence of serum. Cultured cells were then trypsinized using 5 mL of 2 X Trypsin/ EDTA. Following centrifugation at a rate of 2,000 rpm for 1 min, the medium was kept for further analysis of secreted proteins associated with drug treatment. The cell pellet was washed with 1 X PBS (pH 7.2) three times and pelleted by centrifugation at 2,000 rpm for 5 min at 4 °C. This was followed by an addition of 200-600 μ L of lysis buffer comprised of 8 M urea, 4% (w/v) CHAPS, and 0.05% SDS (w/v) (GE Healthcare). Briefly, the cells in lysis buffer were vortexed for 1 min vigorously and kept on ice for 20 min with frequent vortexing. After subjected to clarification by centrifugation at 15,300 rpm, 4 °C for 1 h, the supernatant containing total proteins were aliquoted and stored at -80 °C for future use.

2.5 Conditioned Medium

After incubation with individual enantiomers of propranolol (atenolol) for 24 h, the conditioned medium was carefully extracted, and then filtered using a 0.20 μ M syringe filter (Sartorius) to remove suspended cells. The filtrate was concentrated in Amicon ultra-4 centrifugal filter unit with ultracel-3 membrane (Millipore) at 4000g with swinging bucket rotor for 20 min and kept for further analysis.

2.6 Protein Quantification

The protein concentrations in samples were quantified using the 2-D Quant Kit (GE Healthcare). Specifically, an appropriate volume of working colour reagent (mixing colour reagent A with colour reagent B at a ratio of 100:1) was prepared based on the number of assays, each of which required 1 ml. The standard curve was plotted by addition of the 2 mg/ml BSA standard solution to six tubes separately with the corresponding amounts of 0, 5, 10, 15, 20, 25 μ L, respectively. The lysed sample was taken 5, 10, 20 μ L to three separate tubes and duplicates are recommended. For each of 9 tubes a volume of 500 μ l precipitant was added, vortexed briefly and incubated for 2–3 min at ambient temperature, followed by an addition of 500 μ l co-precipitant. Then the tubes were centrifuged at $13,000 \times g$ for 5 min to sediment the protein. After centrifugation, the supernatants were decanted and protein pellet should be visible. The tubes were then performed centrifugation again to remove any remaining supernatant, followed by an addition of 100 μ l of copper solution and 400 μ l of distilled water to each tube. Finally 1 ml of working colour reagent was added to each tube, followed by vortexing. The tubes were then incubated at room temperature for 15 min before the absorbance of each tube was read at a wavelength of 480 nm using

water as the reference.

2.7 Protein Precipitation

Protein samples commonly contain substances that interfere with downstream applications. In this study a total of 100 μg of each sample was precipitated by the addition of four times the sample volume of cold (-20°C) acetone. Following vortexing, the sample tubes were incubated at -20°C for 2 h. Then after centrifugation for 10 min at 13,000-15,000 $\times g$, the supernatant was discarded and the protein pellet was kept at room temperature for 10 min until the remaining acetone evaporated from the uncapped tube.

2.8 Protein Digestion, and Labeling with iTRAQ Reagents

A typical schematic of workflow for subsequent preparation of the samples is displayed in Figure 2.1. Specifically, 20 μL of dissolution buffer was added to each of up to four sample tubes containing 100 μg of precipitated protein pellet of each sample from acetone precipitation to dissolve the proteins. The 1 μL of the denaturant was added to each tube to disrupt the hydrogen, hydrophobic, and electrostatic bonds of the proteins, followed by an addition of 2 μL of reducing reagent to reduce the disulfide bonds of the proteins. After they were kept at 60°C for 1 h, 1 μL of cysteine-blocking reagent was added to block the cysteine group of proteins reversibly, followed by another 10 min of incubation at room temperature. The proteins in each tube were digested with 20 μL of 0.25 $\mu\text{g}/\mu\text{L}$ sequence grade modified trypsin (Promega) solution at 37°C overnight. To each vial of iTRAQ reagent, 70 μL of ethanol was added to dissolve the iTRAQ reagent and the whole contents of one iTRAQ reagent vial was transferred to one sample tube for reaction of peptides with

the reactive groups of the iTRAQ molecules. After reaction for approximately 1 h, the contents of each iTRAQ reagent-labeled sample tube were combined into a fresh tube, kept at -80 °C for subsequent experiments.

For the experiment of A7r5 cells treated with individual enantiomers of propranolol, the labeling with the iTRAQ tags are as follows: normal A7r5 = iTRAQ 114; A7r5 incubated with the *S*-enantiomer of propranolol = iTRAQ 115; A7r5 incubated with the *R*-enantiomer of propranolol = iTRAQ 116. For conditioned medium the labeling with the iTRAQ tags was as follows: control A7r5 = iTRAQ 114; A7r5 incubated with the *S*-enantiomer of propranolol = iTRAQ 115; A7r5 incubated with the *R*-enantiomer of propranolol = iTRAQ 117. The labeled samples were then pooled before analysis.

For the experiment of A7r5 cells treated with individual enantiomers of atenolol, the labeling with the iTRAQ tags was as follows: control A7r5, iTRAQ 114; A7r5 incubated with the *S*-enantiomer of atenolol, iTRAQ 115; and A7r5 incubated with the *R*-enantiomer of atenolol, iTRAQ 117. For conditioned medium the labeling with the iTRAQ tags was as follows: control A7r5 = iTRAQ 114; A7r5 incubated with the *S*-enantiomer of atenolol = iTRAQ 115; A7r5 incubated with the *R*-enantiomer of atenolol = iTRAQ 117. All the chemicals and reagent used for iTRAQ labeling in this section are from Applied Biosystems, except for those stated otherwise.

To verify that sample preparation techniques do not interfere with digestion and labeling procedures, the bovine serum albumin (BSA) standard solution (Pierce) was used in the acetone precipitation, followed by enzymatic digestion with trypsin and labeling with the iTRAQ reagents as described above. These differentially labeled digests were mixed at a ratio of 1:1:0.75:1.5 and analyzed by LC-MS/MS.

Figure 2.1: A typical schematic of workflow for the preparation of the samples labeled with iTRAQ reagents.

2.9 On-Line 2D Nano LC-MS/MS Analysis

The analysis was performed on an Agilent 1200 nanoflow LC system (Agilent Technologies) interfaced with an electrospray ionization QSTAR XL mass spectrometer (Applied Biosystems/MDS Sciex). In the first dimension 3 μ L of the combined peptide mixture (approximately 2 μ g peptides) was loaded onto the PolySulfoethyl A SCX column (0.32 x 50 mm, 5 μ m) and was eluted stepwise by injecting salt plugs of 10 different molar concentrations of 10, 20, 30, 40, 50, 60, 80, 100, 300, 500 mM of potassium chloride (KCl) solution. In the second dimension, while the 10-port valve being in position 1 (Figure 2.2A), the sequentially eluted

peptides from SCX column were trapped onto the ZORBAX 300SB-C18 enrichment column 1 (0.3 x 5 mm, 5 μ m) and washed isocratically with buffer A (5% acetonitrile, 0.1% formic acid) at 0.005 mL/min for 100 min to remove any excess reagent. Meanwhile, the peptides bound on the ZORBAX 300SB-C18 enrichment column 2 during the previous run were eluted with buffer B (0.1% formic acid) and buffer C (a nanoflow gradient of 5-80% acetonitrile + 0.1% formic acid) at a flow rate of 500 nL/min. Further separation was achieved onto the analytical Zorbax 300SB C-18 reversed-phase column (75 μ m x 50 mm, 3.5 μ m). In the next run while the 10-port valve being switched to position 2 (Figure 2.2B), the column 1 was switched into the solvent path of the nanopump and the column 2 was used to trap the newly eluted peptides from SCX. Altogether 11 runs were performed to finish one experiment. For MS/MS analysis, survey scans were acquired ranging from m/z 300-1500 with up to two precursors selected for MS/MS ranging from m/z 100-2000 using dynamic exclusion, and the rolling collision energy was applied to enhance fragmentation. To gain statistical evidence for differential expression of proteins another two separate experiments were performed as described above.

Figure 2.1: Flow diagram for online nano 2D LC. (A) The 10-port valve was in position1: bypassing SCX column, washing enrichment column 1, and analyzing peptides from enrichment column 2. (B) The 10-port valve was in position 2: bypassing SCX column, washing enrichment column 2, and analyzing peptides from enrichment column 1.

2.10 Data Analysis and Interpretation

In Chapter 3 peptide identifications were performed using ProID software packages (Applied Biosystems). Each MS/MS spectrum was searched against the Rat IPI protein database, and protein identifications were accepted based on the ProtScore more than 2.0, which gives the confidence value of 99%. The differentially expressed proteins based on single unique peptides were discarded. iTRAQ reagents enabled to react with N-terminal residues, internal Y and K residues, and allowed the fixed modification of cysteine with methylmethanethiosulfate as well as one missed cleavage. The search for the iTRAQ experiments was performed with the software, ProQUANT 1.0, wherein tolerance settings for peptide identification searches were 0.15 Da for MS and 0.1 Da for tandem MS. Pro-QUANT added all the runs with different salt plugs of increasing concentrations of KCL in one experiment. The relative abundance of a peptide in treated sample was calculated by dividing the peak areas appeared at 115.1 and 116.1 m/z by that for 114.1 m/z assigned to control sample. The calculated peak area ratios were further adjusted for overlapping isotopic contributions. It is reasonably postulated that the total mass of proteins in individual samples should be the same. To account for small differences in protein quantitation and loading for a particular sample, the calculated ratios have been normalized by dividing a coefficient, as recommended by Applied Biosystems. In the study the differentially expressed proteins with fold changes greater than 1.2 were chosen for further analysis.

In Chapter 4, 5 & 6, relative abundance quantitation, peptide and protein identification were performed using ProteinPilotTM Software 2.0 (Applied Biosystems, Software Revision 50861). Protein identifications were accepted based on the ProtScore more

than 2.0, which gives the confidence value of 99%. Each MS/MS spectrum was searched for species of *Rattus norvegicus* against the UniProt_sprot_20070123 database. The searches were run using the following parameters: fixed modification of methylmethanethiosulfate-labeled cysteine, fixed iTRAQ modification of free amine in the amino terminus and lysine, variable iTRAQ modifications of tyrosine, and allowing serine and threonine residues undergoing side reaction with the iTRAQ reagent. Other parameters including cleaving specificity by trypsin, tolerance setting for precursor ion and MS/MS accuracy for fragment ion are built-in functions of ProteinPilot software. Relative quantification of peptides labeled with iTRAQ was performed based on the MS/MS scans. The ratio of the areas under the 4 distinct reporter ions, i.e., 114, 115, 116 and 117 Da, will give the relative concentrations of peptides. Sequence coverage was defined as the percentage of the number of amino acids observed in the complete protein amino acid length. Error factor (EF) is a statistic that has been applied in error-reporting in ratios and expressing the 95% confidence interval for an average ratio ($EF = 10^{95\% \text{ confidence interval}}$, where $95\% \text{ confidence interval} = (\text{ratio} \times EF) - (\text{ratio}/EF)$). The following criteria were applied for considering a protein for further statistical analysis: at least two high confidence (>95%) distinct peptides had to be identified, the *p* value in the Protein Quant had to be $p < 0.05$, and the fold difference was set to be greater than 1.2. The candidate proteins were carefully examined in the Protein ID of the ProteinPilot software. The peptides without any modification of free amine in the amino terminus or without iTRAQ modification of free amine in the lysine were excluded from calculation of the protein ratios. Similar as the ProQUANT 1.0, the protein ratios were automatically normalized using the overall ratios for all proteins in the sample as recommended by Applied Biosystems.

The differential expressed proteins were identified based on the criteria that protein score is greater than 2 with associated sequence coverage and the number of distinct peptides identified.

2.11 NAD⁺/NADH Assay

Each of the three types of A7r5 cells (control cells, the cells incubated with *S*-enantiomer of propranolol (atenolol) at a concentration of 20 μ M and the cells incubated with *R*-enantiomer of propranolol (atenolol) at a concentration of 20 μ M) were cultured for 24 h in the absence of serum, for the nicotinamide adenine dinucleotide (NAD⁺)/ nicotinamide adenine dinucleotide plus hydrogen (NADH) quantification assay (BioVision).

Three independent experiments were performed for each type of cells. A total of 4×10^5 cells were used in each assay, and intracellular NAD⁺/NADH was extracted with 400 μ L of NAD⁺/NADH extraction buffer by subjecting the cells to two cycles of freeze/thaw (20 min on dry ice, followed by 10 min at room temperature). To detect total NAD⁺ and NADH (NADt), 50 μ L of each extracted sample was loaded into a 96-well plate. Then the plate was incubated at ambient temperature for 5 min in the presence of 100 μ L of NAD Cycling Mix, to allow the conversion of NAD⁺ to NADH. To detect NADH, 200 μ L of extracted solution was taken from each sample and heated at 60 °C for 30 min in a heating block. Under these conditions, all NAD⁺ was decomposed, while NADH remained intact. Then, 50 μ L of each NADH sample was taken into a 96-well plate. Subsequently, 10 μ L of NADH developing solution was added into each well. After 40 min in the dark, the plates were read at 450 nm wavelength on a microplate reader (Benchmark Plus). The ratio of NAD⁺/NADH was calculated as follows: (NADt - NADH)/NADH.

2.12 ROS Detection

Image-iT LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Invitrogen) was applied to test ROS in living cells treated with different enantiomers of propranolol. The assay is based on 5-(and-6)-carboxy-20, 70-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), a reliable fluorogenic marker for ROS in live cells. In the presence of nonspecific ROS, the reduced fluorescein compound is oxidized and emits bright green.

As described by the optimized protocol provided in the kit, oxidatively stressed and non-stressed cells could be reliably distinguished by fluorescence microscopy. Briefly, the treated and untreated A7r5 cells were gently washed with warm Hank's Buffered Salt Solution (HBSS) buffer, followed by an addition of a sufficient amount of 25 μ M carboxy-H2DCFDA working solution to cover the cells adhering to the plate for a 30 min' incubation at 37 °C, protected from light. Then the cells were washed three times by warm HBSS and added more HBSS buffer immediately before taken image under fluorescence microscopy. The use of tert-Butyl hydroperoxide (TBHP) (provided in the kit) was employed as a positive control for the induction of ROS, by applying 100 μ M TBHP working solution to the cells and incubating for 90 min at 37 °C in an atmosphere of 5% CO₂ before labeling as described above.

2.13 Real-time Reverse Transcription-Polymerase Chain Reaction

2.13.1 RNA Extraction

Control A7r5 cells and treated A7r5 cells were cultured separately for RNA isolation by using RNeasy mini kit (Qiagen). Total RNA was extracted following the steps in

the procedure as described in manufacturer's manual for three independent experiments. Briefly, cells were spun down after being trypsinized and then dissolved in 350 μ L or 600 μ L (if the number of the cells $> 5 \times 10^6$) of Buffer RLT (prepared with 1 mL of Buffer RLT supplemented with 10 μ L of β -mercaptoethanol, as described by Qiagen). After being homogenized for 30 sec, the resuspended solution was added an equal volume of 70 % ethanol (analytical grade). The mixture was then loaded to the spin columns (reloading if volume was greater than 700 μ L) before it was centrifuged at 13,200 rpm for 30 sec. The RNA bound to the columns was firstly washed with 700 μ L of Buffer RW1 (described by Qiagen) and centrifugation at a rate of 13,200 rpm for 30 sec. Another two additional washing with 500 μ L of Buffer RPE were performed, which was followed by centrifugation at a rate of 13,200 rpm for 30 sec and 2 min respectively. The residual Buffer RPE was removed by centrifugation at a rate of 13,200 rpm for 1 min. Finally, the total RNA eluted by an addition of 30 μ L of RNase-free water and kept at a temperature of -70°C for future use with minimal degradation.

2.13.2 RNA Quantitation

Quantitation of total RNA was performed by measuring the absorbance with the UV spectrophotometer at $\text{OD}^{260\text{nm}}$ and $\text{OD}^{280\text{nm}}$, respectively. Yield was determined by the product of $\text{OD}^{260\text{nm}}$ and 40 $\mu\text{g} / \text{mL}$ and the corresponding dilution factor. The value obtained by division of $\text{OD}^{260\text{nm}}$ by $\text{OD}^{280\text{nm}}$ ranges in between 1.9 to 2.1 will indicate a good RNA purity.

2.13.3 One-Step Reverse Transcription – Polymerase Chain Reaction

One-Step RT-PCR kit (Qiagen) was used for the genes of interest and reverse transcription. Specifically, the total RNA was added with a mass ranges between 1 pg

to 2 μL to a master mix comprised of 10 μL 5 X OneStep RT56 PCR buffer (Qiagen), 1.5 μL of 20 mM (0.6 μM) forward and reverse specifically designed oligonucleotides, 2 μL of dNTP mix (consisting of 10mM of each dNTP) and 2 μL of OneStep RTPCR Enzyme mix (Qiagen) and arrived at a final volume of 50 μL by addition of RNase-free water. The prepared RT-PCR mixture was then on ice and placed onto the thermal cycler when the temperature reached 50 °C. The initial reverse transcription occurred at 50 °C for 30 min, followed by 95 °C for another 15 min to activate the HotStar*Taq* DNA polymerase and then inactivate the reverse transcriptases. The 3-step cycling consisting of 1 min denaturation at 95°C, 1 min primer annealing at 50°C and 1 min primer extension at 72°C. It was set up for 30 cycles followed by an additional extension at 72 °C for 10 min. The generated PCR products were then ran on a 1 % agarose gel and observed using ethidium bromide.

2.13.4 Real-time RT-PCR

Real-time RT-PCR was specially designed to monitor the amplification of PCR product during cycling other than measurement of the end product such as traditional RT-PCR. The incorporation of SYBR Green allows for detection of the double stranded DNA by intercalation. Upon excitation, the emitted fluorescence can be detected and quantification is made by calculation of the fluorescence density in the process of amplification cycles. The iScript One-step RT-PCR kit with SYBR Green (Bio-Rad) was applied for the precise real-time quantification of RNA targets. Specifically, the reactions were carried out by an addition of 25 μL of 2 X SYBR green RTPCR reaction mix (consisting of 0.4 mM of each dNTP, *iTaq* DNA and magnesium chloride), 1.5 μL each of forward and reverse primer (10 μM), 1 μL of iScript Reverse Transcriptase (50 X iScript MMLV reverse transcriptase) followed by an addition of 10 ng RNA. A total volume of 50 μL was reached by addition of

appropriate nuclease-free water. The mixture was then transferred to 0.2 mL white strip tubes (Bio-Rad), covered with optical flat strip caps (Bio-Rad) and kept in ice. The real-time PCR was performed on a IQ5 multicolor Real-time PCR detection system (Bio-Rad), as described by Bio-Rad as follows: cDNA synthesis occurred at a temperature of 50 °C for 10 min and iScript Reverse Transcriptase inactivation occurred at a temperature of 95 °C for 5 min. PCR cycling for 40 cycles proceeded with at 95 °C for 10 sec and at 60 °C 30 sec. The dissociation analysis was also acquired by fluorescent reading from 55 °C to 95 °C with one degree increase. Experimental report, melt curve analysis and threshold cycle number were extracted from the DNA IQ5 optical system software (Bio-Rad).

The fold changes were calculated as shown in the following formula:

$$\text{Sample } \Delta\text{Ct} = \text{Ct}_{\text{sample}} - \text{Ct}_{\beta\text{-actin}};$$

$$\Delta \Delta\text{Ct} = \text{Sample } \Delta\text{Ct} - \text{control } \Delta\text{Ct};$$

$$\text{Fold of sample versus control} = 2^{\Delta \Delta\text{Ct}}$$

Primers that were specific to the following cDNAs that displayed changes in the respective protein level (Table 3.1), L-lactate dehydrogenase, peroxiredoxin-2, thioredoxin, deoxyribonuclease-1 precursor, glutamate dehydrogenase 1, and medium chain acyl-CoA dehydrogenase are shown in Table 2.1. Primers that were specific to the following cDNAs that displayed changes in the respective protein level (Table 4.1), calmodulin, protein S100-A4, protein S100-A11, and annexin A6 are shown in Table 2.2. These primers were designed to yield amplification products within 300 bp in size to reduce nonspecific binding of SYBR Green. Quantification was performed by calculating the fluorescence density during the amplification cycle.

Protein name	Primers
L-lactate dehydrogenase	5'- GGTCCAGCGAAACGTGAACAT-3' 5'- ATCAGGTAACGGAACCGAGCC-3'
Peroxiredoxin-2	5'- CCGTGGTGGATGGTGCCTTTA-3' 5'- CACCTCGCAGCCTAGCTTTTCG-3'
Thioredoxin	5'- CCTTCTTTTCATTCCCTCTGTG-3' 5'- AACTCCGTAATAGTGGCTTCG-3'
Deoxyribonuclease-1 precursor	5'- CGATGCCCTCTACGATGTTTA-3' 5'- AAGACCATTGGGAGGAAGTGA-3'
Glutamate dehydrogenase 1	5'- CTGGCGTCCCTAATGACCTAC-3' 5'- CCAAATCCCTGAACAACAAAC-3'
Medium chain acyl-CoA dehydrogenase	5'- CTACTGCGTGACAGAACCCTC-3' 5'- CAATCTTGAAACCTGCTCCTT-3'

Table 2.1: Sequence of DNA oligonucleotides for real time PCR.

Protein name	Primers
Calmodulin	5'- CCGAAGAACAGATTGCAGAGT-3' 5'-ATTGCCATCAGCATCCACCTC-3'
Protein S100-A11	5'-CAGTGGGAAGGATGGAAATAG-3' 5'-AGCCACCAATAAGGTTGAGAA-3'
Protein S100-A4	5'-GCCCTGGATGTAATAGTGTCC-3' 5'-TTCATTGTCCCTGTTGCTGTC-3'
Annexin A6	5'-CACGACTTCGCAGACTTTGAT-3' 5'-GATGAGGTCCTTGCCATAGAG-3'

Table 2.2: Sequence of DNA oligonucleotides for real time PCR.

2.14 Intracellular Ca²⁺ Concentration Measurements

A Fluo-4 NW Calcium Assay kit (Invitrogen) was used to measure intracellular Ca²⁺ concentration on a fluorometer (Tecan) following the manufacturer's protocols. Briefly, each of the three types of A7r5 cells was cultured separately in a 96-well plate. The growth medium was replaced with 100 μL/well Fluo-4 dye solution containing

probenecid to prevent extrusion of the dye out of cells. The plate was maintained at 37 °C for 30 min and then placed at room temperature for an additional 30 min. The assay was done at 494 nm for excitation and at 516 nm for emission.

2.15 Immunostaining and Fluorescence Microscopy

Each of the three types of A7r5 cells were seeded separately on glass coverslips for 24 h, washed with pre-warmed PBS, and fixed with 1 ml of 3% paraformaldehyde/PBS for 20 min. Fixation buffer was then removed and followed by another washing with PBS for 10 min. Cells were then permeabilized with 0.2% Triton X-100/PBS for 10 min and blocked with 10% FBS/0.1% Triton-X100/PBS for 10 min, followed by a wash with PBS. To probe the structure of cytoskeleton, the fixed and permeabilized A7r5 cells were stained with fluorescent phalloidin conjugate solution (FITC-phalloidin) in PBS at room temperature for 1 h. The coverslip was mounted with mounting solution, and the fluorescence imaging of cells stained with actin was performed with a Pascal 5 confocal microscope (Pascal 5, Carl Zeiss). The prepared sample was excited by an argon-ion laser with a wavelength of 488 nm, and the emitted light was detected with a band pass filter of 520 nm.

2.16 Western Blot Analysis

2.16.1 Preparation of water-saturated butanol

10 mL of distilled water mixed with 40 mL of 2-Butanol (Fisher) well and kept for 1 day for the complete separation of two phases. The upper liquid phase comprised of the water-saturated butanol was collected for removing any surface bubbles and leveling of separating gels.

2.16.2 Preparation of SDS-PAGE

SDS-PAGE with concentrations ranging between 8%-12% was performed on the Bio-Rad mini-protean electrophoresis system. Each 1 mm thick gel consists of stacking gel and separation gel. For 12% SDS-PAGE, the separating gel, consisting of 2.5 mL of 4 X separating gel buffer (1.5 M Tris-HCl, pH 8.8), 4 mL of monomer solution (29 % acrylamide vs. 1 % bisacrylamide) (Bio-Rad), 0.1 mL of 10% ammonium persulfate (APS), 0.1 mL of 10% SDS, 3.3 mL of distilled water and 5 μ L of N, N, N', N'- tetramethyl-ethylenediamine (TEMED), was loaded in between the two glass plates. To make a flat solution surface, 0.2 mL of water-saturated butanol was added. After 20 mins' polymerization at room temperature, the water-saturated butanol was washed away by using distilled water. The residual water was drained properly prior to casting of the stacking gel. The stacking gel solution was prepared with 0.67 mL of monomer solution (29 % acrylamide vs. 1 % bisacrylamide), 0.83 mL of 4 X stacking gel buffer (0.5 M Tris-HCl with pH 6.8), 50 μ L of 10 % APS, 50 μ L of 10 % SDS, 3.4 mL of distilled water and 5 μ L of TEMED and loaded onto the top of the solidified separation gel. Comb of corresponding thickness were immersed into the liquid stacked gel solution. After allowing the polymerization reaction for about 15 min at room temperature, the comb was carefully removed and the formed wells were washed thoroughly with distilled water to remove residual unreacted monomer solution.

The casted gel was placed into the inner buffer chamber with short plates facing inwards. The inner buffer chamber was carefully filled with fresh 1 X SDS running buffer (0.192 M glycine, pH 8.3, 0.025 M Tris, 0.1 % SDS). After protein quantitation, they were mixed with 6 X SDS sample buffer and boiled for 5 min. Molecular weight rainbow markers RPN 755 and RPN 756 (GE Healthcare) were used for low

molecular weight detection (< 45 kDa) and high molecular detection (up to 200 kDa) respectively. The individual samples were then loaded slowly into each well. For the stacking run, the voltage was set at a voltage of 80 V and was changed to 150 V for the subsequent separation phase when the samples were observed to enter the separating gel. The power was turned off once the dye was observed to reach the bottom of the casted gel.

2.16.3 Western Transfer

The cast SDS-PAGE gel was transferred onto 0.45 μm nitrocellulose membrane (GE Hybond) via Bio-Rad's semi-dry transfer system. The typical schematic of the setup for the semi-dry system is shown in Figure 2.3. Nitrocellulose membranes were pre-soaked with transfer buffer (25 mM Tris, 192 mM glycine, 10 % (v/v) methanol). In this study the settings for the transfer is fixed at a constant voltage of 21 V while allowing to adjust the timings between 20 min and 50 min based on the molecular weight of the protein of interest. The transferred nitrocellulose membrane was thus exposed to Ponceau S staining consisting of 5 % (v/v) glacial acetic acid, 0.1 % (w/v) Ponceau S and 95 % distilled water to estimate transfer efficiency roughly after completion of the transfer.

2.16.4 Western Analysis

After transferring, the membranes were then blocked in 5 % non-fat milk dissolved in 1 X PBS for 60 min at room temperature or at 4 °C overnight with regular rotation. Following blocking, the specific primary antibody, dissolved in 3 % non-fat milk / 1 X PBS solution, was reacted for 1 h at room temperature. Non-specific reactions were then removed by washing three times, each of which for at least 10 min, with cold 0.005 % Tween-20 / 1X PBS solution. Secondary antibody tagged with Horseradish

Peroxidase (HRP) was dissolved in 3 % nonfat milk / 1 X PBS solution, and reacted for 1 h. This was followed by another 3 washing each of for 10 min by cold 0.005 % Tween-20 / 1X PBS solution. The processed membrane is then ready for enhanced chemiluminescence (ECL) using SuperSignal West Pico chemiluminescent substrate (Pierce). Briefly, an equal amount of stable peroxide solution and luminol /enhancer solution were mixed, evenly applied to the membrane and allowed to perform reaction for 1 min at room temperature. The excess substrates were decanted and the membrane was wrapped in Cling wrap for detection of ECL using CLxposure X-ray film (Pierce) or ECL hyperfilm (GE healthcare). The signals on the membrane were measured on the X-ray film with varying durations, considering the concentration of the protein of interest or the trend resulting from different exposure time. Both developing and fixing reagents (Kodak) were diluted 5 times by distilled water prior to use. Film was developed manually and the time of duration was adjusted to obtain well exposed results. After exposure, the films were positioned in the diluted developer solution and continuously agitated until the appearance of target band was observed. The films were then removed and washed with distilled water to remove residual developer. The films were then fixed with agitation in the fixing solution for about 2 min.

The antibodies used in this study were as follows: 1) goat anti-annexin VI (Santa Cruz Biotechnology, Inc. SC-30763), 2) mouse anti-calmodulin (GeneTex GTX22860), 3) mouse anti- β -actin (Sigma A5441) and 4) goat-anti kininogen (Santa Cruz SC-25889).

Figure 2.3: A typical schematic diagram of the setup of Bio-RAD semi-dry system. (Reproduced from: Tan T. L., Ph.D. dissertation, Role of Hepatitis B virus X protein (HBx) in cell adhesion and cytoskeletal reorganization-implications on virus replication, (2007)).

2.17 Data Analysis and Statistics

In this study Student's *t* tests were performed if the quantitative information met the criteria for further statistical analysis as described above and was in a minimum of two independent experiments. In addition, the statistical difference for the real time PCR analysis, the quantification of the protein level by Western blot analysis, and the measurement of intracellular Ca^{2+} concentration was also evaluated by Student's *t* test with $p < 0.05$ denoting a statistical difference.

3. Protein Profile in A7r5 Cells Incubated with S- and R-enantiomers of Propranolol

3.1 Introduction

Propranolol was the first successful beta blocker developed and currently available in generic form as propranolol hydrochloride. Upon oral administration it is rapidly and completely absorbed, however, the bioavailability is variable due to extensive first-pass metabolism with the main metabolite, 4-hydroxypropranolol, being pharmacologically active. Propranolol is a nonselective β -receptor blocking agent, competing with β -adrenergic receptors stimulating agents to reside on available β -receptor sites. Blocking of the access to β -adrenoceptor sites by propranolol will decrease the inotropic, chronotropic and vasodilator responses to β -adrenergic stimulation proportionately.

In the past 40 years, propranolol, a nonselective β -blocker, is widely used in the treatment of hypertension (Vlachakis & Aledort 1980, Witte *et al.* 2004), angina (Detry *et al.* 1994, Subramanian *et al.* 1982), and to prevent patients who have suffered from myocardial infarction from re-infarction (Norris *et al.* 1984, Shivkumar *et al.* 1998). However until now the potential mechanism underlying the antihypertensive effects of propranolol has not been well established. A few factors may be used to explain it, including the inhibition of renin release by the kidneys, the reduced cardiac output, and decreased tonic sympathetic nerve activity out of vasomotor outflow centers in the brain.

In addition, propranolol is often taken by musicians and other performers to avoid stage fright and is currently being explored to serve as a potential treatment for post-traumatic stress disorder (Brunet *et al.* 2008). It was reported that propranolol

was used clinically as a prophylactic drug to prevent oesophageal variceal bleeding in cirrhotic patients with portal hypertension(Pagliari *et al.* 1989). On the other hand, propranolol has an additive effect when used with other drugs which decrease blood pressure, particularly thiazide diuretic(Goto *et al.* 1987) or peripheral vasodilators(Barrios *et al.* 1979), showing compatible and more effective than propranolol alone. Like other β -blockers propranolol is able to reduce perfusion of the placenta and possible to induce negative outcomes for the neonate such as pulmonary, cardiac complications or premature birth. For the infants the additional adverse effects including hypoglycemia and bradycardia may arise (Lin *et al.* 2004).

There is an increasingly scientific data showing that the interaction of propranolol with β -adrenoceptors is highly stereoselective (Stoschitzky *et al.* 1995, Vermeulen *et al.* 1990). Generally the *S*-enantiomer of propranolol is more potent as antagonist of β -adrenergic receptors and accounts for most of the β -blocker effect. However, both have local anesthetic effect. Despite the well documented beneficial effects from the propranolol treatment, concerns regarding its potential adverse metabolic effects and particularly the underlying molecular mechanism remain to be addressed.

In this study, we used a 4-plex multiplex strategy to simultaneously detect and quantify differences in expression levels of proteins in untreated vascular smooth muscle cells and those incubated with *S*- and *R*-enantiomer, respectively, which reflect pharmacologic action of enantiomers. To identify proteins from a complex mixture, the 2D LC setup was applied. Briefly, a strong cation exchange (SCX) column was set up for the first dimension followed by a reversed-phase (RP) column used for the second dimension. To trap the subsequently eluted peptides from SCX, two identical enrichment columns were used between the SCX and RP. The sample peptides

attached on the SCX columns were then eluted by subsequently injected salt solution plugs of increasing concentrations and further bound on a short enrichment column before they were analyzed on a nano RP column interfaced with ESI-MS/MS. Our results indicated that protein profile in cells incubated with the *S*-enantiomer of propranolol was different from that for *R*-enantiomer of propranolol incubated cells or control cells.

3.2 Results

3.2.1 MTT Assay and Concentration of Propranolol

Drugs have been shown to impact on cell growth *in vitro*, as the cell death has been observed depending on the concentration of drugs and the time of incubation (Witczak *et al.* 2003). Disturbances in metabolism and cell signaling as a result of the cell death may however be different in nature to normal cellular responses to external stimulation. In the case of drugs treatment, such cell death related signaling would not be relevant for drugs that were designed and synthesized for non-anticancer applications. As the purpose of this study was to establish the cellular protein profile in response to the non-anticancer drug, it was critical to minimize the cell death associated with the incubation of drugs. To determine the appropriate concentration of propranolol used in our study, A7r5 cells were incubated with an increasing concentration of the respective enantiomer of propranolol up to 200 μ M and the viability was examined by the MTT assay.

Results shown in Figure 3.1 indicated that either of the two enantiomers of propranolol was toxic to the cells, with a drastic decrease in cell viability for any concentration higher than 100 μ M. In addition, the overall comparison of the MTT values obtained showed that the *R*-enantiomer of propranolol was somewhat less toxic

than the *S*-enantiomer of propranolol. Our results also indicated that no significant effect on the cell viability was observed for those concentrations below 20 μ M. The concentration of 20 μ M was therefore chosen in all experiments in this study.

3.2.2 iTRAQ Analysis of BSA

To ensure the feasibility of our adopted approach, the standard protein BSA (2 μ g/ μ L) from Pierce was used. Briefly, four sets of BSA solution each containing 100 μ g BSA were precipitated by adding four times the sample volume of cold (-20°C) acetone, respectively. The precipitated BSA was then dissolved in the solution buffer, denatured, and cysteines were blocked by blocking agent as described by the iTRAQ protocol (Applied Biosystems). Four identical BSA tryptic peptides were each labeled with one of the four isobaric iTRAQ reagents and the labeled peptides were then mixed in a ratio of 1:1:0.75:1.5. Following CID, the four reporter group ions separated as distinct masses between 114–117 Da. The measured ratios were compared with the supposed ratios, i.e., 115/114 ratio of 1.00, 116/114 ratio of 0.75, and 117/114 ratio of 1.5. A representative spectrum showing a peptide, NECFLSHK (J), from BSA was displayed in Figure 3.2, in which the measured 115/114 ratio of 0.96, 116/114 ratio of 0.78, and 117/114 ratio of 1.47 were obtained by the ProQUANT software, where J is assigned to iTRAQ-lysine and U is assigned to iTRAQ-tyrosine.

It was found that no peptides were left unlabeled by carefully examining the resulting parent ion masses for the MS analysis and comparing with theoretical tryptic digests of the BSA. Each member of the four iTRAQ reagent tags was labeled with equal efficiency, and this indicated that our approach was feasible.

Figure 3.1: Cell viability of vascular smooth muscle cells after 24 h of exposure to *R*- and *S*-enantiomer of propranolol, respectively. Three independent experiments were carried out for each enantiomer of propranolol.

Figure 3.2: Tandem mass spectra of BSA tryptic peptides prepared by labeling with each of the four isobaric iTRAQ tags and pooling the reaction mixtures in a 1:1:0.75:1.5 ratio. The enlarged low-mass region shows the report ions m/z 114, 115, 116, and 117 in an approximate 1:1:0.75:1.5 ratio. 'I' is the iTRAQ-modified lysine residue.

3.2.3 iTRAQ Analysis of Differentially Expressed Proteins

To establish the protein profile in the vascular smooth muscle cells incubated with individual enantiomers of propranolol, incubated cells were harvested and lysed prior to their analysis. In contrast to the control BSA protein, iTRAQ tags were applied as follows: normal A7r5 = iTRAQ 114; A7r5 incubated with the *S*-enantiomer of propranolol = iTRAQ 115; A7r5 incubated with the *R*-enantiomer of propranolol = iTRAQ 116.

Three separate experiments were carried out for each enantiomer of propranolol. ProQUANT 1.0 was used to identify and quantify the protein expression levels among different samples. A total of 361 unique proteins were identified (supplementary Table 1) in the three independent experiments. In statistical analysis of the values of ratios of these proteins, the most common assumption made is that the probability of a specific value x deviating from the mean value μ is in inverse proportionality to the square of its standard deviation from the mean, which gives rise to the fundamental meaning of normal distribution of all values. The normal probability density function is defined as follows:

$$f(x) = \frac{1}{\sigma\sqrt{2\pi}} e^{-(x-\mu)^2 / 2\sigma^2} \quad (2.1)$$

where σ^2 is the population variance, i.e., the mean of all calculated values of $(x - \mu)^2$. The factor is $1/\sigma\sqrt{2\pi}$ and chosen for $\int_{-\infty}^{+\infty} f(x)dx = 1$. Integrating equation 2.1 gives rise to the cumulative distribution defined as in equation 2.2.

$$F = \int_{-\infty}^x f(t)dt = \frac{1}{2} \left(1 + \operatorname{erf} \frac{x-\mu}{\sqrt{2}\sigma} \right) \quad (2.2)$$

where $\text{erf}(z) \equiv \frac{2}{\sqrt{\pi}} \int_0^z e^{-t^2} dt$ is called the error function. To test to see if a population of the values of the ratios of different protein expression level in *R*- or *S*-enantiomer incubated cells relative to the control cells is normally distributed, one of the approaches is to graph the cumulative distribution at a scale that would produce a straight line if the population distribution were normal. It is accomplished by making the vertical scale as $\text{erfinv}(2*F-1)$, in which erfinv is the inverse error function, defined as $x = \text{erfinv}(y)$ which satisfies $y = \text{erf}(x)$.

The MATLAB program (adopted codes from W.R. Wilcox, Clarkson University in appendix 1 & 2) was used to compare the resulting plot with the cumulative distribution for the normal probability density function. Our results displayed in Figure 3.3 & 3.4 indicated that the resulting values of ratios were very close to the straight line for a normal distribution, with the exception of very high and the very low values.

Identification of protein with significant expression level was based on the ProtScore with the cutoff at 2.0, which gave the confidence value of 99%. A total of 20 unique proteins with fold change greater than 1.2 were tabulated in Table 3.1 for further analysis. These 20 proteins which were differentially expressed between the propranolol-incubated and normal sample were subsequently categorized into separate tables according to their cellular functions. These included 6 metabolic enzymes, 3 signaling molecules, 7 cytoskeletal proteins and 4 proteins involved in DNA synthesis/protein translation.

By Overall comparison of the 20 differentially expressed proteins, it was observed that most of the tabulated proteins showed relatively more dramatic changes in levels in

response to the incubation with *S*-enantiomer of propranolol than those in response to *R*-enantiomer of propranolol. Close examining Table 3.1 showed that for A7r5 cells incubated with *S*-enantiomer of propranolol most of the metabolic enzymes were up-regulated with the protein, L-lactate dehydrogenase being the exceptionin. Unlike the metabolic enzymes, the listed cytoskeletal proteins, signaling proteins and those cell growth proteins in Table 3.1 did not present a specific trend, but with protein levels being randomly up-regulated or down-regulated.

Accession	Name	No. peptides	Sequence coverage	Avg. S:C (±SD)	Avg. R:C (±SD)	p (S:C)	p (R:C)
IPI00197711.1	L-lactate dehydrogenase	6	45.7%	1.03 ± 0.07	0.81 ± 0.06	>0.05	0.0207
IPI00201561.2	Peroxisomal protein 2	5	58.1%	1.16 ± 0.18	0.86 ± 0.07	>0.05	0.0278
IPI00231368.4	Thioredoxin	4	70.5%	1.25 ± 0.12	0.92 ± 0.11	0.0421	>0.05
IPI00230876.4	Deoxyribonuclease-1 precursor	2	31.4%	1.63 ± 0.30	1.16 ± 0.18	0.0307	>0.05
IPI00324633.2	Glutamate dehydrogenase 1, mitochondrial precursor	3	35.5%	1.29 ± 0.11	1.02 ± 0.13	0.0305	>0.05
IPI00212015.1	Medium chain acyl-CoA dehydrogenase, mitochondrial precursor	2	41.8%	1.37 ± 0.16	1.18 ± 0.14	0.0328	>0.05
IPI00421888.2	Annexin A6	2	37.3%	0.75 ± 0.11	0.81 ± 0.08	0.0304	0.0272
IPI00230907.4	Macrophage migration inhibitory factor	3	29.0%	0.79 ± 0.11	1.06 ± 0.14	0.0466	>0.05
IPI00324893.4	14-3-3 protein zeta/delta	2	23.1%	1.04 ± 0.06	0.83 ± 0.05	>0.05	0.0148

Continued

Table 3.1: List of differential expressed proteins in A7r5 cells incubated with propranolol^a

^a S:C is the ratio of different protein expression level in the S-enantiomer incubated cells relative to the control cells; R:C is the ratio of different protein expression level in the R-enantiomer incubated cells relative to the control cells. The p values are listed if predefined criteria for statistical testing were met.

Table 3.1 continued

Accession	Name	No. peptides	Sequence coverage	Avg. S:C (±SD)	Avg. R:C (±SD)	<i>p</i> (S:C)	<i>p</i> (R:C)
Cytoskeletal protein							
IPI00421517.6	Desmin	5	41.2%	0.79 ± 0.11	0.83 ± 0.13	0.0412	>0.05
IPI00327144.6	Cofilin-1	4	25.5%	0.84 ± 0.09	0.87 ± 0.10	0.0452	>0.05
IPI00230775.4	Isoform 1 of Tropomyosin beta chain	2	30.6%	0.85 ± 0.05	0.79 ± 0.04	0.0189	0.0152
IPI00197129.1	Actin, aortic smooth muscle	3	34.3%	0.86 ± 0.07	0.91 ± 0.08	0.0371	>0.05
IPI00763796.1	(similar to) Plastin-3	4	28.2%	1.06 ± 0.06	0.63 ± 0.15	>0.05	0.0325
IPI00362160.1	Tubulin beta-3 chain	5	42.3%	0.86 ± 0.11	0.83 ± 0.08	>0.05	0.0372
IPI00327630.1	Dynein heavy chain, cytosolic	2	25.1%	1.09 ± 0.11	0.80 ± 0.10	>0.05	0.0451
Cell growth proteins							
IPI00559188.1	11 kDa protein	4	73.5%	0.69 ± 0.04	1.07 ± 0.07	0.0048	>0.05
IPI00475474.2	40S ribosomal protein S4, X isoform	3	53.4%	0.88 ± 0.10	0.84 ± 0.08	>0.05	0.0374
IPI00191142.1	40S ribosomal protein S10	3	47.8%	1.20 ± 0.11	0.88 ± 0.08	0.0480	>0.05
IPI00767277.1	(similar to) NonO/p54nrh homolog	2	40.2%	1.45 ± 0.24	1.06 ± 0.13	0.0496	>0.05

Figure 3.3: Normally distributed "data" from the calculated values of S:C ratios with a mean of 0.99 and a variance of 0.12. S:C is the ratio of different protein expression level in the *S*-enantiomer of propranolol-incubated A7r5 cells relative to the control cells.

Figure 3.4: Normally distributed "data" from the calculated values of R:C ratio with a mean of 0.99 and a variance of 0.11.
R:C is the ratio of different protein expression level in the *R*-enantiomer of propranolol-incubated A7r5 cells relative to the control cells.

3.2.4 Analysis of Cytoskeletal Proteins

It was observed that the cytoskeletal proteins listed in Table 3.1 did not exhibit a specific behavior, i.e., some of them showed more dramatic changes in protein level in response to *R*-enantiomer of propranolol than those in response to the *S*-enantiomer of propranolol, which remains to be addressed as the generally accepted point is that *R*-enantiomer of propranolol is less active as compared with *S*-enantiomer of propranolol.

Plastins are a family of actin-bundling proteins. Recently it was reported that asymptomatic females carrying the same survival motor neuron 1 gene mutation as their affected siblings exhibited significantly higher expression of plastin 3 than their counterparts with spinal muscular atrophy caused by the survival motor neuron 1 gene mutation, therefore demonstrating that plastin 3 plays an important role in axonogenesis via increasing the F-actin level (Oprea *et al.* 2008).

The dynein heavy chain belongs to dyneins family, which moves processively along the surfaces of microtubules and facilitates the microtubule-based transport in eucaryotic cells (Paschal & Vallee 1987).

Cofilin-1 is the major component of intranuclear and cytoplasmic actin rods and able to bind G- and F-actin in a 1:1 ratio of cofilin to actin, playing an important role of controlling reversibly actin polymerization and depolymerization in a pH-sensitive manner. In contrast to members of the actin-depolymerizing factor (ADF) group, which are more effective in filament depolymerization (including severing, offrate enhancement, and monomer sequestering), the members of the cofilin group promote filament assembly by severing without substantially increasing the critical

concentration (Chen *et al.* 2004).

Among those cytoskeletal proteins listed in Table 3.1 are some muscle regulating proteins such as the isoform 1 of tropomyosin beta chain, desmin, and the actin in the aortic smooth muscle, which were associated with the contractile/cytoskeletal system. It was also observed that these cytoskeletal proteins exhibited no significant changes in protein level in *S*-enantiomer of propranolol-treated cells as compared with those in *R*-enantiomer of propranolol-treated ones.

3.2.5 Analysis of Signaling Proteins

Of all the signaling proteins listed in Table 3.1, annexin A6, the calcium-binding protein was down-regulated, with iTRAQ ratio being 0.75 fold for *S*-enantiomer of propranolol-incubated cells. 14-3-3 protein zeta/delta is also called protein kinase C inhibitor protein 1, which is implicated in the regulation of a number of signaling pathways. Thandavarayan and co-workers recently reported that enhancement of 14-3-3 protein might provide an innovative therapeutic approach against hyperglycemia-induced left ventricular dysfunction and through regulating apoptosis signal-regulating kinase 1 signaling the progression of diabetic cardiomyopathy can be limited (Thandavarayan *et al.* 2008). Another example was demonstrated by Kim, *et al.*, showing that a reduction in 14-3-3 zeta was able to stimulate protein kinase C activation and suggesting this is a main cause of retina visual dysfunction during diabetes (Kim *et al.* 2005).

In addition, the protein, macrophage migration inhibitory factor was down-regulated in *S*-enantiomer of propranolol-treated A7r5 cells. It is recognized as a pivotal regulator of innate immunity and an integral component of the host antimicrobial alarm system and stress response that promotes the pro-inflammatory functions of

immune cells. A rapidly increasing amount of literature indicates that macrophage migration inhibitory factor is implicated in the pathogenesis of sepsis, and inflammatory and autoimmune diseases (Lue *et al.* 2002). For example, the gene coding for the ventricular myosin light chain associated with skeletal muscle regeneration has been proved to be down-regulated in burned children receiving propranolol treatment (Herndon *et al.* 2003).

3.2.6 Up-Regulation of Metabolic Enzymes

Among the 6 metabolic enzymes which showed differential expression level, a number of them had increased levels in the *S*-enantiomer-incubated cells compared with the levels in the *R*-enantiomer-incubated cells. Interestingly, these represented enzymes which are involved in the anabolic (or biosynthetic) pathways.

One common feature of anabolic metabolic pathways, as opposed to the catabolic pathways, is the oxidation of cofactors such as NADH to NAD⁺. Anabolic enzymes with such a feature in our study included L-lactate dehydrogenase involved in the conversion of lactate to pyruvate (Friedrich *et al.* 1998), and the mitochondrial glutamate dehydrogenase 1 for the oxidative deamination (Patel 2000). Both of these enzymes also oxidized NADH to NAD⁺.

Deoxyribonuclease-1 is an enzyme that catalyzes the hydrolysis of deoxyribonucleic acid. Among other functions it seems to bind to the G-actin specifically and subsequently block the process of the actin polymerization (Wawro *et al.* 2005). In this study it was shown to be up-regulated more drastically for *S*-enantiomer of propranolol-treated A7r5 cells than for *R*-enantiomer of propranolol treatment.

Medium-chain acyl-CoA dehydrogenase is a nucleus-encoded mitochondrial matrix enzyme which catalyses the initial dehydrogenation step in the β -oxidation of medium-chain fatty acids, from which 40% of our overall energy requirements come (Matsubara *et al.* 1992).

Peroxiredoxin-2 is involved in reducing peroxides with reducing equivalents provided through the thioredoxin system and thus may play an important role in eliminating peroxides generated during metabolism by regulating the intracellular concentrations of H_2O_2 .

Thioredoxin is a small redox active protein induced by oxidative stress, which was distributed ubiquitously in various mammalian tissues and cells, and found to act as an antioxidant, an anti-inflammatory and an antiapoptotic protein. To validate if the expression levels for thioredoxin were correlated with corresponding ROS levels in A7r5 cells incubated with individual enantiomers of propranolol, the ROS experiments were performed. The results displayed in Figure 3.5 indicated that ROS level in *S*-enantiomer of propranolol-incubated A7r5 cells was much lower than that for control cells or *R*-enantiomer of propranolol-incubated ones, as illustrated by the fluorescence intensities in corresponding samples.

To strengthen our findings on the involvement of anabolic (biosynthetic) enzymes in cellular in response to the *S*-enantiomer of propranolol, the level of intracellular NAD^+ and NADH was measured. Results shown in Table 3.2 indicated that the ratio of $NAD^+/NADH$ was significantly higher in cells treated with the *S*-enantiomer of propranolol (with an average of 1.545), compared with the level in either control cells (with an average of 0.763) or cells incubated with the *R*-enantiomer of propranolol (with an average of 0.788).

	Content of NADt [pmol]	Content of NADH [pmol]	NAD ⁺ /NADH Ratio
Control cells	37.2 ± 0.885	21.1 ± 0.676	0.763 ± 0.074
Cells with <i>S</i> -enantiomer	34.1 ± 0.496	13.4 ± 0.332	1.545 ± 0.098
Cells with <i>R</i> -enantiomer	32.0 ± 0.623	17.9 ± 0.519	0.788 ± 0.092

Table 3.2: Ratios of intracellular NAD⁺/NADH in control cells, *S*-enantiomer of propranolol-incubated cells and *R*-enantiomer of propranolol-incubated cells.

To assess if the changes in the mRNA levels were significant, real-time PCR was carried out for the L-lactate dehydrogenase, peroxiredoxin-2, thioredoxin, deoxyribonuclease-1 precursor, glutamate dehydrogenase 1 and medium chain acyl-CoA dehydrogenase. For real-time PCR, the results based on three independent experiments for each enantiomer indicated significant changes ($p < 0.05$) at the mRNA level for *S*-enantiomer of propranolol (Figure 3.6).

Figure 3.5: ROS levels in A7r5 cells incubated with *R*-, *S*-enantiomer of propranolol, respectively. The pictures on the left-handed panels were taken under fluorescent filter. ROS in untreated and treated cells incubated with carboxy-H₂DCFDA can cause the green fluorescence; the cells incubated with *S*-enantiomer of propranolol (D) emitted the lowest fluorescence intensity as compared with control cells or with *R*-enantiomers of propranolol-treated cells. The pictures on the right-handed panels were taken at phase-contrast setting and overlaid with the corresponding images on the left. The control cells without treatment were used as the negative control (B), the cells with TBHP treatment as positive control (A). The cells incubated with the *R*-enantiomer of propranolol are shown in (C).

Figure 3.6: Real-time PCR analysis of mRNA levels of genes coding for metabolic enzymes in *S*-enantiomer of propranolol- and *R*-enantiomer of propranolol incubated cells. Ratio between the *S*-enantiomer incubated and control cells (S:C), as well as between the *R*-enantiomer incubated and control cells (R:C) was shown.

3.3 Discussion

3.3.1 Enzymes Involved in Anabolism and Actin Polymerization

Metabolic enzymes are part of short-term cellular responses to external stimulation, and the activation and deactivation of many are under the regulation of phosphorylation. One well-known example is the regulation of the glycogen phosphorylase and the glycogen synthase in the process of glycogen metabolism (Brushia & Walsh 1999). Both enzymes are already present in the cytoplasm, and their mutually exclusive activation is under the control of phosphorylation by protein kinase A. Close examining the real-time PCR results shown in Figure 3.6 indicated that the gene expression levels in most of those metabolic enzymes were in line with the corresponding protein expression levels, except for peroxiredoxin-2, which showed more intense change in mRNA level than protein level in *S*-enantiomer of propranolol-incubated cells. This is quite understandable as many other factors could determine the levels and activities of proteins such as regulated destruction of proteins. The results in our study shown indicated that the changes in protein levels were less than 100%, and the less than drastic changes in protein levels as observed have recently been found in a recent report on pharmacodynamic protein markers where the changes in protein levels fluctuated below 50% between the drug-administered individuals and the control individuals (Patil *et al.* 2007). The identification of anabolic metabolic enzymes in *S*-enantiomer of propranolol-incubated cells was also in line with earlier clinical reports on the reversal of catabolism by propranolol treatment after severe burn (Herndon *et al.* 2001), reduction of catabolism rate in patients receiving propranolol (Lamont *et al.* 2000), and increase in body fat which is considered as one of the side effects of propranolol treatment (Lamont 1995).

The higher ratio of NAD^+/NADH would correlate with higher proportion of NAD^+ , indicative of higher anabolic activity. Interestingly, the NAD^+/NADH ratio in cells treated with the *S*-enantiomer of propranolol was 102% higher than that for the other two types of cells, which was significantly higher than the level of the anabolic enzymes which oxidize NADH to NAD^+ . For example, the levels of the L-lactate dehydrogenase and the glutamate dehydrogenase were, respectively, 20% and 26% higher in cells treated with the *S*-enantiomer of propranolol. Thus, the changes in metabolic reactions intermediates (NAD^+/NADH) were 4 to 5 fold higher than those in the levels of metabolic enzymes. This was consistent with our hypothesis that metabolic activity may not be always reflected by the protein level of the involved enzymes, but the measurement of metabolic reactions intermediates (in this case the cofactor NAD^+/NADH) should provide a more accurate information.

The protein which showed the most significantly increased level in the *S*-enantiomer of propranolol-incubated cells compared with that for the *R*-enantiomer of propranolol-incubated cells was deoxyribonuclease-1, a protein served to bind G-actin specifically and subsequently block the process of the actin polymerization (Wawro et al. 2005). It has yet to be determined whether the up-regulation of deoxyribonuclease-1 in the *S*-enantiomer of propranolol-incubated cells was linked to the β -adrenergic receptor-mediated signaling pathway. However, close observation of Table 3.1 revealed that the protein, actin aortic smooth muscle showed a slight lower change in protein level in *S*-enantiomer of propranolol-incubated cells as compared with control ones, which was likely due to the negative effects of deoxyribonuclease-1 on actin polymerization and could provide a molecular explanation for the clinically observed effects of propranolol on reducing cardiac output, reducing the heart beat and preventing the cardiomyopathy (Asai et al. 1999).

3.3.2 Enzymes Involved in Antioxidant Activity

Of the metabolic enzymes listed in Table 3.1, both the peroxiredoxin and the thioredoxin showed higher expression levels in cells treated with the *S*-enantiomer of propranolol as compared with the levels in those incubated with the *R*-enantiomer of propranolol.

As is well documented, cellular reduction/oxidation (redox) status plays a very important role in maintaining normal cellular functions. The impairment of cell redox state is able to induce the changes in ROS levels and thus alters multiple cell pathways, which may contribute to the pathogenesis of cardiovascular disorders including atherosclerosis, hypertension, and heart failure (World *et al.* 2006). The thioredoxin system, a ubiquitous thiol oxidoreductase system, includes thioredoxin, thioredoxin reductase and NADPH, which modulates cellular redox status. Thioredoxin plays essential roles in limiting oxidative stress directly through antioxidant effects and indirectly by interactions with thioredoxin interacting protein. For example, it was reported that in ex vivo working rat heart that thioredoxin was down-regulated due to reperfusion of ischemic myocardium. Furthermore, thioredoxin-over-expressing mouse hearts had improved post-ischemic ventricular recovery and decreased myocardial infarct size compared to wild-type hearts (Turoczi *et al.* 2003). Conversely, the generated transgenic mice with cardiac-specific overexpression of a dominant negative mutant (C32S/C35S) of thioredoxin exhibited increased cardiac oxidative stress and hypertrophy both under basal conditions and in response to pressure overload through redox-sensitive mechanisms (Yamamoto *et al.* 2003). Recently Kuster *et al.* found that thioredoxin overexpression prevented α -adrenergic receptor-stimulated hypertrophy by inhibiting Ras activation in adult rat

cardiomyocyte (Kuster *et al.* 2005). These results indicated that endogenous thioredoxin plays a protective role in failing heart associated with oxidative stress.

In addition, it is clear that both peroxiredoxin and the thioredoxin showed higher levels in the *S*-enantiomer of propranolol-incubated cells than in *R*-enantiomer of propranolol incubated-ones. Both of the two proteins are part of a cellular complex involved in the regulation of intracellular level of hydrogen peroxide (H_2O_2) (Nakamura 2005). Thioredoxin is involved in reducing peroxide into H_2O and becomes oxidized in the process, while peroxiredoxin is a thioredoxin reductase and is responsible for generating the reduced form of thioredoxin which can be used for further reduction of other peroxide molecules (Rhee *et al.* 2005a). H_2O_2 has been implicated in various cellular activities including cell signaling, metabolism, and apoptosis (Rhee *et al.* 2005b). While the therapeutic effects of β -blockers have usually been explained by their capacity to block the β -adrenoceptors, a recent study has suggested their antioxidant activity by reducing cellular apoptosis associated with H_2O_2 (Gomes *et al.* 2006). The ROS results shown in Figure 3.5 also demonstrated that *S*-enantiomer of propranolol could induce lower ROS levels than *R*-enantiomer of propranolol for A7r5 cells, indicative of increased antioxidant activity.

4. Comparative Proteomics Analysis of A7r5 Cells Incubated with S- and R-Enantiomers of Atenolol

4.1 Introduction

Chemically, atenolol is a phenylacetamide ((4–2'-hydroxy-3'-isopropyl-aminopropoxy) phenylacetamide). It is a relatively polar, hydrophilic β_1 -selective drug, which exerts greater blocking activity on β_1 adrenergic receptors than on β_2 ones, with the S-enantiomer being more active than the R-enantiomer. Upon oral administration of the drug the intestinal absorption in humans and most laboratory animal species is rapid but incomplete. It was reported that the systemic bioavailability is about 50% to 60% in the human as well as in the rat, mouse, rabbit, and monkey (Tabacova & Kimmel 2002). Following absorption, atenolol is widely distributed to most body tissues in humans and animals (Brown *et al.* 1976, Reeves *et al.* 1978a, Reeves *et al.* 1978b). However, only a small proportion of the administered dose reaches the brain in both humans (Cruickshank *et al.* 1980) and animals (van Zwieten & Timmermans 1979, Reeves *et al.* 1978a). The blood concentration ratio in the human brain tissue is about 0.2:1 compared to a much larger proportion (33:1) for another widely used β -blocker, propranolol (Cruickshank *et al.* 1980).

In clinical practice, atenolol is used for treatment of hypertension, including hypertension in pregnancy. The beneficial property for use of β_1 -receptor selective blockers in therapeutic doses is that the effects via β_2 -receptor stimulation (vasodilation, bronchodilation and glycogenolysis) should not be affected. Therefore, during treatment with a β_1 -receptor selective blocker, less vasoconstriction, bronchoconstriction and (in patients with diabetes mellitus) hypoglycemias should occur (Brodde 2007). However, although it has been reported that patients with

chronic obstructive pulmonary diseases and cardiovascular diseases benefit from β_1 -receptor selective blocker treatment in a very similar fashion as patients with cardiovascular diseases without airway disease, the use of β_1 -receptor selective blockers should be avoided in patients with asthma bronchiale (Gottlieb *et al.* 1998). For pregnant women, atenolol is available for treatment of pre-existing or pregnancy-associated hypertensive disorders, which are known to be among the most frequent complications of pregnancy. It has been reported that in humans, a plasma level of 1 $\mu\text{g/mL}$ atenolol is associated with a 30% reduction of exercise-accelerated heart rate and that a clear linear relationship could be obtained between log plasma concentration and percentage reduction in heart rate (Brown *et al.* 1976). Besides exerting blocking effects on cardiac adrenoreceptors, atenolol acts on adrenoreceptors that regulate renin release and metabolic responses. Stimulation of β -adrenoreceptors is able to enhance the release of renin from the kidneys and conversely suppressed by atenolol, as demonstrated in vitro in isolated rat kidney slices (Capponi *et al.* 1977), and in vivo in animal experiments (Johns & Singer 1974). Similarly, in the human, atenolol reduces circulating levels of active renin (Sassard *et al.* 1976).

In this study, we used a 4-plex multiplex strategy to simultaneously detect and quantify differences in expression levels of proteins in untreated vascular smooth muscle cells and those incubated with the *S*- and *R*-enantiomers of atenolol, respectively, which reflect the pharmacologic action of enantiomers. To identify proteins from a complex mixture, a 2D method was applied. In this approach, a SCX column was used for the first dimension, a RP column was used for the second, and two identical enrichment columns were used for trapping the digest peptides.

4.2 Results

4.2.1 MTT Assay and Concentration of Atenolol

To determine the appropriate concentration of atenolol used in our study, A7r5 cells were incubated with an increasing concentration of the respective enantiomer of atenolol, and the viability was examined by the MTT assay. Results shown in Figure 4.1 indicated that either of the two enantiomers of atenolol was toxic to the cells with a drastic decrease in cell viability for any concentration higher than 50 μM . In addition, the overall comparison of the MTT values obtained showed that the *R*-enantiomer of atenolol was somewhat less toxic than the *S*-enantiomer of atenolol. Our results also indicated that no significant effect on the cell viability was observed for those concentrations below 25 μM . For this study the concentration of 20 μM was chosen in all experiments.

Figure 4.1: Cell viability of A7r5 cells after 24 h of exposure to *R*- and *S*-enantiomer of atenolol, respectively.

Figure 4.2: Tandem mass spectra of BSA tryptic peptides prepared by labeling with each member of the four isobaric iTRAQ reagent tags and pooling the reaction mixtures in a ratio of 1:1:0.75:1.5. The enlarged low-mass region shows the report ions m/z 114, 115, 116, and 117 an approximate 1:1:0.75:1.5 ratio.

4.2.2 iTRAQ Analysis and Differentially Expressed Proteins

Compared with the software, ProQUANT which was utilized for quantitation of the differentially expressed proteins in cells response to individual enantiomers of propranolol, the newly purchased ProteinPilot software was used for atenolol incubation experiments. To test if a combination of four labeled BSA peptides, with the same ratio of 1:1:0.75:1.5 as the one discussed in section 3.2.2 in chapter 3, can be quantitated with the similar results by ProteinPilot software, another independent experiment with iTRAQ analysis of digested BSA mixture was performed. A representative spectrum showing a peptide, NECFLSHK, from BSA was displayed in Figure 4.2, in which, the measured 115/114 ratio of 0.98, 116/114 ratio of 0.76, and 117/114 ratio of 1.53 were obtained, indicative of the similar results as previously obtained by ProQUANT software.

To establish the biological difference between atenolol-incubated A7r5 cells and control A7r5 cells, the protein profile in A7r5 cells incubated with individual enantiomers of atenolol was analyzed by 2D LC-MS/MS. Cells incubated with individual enantiomers of atenolol as well as the control cells were collected, lysed, and labeled prior to 2D LC-MS/MS. In this study, iTRAQ 114 represented control A7r5 cells, iTRAQ 115 represented A7r5 cells incubated with the *S*-enantiomer of atenolol, and iTRAQ 117 represented A7r5 cells incubated with the *R*-enantiomer of atenolol (Figure 4.3 and Figure 4.4).

More than 200 proteins were identified from each of the three independent experiments conducted (268, 249, and 257 proteins, respectively) by the following criteria: unused protein score was more than 2 (99% confidence) per experiment, and one or more peptide hits were found per protein at >95% confidence per peptide.

Combining these three experiments, a total of 323 unique proteins were identified (supplementary Table 2) in the three independent experiments and were classified into distinct categories (Figure 4.5) according to their molecular functions and represented by percentage of proteins found for each category.

It is apparent that metabolism and structural proteins account for a large proportion of the detected proteins. For each experiment, we examined the original protein list generated by ProteinPilot software and filtered it according to the criteria considering a candidate protein for further statistical analysis as described in section 2.10 (Data Analysis and Interpretation). Among the three experiments, the range of proteins in the filtered list was 19–27. Testing for multiple comparisons from quantitative information obtained from the three experiments eventually led to the 13 proteins listed in Table 4.1. All 13 proteins showed statistically significant changes ($p < 0.05$) in cells incubated with *S*-enantiomer of atenolol compared with control cells. In contrast, in *R*-enantiomer of atenolol-incubated cells the changes in most of the proteins were not significant ($p > 0.05$).

Figure 4.3: A MS/MS spectrum representing a peptide, ADQLTEEQIAEFK, from calmodulin. The ion assignments are as follows: control A7r5 = iTRAQ 114; A7r5 incubated with the *S*-enantiomer of atenolol = iTRAQ 115; A7r5 incubated with the *R*-enantiomer of atenolol = iTRAQ 117.

Figure 4.4: A MS/MS spectrum representing a peptide, DLESDIIGDTSGHFQK , from annexin A6. The ion assignments are as follows: control A7r5 = iTRAQ 114; A7r5 incubated with the *S*-enantiomer of atenolol= iTRAQ 115; A7r5 incubated with the *R*-enantiomer of atenolol = iTRAQ 117.

Figure 4.5: Distribution of all proteins identified after iTRAQ labeling and tandem mass spectrometry into different functional categories.

Accession	Name	Sequence coverage	No. peptide	Avg. S:C (±SD)	Avg. R:C (±SD)	<i>p</i> (S:C)	<i>p</i> (R:C)
Calcium-binding							
P48037	Annexin A6	31.7%	3	0.71±0.11	0.96±0.13	0.0194	>0.05
P62161	Calmodulin	58.1%	8	0.68±0.16	0.84±0.17	0.0371	>0.05
Q6B345	Protein S100-A11	76.5%	6	0.81±0.11	0.97±0.09	0.0491	>0.05
P05942	Protein S100-A4	77.2%	5	0.76±0.10	0.91±0.16	0.0266	>0.05
Cellular metabolism							
Q66H12	Alpha-N-acetylgalactosaminidase precursor	25.9%	2	1.71±0.43	1.08±0.09	0.0482	>0.05
P00507	Aspartate aminotransferase, mitochondrial precursor	54.4%	15	1.25±0.13	1.07±0.07	0.0380	>0.05
P04906	Glutathione S-transferase P	57.1%	13	1.35±0.15	1.16±0.10	0.0281	0.0468
P20070	NADH-cytochrome b5 reductase	39.4%	5	1.65±0.29	1.16±0.19	0.0321	>0.05
Cytoskeletal protein							
P21263	Nestin	35.3%	5	0.80±0.14	0.96±0.15	0.0371	>0.05
P58775	Tropomyosin beta chain	32.4%	2	0.67±0.28	0.89±0.25	0.0303	>0.05
Q6P9V9	Tubulin alpha-2 chain	79.6%	17	1.30±0.17	1.13±0.11	0.0462	>0.05
Signal transduction							
P61589	Transforming protein RhoA precursor	36.1%	3	0.80±0.19	0.92±0.17	0.0425	>0.05
P63102	14-3-3 protein zeta/delta	62.4%	12	1.21±0.11	1.02±0.06	0.0403	>0.05

Table 4.1: List of differential expressed proteins in A7r5 cells incubated with atenolol^a

^a S:C is the ratio of different protein expression level in the S-enantiomer incubated cells relative to the control cells; R:C is the ratio of different protein expression level in the R-enantiomer incubated cells relative to the control cells. The sequence coverage was calculated from one typical experiment. The *p* values are calculated for those meeting the predefined criteria for statistical testing.

These proteins were subsequently categorized into four groups according to their cellular functions (Table 4.1). These included four calcium-binding proteins, four proteins involved in cellular metabolism, three cytoskeletal proteins, and two proteins involved in cellular signal transduction. In general, proteins in A7r5 cells treated with the *S*-enantiomer of atenolol showed differential levels from those in cells incubated with the *R*-enantiomer of atenolol. These results were consistent with the fact that the *S*-enantiomer of atenolol is more biologically active than the *R*-enantiomer of atenolol. The metabolic enzymes listed in Table 4.1 were significantly up-regulated in A7r5 cells treated with the *S*-enantiomer of atenolol compared with those incubated with the *R*-enantiomer of atenolol, whereas the four calcium-binding proteins were significantly down-regulated in A7r5 cells incubated with the *S*-enantiomer of atenolol compared with those incubated with the *R*-enantiomer of atenolol. In contrast to the metabolic enzymes and calcium-binding proteins, cytoskeletal proteins and signal proteins in Table 4.1 showed random changes with some down-regulated and others up-regulated regardless of the enantiomer of atenolol used in the experiment.

4.2.3 Up-regulation of Metabolic Enzymes

Anabolism is the set of metabolic pathways that construct molecules from smaller units, which is the opposite of catabolism. One common feature of anabolic metabolic pathways is the oxidation of cofactors such as NADH to NAD⁺. Anabolic enzymes with such a feature in our study included aspartate aminotransferase (mitochondrial), which facilitates the conversion of aspartate and α -ketoglutaric acid to oxaloacetate and glutamate (Dennis & Clark 1977); glutathione *S*-transferase P, which catalyzes the conjugation of reduced glutathione via the sulfhydryl group to electrophilic centers on a wide variety of substrates (Douglas 1987) and is considered to contribute to the phase II biotransformation of xenobiotics (Oshino *et al.* 1971); and NADH-cytochrome *b*₅

reductase, which functions in the desaturation and elongation of the fatty acids(Hales & Huang 1994, Keyes & Cinti 1980), cholesterol biosynthesis(Reddy *et al.* 1977), and drug metabolism(Hildebrandt & Estabrook 1971). NADH-cytochrome *b*₅ reductase is involved in the oxidation of NADH to NAD⁺ in the following catalytic reaction:
$$\text{NADH} + 2 \text{ ferricytochrome } b_5 = \text{NAD}^+ + \text{H}^+ + 2 \text{ ferrocytochrome } b_5.$$

To strengthen the involvement of NADH-cytochrome *b*₅ reductase in cellular response to the individual enantiomers of atenolol, the level of intracellular NAD⁺ and NADH was measured. Results shown in Table 4.2 indicated that the ratio of NAD⁺/NADH was significantly higher in cells treated with the *S*-enantiomer of atenolol (with an average of 1.248) compared with the level in either control cells (with an average of 0.773) or cells incubated with the *R*-enantiomer of atenolol (with an average of 0.863). The higher ratio of NAD⁺/NADH would correlate with a higher proportion of NAD⁺, indicative of higher anabolic activity.

	Content of NADt [pmol]	Content of NADH [pmol]	NAD ⁺ /NADH Ratio
Control cells	36.7± 0.788	20.7± 0.534	0.773± 0.086
Cells with <i>S</i> -enantiomer	35.3± 0.571	15.7± 0.615	1.248± 0.075
Cells with <i>R</i> -enantiomer	34.1± 0.609	18.3± 0.462	0.863± 0.094

Table 4.2: Ratios of intracellular NAD⁺/NADH in control cells, S-enantiomer of atenolol-incubated cells and R-enantiomer of atenolol-incubated cells.

4.2.4 Down-regulation of Calcium-binding Proteins

A closer analysis of the proteins in Table 4.1 revealed that the calcium-binding proteins such as calmodulin, protein S100-A11, protein S100-A4, and annexin A6 were all down-regulated with iTRAQ ratios ranging from 0.68- to 0.81-fold for *S*-enantiomer-incubated cells. Further evaluation by Student's *t* tests indicated that the down-regulation of these calcium-binding proteins was statistically significant (*p* <

0.05) in cells treated with the *S*-enantiomer of atenolol compared with the control cells (Table 4.1). Although a down-regulation of the same calcium-binding proteins was also observed in cells incubated with the *R*-enantiomer of atenolol, evaluation by Student's *t* tests suggested that the changes were not of significant difference ($p > 0.05$) compared with the control cells (Table 4.1).

To substantiate the down-regulation as quantitated by iTRAQ-coupled 2D LC-MS/MS, the level of two proteins (calmodulin and annexin A6) was examined by Western blot analysis. Similar to the iTRAQ analysis, results shown in Figure 4.6 indicated that the level of the calmodulin and annexin A6 was lower in cells treated with the *S*-enantiomer of atenolol compared with the level in the control cells. In addition, the level of annexin A6 showed no statistically significant changes in *R*-enantiomer of atenolol-incubated cells compared with control cells ($p > 0.05$), whereas the level of calmodulin in cells incubated with the *R*-enantiomer of atenolol was found to be significantly different compared with the control cells ($p < 0.05$). Quantification of the protein level based on Western blot analysis indicated that the decrease in these two proteins was more dramatic than that in iTRAQ analysis (Figure 4.6B). The possible cause for the observed discrepancy between iTRAQ and Western blot analyses could be due to the normalization of total protein content leading to less dramatic values for lower abundance proteins in iTRAQ quantitation.

To confirm the expression of the genes encoding the four identified calcium-binding proteins, real time PCR was carried out for calmodulin, protein S100-A11, protein S100-A4, and annexin A6. Results shown in Figure 4.7 indicated that the changes in gene expression for these four proteins in cells treated with the *S*-enantiomer of atenolol were statistically significantly lower than those in control cells ($p < 0.05$). On

the other hand, only the gene coding for calmodulin in cells incubated with the *R*-enantiomer of atenolol showed statistically significant changes in its expression ($p < 0.05$) compared with that in the control cells. The expression of the other three genes (coding for protein S100-A11, protein S100-A4, and annexin A6, respectively) in cells incubated with the *R*-enantiomer of atenolol was found to show no significant difference ($p > 0.05$) compared with the control cells. Our real time PCR results were consistent with the Western blot analysis at two levels. First, all calcium-binding proteins showed statistically significant down-regulation in cells treated with the *S*-enantiomer of atenolol (at both gene and protein levels). Second, calmodulin was the only protein showing down-regulation in cells incubated with the *R*-enantiomer of atenolol (at both gene and protein levels).

Figure 4.6: Western blot analysis of protein levels of calmodulin and annexin A6 in *S*-enantiomer- and *R*-enantiomer-incubated cells. (A) *Lane Control*, cells in the absence of atenolol; *Lane S*, cells treated with the *S*-enantiomer of atenolol; *Lane R*, cells incubated with the *R*-enantiomer of atenolol. (B), quantification of the protein level based on Western blot analysis. The ratio between *S*-enantiomer-incubated cells and control cells (S:C) as well as between *R*-enantiomer-incubated cells and control cells (R:C) is shown. The *p* values indicated statistical significance of the observed differences with *p* < 0.05 considered as statistically significant and *p* > 0.05 considered as not statistically significant.

Figure 4.7: Real time PCR analysis of mRNA levels of genes coding for calcium-binding proteins in *S*-enantiomer and *R*-enantiomer of atenolol-incubated cells. The ratio between the *S*-enantiomer-incubated and control cells (S:C) as well as between the *R*-enantiomer-incubated and control cells (R:C) is shown. The p values indicated statistical significance of the observed differences with $p < 0.05$ considered as statistically significant and $p > 0.05$ considered as not statistically significant.

To investigate the Ca^{2+} entry into the cytoplasm intracellular Ca^{2+} concentration was measured. The results in Table 4.3 showed that intracellular Ca^{2+} concentration in A7r5 cells treated with *S*-enantiomer of atenolol was about 8% lower than that for control cells and about 3% lower in the *R*-enantiomer of atenolol-incubated cells compared with the control cells. Statistical analysis based on three independent experiments indicated statistically significant changes ($p < 0.05$).

Combining our results from real time PCR, Western blot, and the measurement of intracellular Ca^{2+} concentration, it is plausible that the reduction of intracellular Ca^{2+} concentration in cells incubated with the *R*-enantiomer of atenolol was possibly due to the reduced level of calmodulin. The immunofluorescence images in Figure 4.8 showed that the A7R5 cells incubated with the *S*-enantiomer of atenolol spread more than the control cells and those cells treated with the *R*-enantiomer of atenolol.

	S:C ($\pm$ SD)	R:C ($\pm$ SD)	p (S:C)	p (R:C)
Ratio of intracellular Ca^{2+} concentration	0.919 $\pm$ 0.016	0.968 $\pm$ 0.018	0.0064	0.0456

Table 4.3: Quantification of the ratio of intracellular Ca^{2+} concentration in *S*-enantiomer- and *R*-enantiomer-incubated cells as compared with that of control cells. Ratio between *S*-enantiomer-incubated cells and control cells (S:C) as well as between *R*-enantiomer-incubated cells and control cells (R:C) is shown.

Figure 4.8: The immunofluorescence images of control A7r5 cell (A), *S*-enantiomer of atenolol-incubated A7r5 cells (B), and *R*-enantiomer of atenolol-incubated A7r5 cells (C). The scale bar represents 20 μm .

4.3 Discussion

Atenolol is a cardioselective β_1 -receptor blocking agent without intrinsic sympathomimetic activity. One explanation of its hypotensive effect is by decreasing heart rate and cardiac contractility (Rasanen & Jouppila 1995). The most beneficial effect of atenolol for treatment of hypertension lie in its selectively blocking effect on β_1 -receptor, thus reducing vasoconstriction and bronchoconstriction, as described in the introduction of this chapter. Currently developments in industrial chemical processes have successfully separated the racemic atenolol into individual enantiomers, i.e., *S*-enantiomer of atenolol and *R*-enantiomer of atenolol economically and marketed them commercially, with each enantiomer exhibiting unique properties. Investigation of the protein profiles in A7r5 cells incubated with individual enantiomers of atenolol and thus identification of signaling effectors downstream of the β_1 -receptor will provide a better understanding of the β -block effect and potential adverse effects.

One up-regulated metabolic enzyme in Table 4.1 is glutathione *S*-transferase P. The rat glutathione *S*-transferase P has been found to be dramatically up-regulated in its expression in preneoplastic and neoplastic cells (Sato 1989, Satoh *et al.* 1985) and is widely used as a specific marker in the basic analysis of chemical carcinogenesis (Ito *et al.* 1996). Another metabolic enzyme associated with lipid and drug metabolism is NADH-cytochrome *b*₅ reductase. It has been well documented that the membrane-bound cytochrome *b*₅ is located in the endoplasmic reticulum where it can accept an electron from NADH-cytochrome *b*₅ reductase. Reduced cytochrome *b*₅ then provides reducing equivalents for the biosynthesis of selected lipids and

drugs(Canova-Davis & Waskell 1984). We performed the NAD^+/NADH assay comparing the cellular anabolism in *S*-enantiomer of atenolol-incubated cells with that in *R*-enantiomer of atenolol-incubated cells and control cells. Interestingly the NAD^+/NADH ratio in cells incubated with the *S*-enantiomer of atenolol was about 61% higher than that for control cells, and the ratio in *R*-enantiomer-treated cells was 12% higher than that for control cells; these results were in line with the protein level for NADH-cytochrome *b*₅ reductase in individual type of cells (about 65 and 16% higher in *S*-enantiomer-treated cells and *R*-enantiomer-treated cells, respectively). The increase in the anabolic activity preceded by higher levels of metabolic enzymes such as NADH-cytochrome *b*₅ reductase therefore provided molecular evidence on the metabolic effect associated with atenolol treatment.

The Ca^{2+} ion is a highly multipurpose intracellular signal molecule involved in many different cellular functions, including fertilization, cell cycle, apoptosis, muscle contraction, vision, and memory(Carafoli 2005). In eukaryotic cells there exist two sources governing the cytoplasmic Ca^{2+} entry and outflow, i.e., the intracellular stores including the endoplasmic reticulum and the extracellular Ca^{2+} which goes into the cell with the help of various transporters on the plasma membrane(Bootman & Berridge 1995). It is generally established that the Ca^{2+} flux machinery comprised of ion channels, pumps and exchangers can facilitate the highly localized and transient Ca^{2+} signals in transducing by calcium-binding proteins and in turn acting on other enzymes and downstream effector proteins. The ubiquitous calcium-binding protein calmodulin plays a crucial role in a variety of cellular signaling cascades via regulation of a number of target proteins in a Ca^{2+} -dependent manner. It can activate the Ca^{2+} pump of plasma membranes by interacting with a domain next to its carboxyl terminus(Shull & Greb 1988) and can interact with GRK5, a member of the GRK

family that is associated with homologous desensitization of G protein-coupled receptor, to reduce GRK5 binding to the membrane (Sallese *et al.* 2000). Annexins are a family of proteins that bind in a calcium-dependent manner to phospholipid membranes. It was reported that annexin A6 increased the activity of the sarcoplasmic reticulum Ca^{2+} -ATPase (Krasavchenko *et al.* 2006). S100-A11 is one member of the S100 family of calcium-binding proteins that is expressed mostly in smooth muscle and other tissues. The Ca^{2+} binding to S100-A11 induces a conformational change that exposes the hydrophobic surface for interaction with target proteins such as annexin A6. It was hypothesized that an increase in cytosolic free Ca^{2+} facilitates the formation of a complex of S100-A11 and annexin A6, which is able to form a physical connection linking the plasma membrane and the cytoskeleton, or in some cases plays a role in the generation of signaling complexes at the level of the sarcolemma (Chang *et al.* 2007). S100-A4 is another member of the S100 family, which is associated with active stress fibers (probably regulating contraction) and the sarcoplasmic reticulum (regulating Ca^{2+} homeostasis) (Heizmann & Cox 1998). The down-regulation of the Ca^{2+} -binding proteins was therefore closely correlated with the decrease in the intracellular Ca^{2+} concentration. The relatively lower intracellular Ca^{2+} concentration in A7r5 cells treated with *S*-enantiomer of atenolol would induce less control of actin-myosin-based contraction in A7r5 cells that was mediated by the myosin light chain kinase-calmodulin complex (Holzapfel *et al.* 1983). The cells incubated with *S*-enantiomer of atenolol spread more (Figure 4.8) compared with the other two types of cells, indicative of less contraction and more relaxation.

Nestin is one of the filament proteins and is expressed in dividing cells at the early stages in the process of development in the central nervous system, as well as myogenic and other tissues, playing a multipart role in regulation of the assembly and

disassembly of intermediate filaments with vimentin or internexin, and thus remodeling the cell (Michalczyk & Ziman 2005). Tropomyosin in muscle and non-muscle occurs in tight association with actin filaments, and in skeletal and cardiac muscle tropomyosin plays a central role in regulation of contraction through mediation of the calcium response of the troponin complex to actin filaments (Lees-Miller & Helfman 1991). However, the physiological function of tropomyosin in smooth muscle cells and non-muscle cells is not fully understood due in part to the absence of troponin. It has been reported that tropomyosin is involved in stabilization of actin filaments, cytoskeletal modeling, and cell motility (Lin *et al.* 1997), and β -tropomyosin plays a significant role in the process of phenotypic modulation of smooth muscle cells (Girjes *et al.* 2002). Unlike nestin and β -tropomyosin, which were down-regulated in A7r5 cells incubated with atenolol, tubulin-2 chain was up-regulated. Cooper and co-workers (Tagawa *et al.* 1997, Tsutsui *et al.* 1993, Tsutsui *et al.* 1994) reported that the isolated myocytes with contractile dysfunction demonstrated augmented cytoskeletal stiffness featured by an increased amount of total tubulin and an enhanced degree of polymerization. However, the role of tubulin involved in the development of hypertrophy and heart failure was questioned by Collins *et al.* (Collins *et al.* 1996) and Bailey *et al.* (Bailey *et al.* 1997).

Transforming protein RhoA is a small GTPase protein that directly stimulates actin polymerization through activation of diaphanous-related formins (DRF proteins), which can play an important role in stimulating the addition of actin monomers to the ends of rapid growing actin filaments. In addition DRFs can act together with Rho kinases (ROCKs) to mediate Rho-induced stress fiber formation (Ridley *et al.* 2003). Moreover ROCKs are able to induce actomyosin-based contractility and phosphorylate a number of proteins involved in regulating myosins and some other

actin-binding proteins(Riento & Ridley 2003). It was reported that inhibition of the RhoA/Rho kinase pathway is effective in reducing pulmonary hypertension (Guilluy *et al.* 2005). Bi *et al.*(Bi *et al.* 2005) reported that the expression level of RhoA plays a crucial role in regulating the contractility of cultured vascular smooth muscle cells. 14-3-3 protein zeta/delta was also found to be differentially expressed in response to individual enantiomers of atenolol, with the protein level being higher in *S*-enantiomer of atenolol-incubated cells than that for *R*-enantiomer of atenolol-incubated cells.

Collectively, calmodulin plays a crucial role in activating the Ca^{2+} pump of plasma membranes, and the lower calmodulin expression level in A7r5 cells incubated with *S*-enantiomer of atenolol correlated with the lower intracellular calcium concentration in those cells compared with the other two types of cells. It can be inferred from the discussion above that the lower calmodulin expression level can increase GRK5 binding to the membrane, resulting in over-desensitization of G protein-coupled receptor. In turn, a relative lower intracellular Ca^{2+} concentration in *S*-enantiomer-incubated cells would lead to less interaction of S100-A11 with annexin A6, resulting in less physical link between the plasma membrane and the cytoskeleton. The Ca^{2+} signals transduced by calcium-binding proteins acted on cytoskeletal proteins such as nestin and β -tropomyosin and therefore played a complex role in regulation of the cytoskeletal modeling and cell contraction in conjunction with S100-A4. In addition a lower RhoA expression level in *S*-enantiomer-incubated cells might result in less actin polymerization and thus induce less contractility with DRF proteins and ROCKs compared with control cells and *R*-enantiomer of atenolol-incubated cells.

5. Secretion of T-Kininogen from A7r5 Cells in Response to *S*-Enantiomer of Propranolol

5.1 Introduction

Propranolol, a nonselective β blocker, exerts blocking activity both on β_1 adrenoceptors and β_2 ones, with the *S*-enantiomer being more effective than the *R*-enantiomer. Biochemical responses to disease or drug action are likely to be reflected in the patterns of protein expression and turnover in affected cells, tissues and, presumably, biological fluids such as blood, cerebrospinal fluid, saliva. Mass spectrometry-based proteomics has been widely used to offer a nontargeted way to identify protein profiles or drug-activity markers.

Our previous work focused on the comparative analysis of protein profile within the vascular smooth muscle cells treated with single enantiomers of propranolol respectively. For a better understanding of the blocking effect exerted by these two enantiomers it will be of great interest to detect changes in secretory proteins and to infer biological function combining with the observed intracellular patterns. In this study, we use a 4-plex multiplex strategy to simultaneously detect and quantitate differences in secretory proteins in untreated vascular smooth muscle cells and those treated with *S*- and *R*-enantiomers of propranolol respectively, which reflect pharmacologic action of enantiomers. To identify proteins from a complex mixture, the online 2D-nano LC/MS/MS coupled with iTRAQ quantitation approach is used.

5.2 Results

To obtain more information about the mechanisms of action of individual enantiomers of propranolol, the secreted protein profile in A7r5 cells incubated with individual

enantiomers of propranolol was established. iTRAQ tags shown in Figure 5.1 were as follows: control A7r5 cells = iTRAQ 114; A7r5 cells treated with the *S*-enantiomer of propranolol = iTRAQ 115; A7r5 incubated with the *R*-enantiomer of propranolol = iTRAQ 117. In three independent experiments a number of proteins were identified with unused protein score more than 2 (99% confidence) on the basis of one or more peptide hits per protein at >95% confidence per peptide. A total of 12 unique secreted proteins identified in the three independent experiments were tabulated in Table 5.1. For further analysis, we examined the original protein list generated by ProteinPilot software and filtered it according to the criteria for considering a protein significant discussed in section 2.10, leading to 1 protein, T-kininogen 1 precursor, which was considered statistically significant ($p < 0.05$) in conditioned culture medium of cells incubated with *S*-enantiomer of propranolol, showing higher protein levels than that for control medium or conditioned medium of *R*-enantiomer of propranolol-incubated cells (Table 5.2).

To verify the differential protein levels quantitated by iTRAQ coupled 2D-LC/MS/MS Western blot analysis was performed. The results in Figure 5.2 showed a similar behavior as the iTRAQ analysis, which indicated that the level of the T-kininogen 1 was higher in culture medium of *S*-enantiomer of propranolol incubated cells compared with the level in those two types of culture medium ($p < 0.05$). However by close examining the quantification of the protein level based on Western blot analysis in Figure 5.2B, the changes in the protein were more dramatic than those in iTRAQ analysis. The cause for this discrepancy is most likely due to a bias when performing normalization of total protein content, which is much more suitable for high abundant proteins, leading to relatively lower values for less abundant proteins in the iTRAQ quantitation. The experimental molecular weight was 66 kDa determined by Western

blot analysis, higher than the theoretical molecular weight of 47 kDa. It was most possibly caused by the glycosylation of T-kininogen 1.

5.3 Discussion

In rats, T-kininogen is a major positive acute phase protein (Esnard & Gauthier 1983), a cysteine proteinase inhibitor (Mulleresterl *et al.* 1986) with an Ile-Ser-bradykinin (BK) (T-kinin) sequence in its structure (Okamoto & Greenbaum 1983). The kallikrein-kinin system is one of the endogenous vasodepressor systems with a variety of functions in regulating blood pressure and generates potent vasodilating peptides, kinins, by a proteolytic activity of kallikreins on specific substrates kininogens (Bhoola *et al.* 1992). The responses to vasoactive kinin peptides are mediated by the activation of bradykinin receptor B1 (B1R) and B2 (B2R) according to the relative potencies and affinities to their agonists (Park *et al.* 2006). B2R is constitutively expressed in most tissues and responsive to intact BK and kallidin (Lys-BK). In contrast, B1R which has a higher affinity for des-Arg⁹-BK (DBK) and des-Arg¹⁰-kallidin, is weakly expressed under physiological conditions but induced by pathological stimuli such as inflammation and tissue injury (Regoli *et al.* 1998). Hang Yin and co-workers reported that the B2 receptor, but not the B1 receptor, protects against ischemia/reperfusion (I/R) induced cardiac dysfunction by inhibiting apoptosis and limiting ventricular remodeling (Yin *et al.* 2007). In this study, we demonstrated for the first time that A7r5 cells can secrete T-kininogen by using the iTRAQ-coupled 2D LC-MS/MS and Western blot analysis. However, the secretion of T-kininogen by *S*-enantiomer of propranolol-incubated cells was enhanced as compared with that of *R*-enantiomer of propranolol-incubated cells or control cells. Since T-kininogen is the precursor of active peptide BK, *S*-enantiomer of propranolol-incubated A7r5 cells will

produce more BK or active metabolites of BK as compared with other two types of cells, indicative of more pharmacologically.

Accession	Name	Avg. S:C (±SD)	Avg. R:C (±SD)	No. experiment
P12346	Serotransferrin precursor	0.89±0.06	0.96±0.05	3/3
P02767	Transthyretin precursor	1.01±0.11	1.03±0.09	3/3
P24090	Alpha-2-HS-glycoprotein precursor	0.91±0.16	0.95±0.13	3/3
P01026	Complement C3 precursor	0.98±0.08	0.98±0.14	3/3
P11762	Galectin-1	1.15±0.17	1.18±0.20	3/3
P01048	T-kininogen 1 precursor	1.39±0.11	1.07±0.06	3/3
Q64268	Heparin cofactor 2 precursor	0.97±0.24	0.99±0.15	2/3
P49745	Thrombopoietin precursor	0.81±0.21	0.89±0.12	2/3
P12843	Insulin-like growth factor-binding protein 2 precursor	1.02±0.17	1.03±0.11	2/3
O88959	Inhibin beta E chain precursor	0.97	0.86	1/3
5X167	F-box only protein 30	1.06	1.03	1/3
Q07936	Annexin A2	1.05	1.27	1/3

Table 5.1: List of secreted proteins in conditioned culture medium of A7r5 cells treated with single enantiomers of propranolol respectively^a
^a S:C is the ratio of the level of each protein secreted by S-enantiomer of propranolol incubated cells relative to that by the control cells; R:C is the ratio of the level of each protein secreted by R-enantiomer of propranolol incubated cells relative to that by control cells. “no. of experiments” indicates the number of times the protein was detected in the three independent experiments.

Accession	Name	Sequence coverage	No. peptides	Avg. S:C (±SD)	Avg. R:C (±SD)	<i>p</i> (S:C)	<i>p</i> (R:C)	No. experiment	
P01048	T-kininogen precursor	1	64.9%	2	1.39±0.11	1.07±0.06	0.0175	>0.05	3/3

Table 5.2: Differentially expressed protein, T-Kininogen 1 in conditioned culture medium of A7r5 cells treated with single enantiomer of propranolol respectively ^a

^a S:C is the ratio of the level of each protein secreted by S-enantiomer of propranolol incubated cells relative to that by the control cells; R:C is the ratio of the level of each protein secreted by R-enantiomer of propranolol incubated cells relative to that by control cells. “no. of experiments” indicates the times the protein was detected in the three independent experiments. The *p* values indicated statistical significance of the observed differences.

Figure 5.1: A MS/MS spectrum representing a peptide, RPPGFSPFR, from T-kininogen 1. The ion assignments are as follows: control A7r5 = iTRAQ 114; A7r5 incubated with the S-enantiomer of propranolol = iTRAQ 115; A7r5 incubated with the R-enantiomer of propranolol = iTRAQ 117.

Figure 5.1: Western blot analysis of T-kininogen 1 in the conditioned medium of A7r5 cells incubated with individual enantiomers of propranolol. Lane C, control, cells in the absence of propranolol. Lane S, cells incubated with the *S*-enantiomer of propranolol. Lane R, cells incubated with the *R*-enantiomer of propranolol. The quantification of the protein level based on Western blot analysis was displayed in the table below the Western blot results. Ratio between *S*-enantiomer incubated cells and control cells (S:C), as well as between *R*-enantiomer incubated cells and control cells (R:C) was shown.

6. Secretion of T-Kininogen from A7r5 Cells in Response to S-Enantiomer of Atenolol

6.1 Introduction

Atenolol is a β_1 -selective blocker, exhibiting greater blocking activity on β_1 -adrenoreceptors than on β_2 ones with the *S*-enantiomer being more active than *R*-enantiomer. In chapter 4 the protein profile in the proteome of A7r5 cells incubated separately with individual enantiomers of atenolol was compared and showed that *S*-enantiomer of atenolol was able to induce more drastic change in a number of proteins, which was further validated by real time PCR and Western blot analysis. In chapter 4 the comparative proteomic analysis of conditioned medium of cells treated with different enantiomers of propranolol as well as control medium was performed and T-kininogen 1 was found to be secreted more in response to *S*-enantiomer of propranolol. In this experiment that follows, the control medium and conditioned medium of cells treated respectively with different enantiomers of atenolol will be investigated by online 2D LC/MS/MS approach to compare the results obtained in response to propranolol treatment described in chapter 4.

6.2 Results and Discussion

In three independent experiments a total of 9 unique secreted proteins were identified in the three independent experiments displayed in Table 6.1. For further analysis, we examined the original protein list generated by ProteinPilot software and filtered it according to the criteria for considering a protein significant discussed in section 2.10, leading to 1 protein, T-kininogen 1 precursor, which exhibited statistical significance ($p < 0.05$) in conditioned culture medium of cells incubated with *S*-enantiomer of

propranolol, showing higher protein levels than that for control medium or conditioned medium of *R*-enantiomer of propranolol-incubated cells (Table 6.2).

It seemed that the secretion of T-kininogen by *S*-enantiomer of atenolol-incubated cells was stimulated as compared with that for *R*-enantiomer of atenolol incubated cells or control cells. Since T-kininogen is the precursor of active peptide BK, *S*-enantiomer of atenolol incubated A7r5 cells might lead to more BK or active metabolites of BK compared with other two types of cells as do *S*-enantiomer of propranolol-incubated A7r5 cells.

Accession	Name	Avg. S:C (±SD)	Avg. R:C (±SD)	No. experiment
P01026	Complement C3 precursor	0.95±0.12	1.02±0.09	3/3
P12346	Serotransferrin precursor	0.84±0.07	0.98±0.10	2/3
P24090	Alpha-2-HS-glycoprotein precursor	0.95±0.11	1.04±0.15	3/3
Q64268	Heparin cofactor 2 precursor	0.92±0.09	1.00±0.07	3/3
P01048	T-kininogen 1 precursor	1.22±0.11	1.04±0.08	3/3
Q9JKS6	Protein piccolo	1.05±0.11	0.96±0.06	3/3
P11762	Galectin-1	0.98±0.15	1.02±0.12	3/3
P02767	Transthyretin precursor	1.04±0.07	1.06±0.13	2/3
P12843	Insulin-like growth factor-binding protein 2 precursor	1.08±0.14	1.09±0.12	2/3

Table 6.1: List of secreted proteins in conditioned culture medium of A7r5 cells treated with single enantiomer of atenolol respectively^a

^a S:C is the ratio of the level of each protein secreted by S-enantiomer of atenolol incubated cells relative to that by the control cells;
R:C is the ratio of the level of each protein secreted by R-enantiomer of atenolol incubated cells relative to that by control cells.
“no. of experiments” indicates the number of times the protein was detected in the three independent experiments.

Accession	Name	Sequence coverage	No. peptides	Avg. S:C (±SD)	Avg. R:C (±SD)	p (S:C)	p (R:C)	No. experiment
P01048	T-kininogen 1 precursor	61.4%	2	1.22±0.11	1.04±0.08	0.0423	>0.05	3/3

Table 6.2: Differentially expressed protein, T-Kininogen 1 in conditioned culture medium of A7r5 cells treated with single enantiomer of atenolol respectively^a

^a S:C is the ratio of the level of each protein secreted by S-enantiomer of atenolol incubated cells relative to that by the control cells; R:C is the ratio of the level of each protein secreted by R-enantiomer of atenolol incubated cells relative to that by control cells. “no. of experiments” indicates the times the protein was detected in the three independent experiments. The p values indicated statistical significance of the observed differences.

7. Conclusions

Currently proteomics has become a more powerful technique to provide more direct and useful information about an organism than genomics as it directly addresses the level of genome products and their interactions. In this project the increasingly popular MS/MS-based quantitation method (iTRAQ) was utilized on the sophisticated platform of ESI LC/MS/MS system. The multiplex iTRAQ allows up to four samples to be analyzed in one experiment. The cleaved peptides from each of the samples were differentially isotopically labeled and all derivatized peptides will have an identical mass and LC retention time after tagging. Following CID MS/MS analysis of the precursor ion, the previously introduced four reporter groups show as distinct ions (m/z 114–117). The relative concentration of the peptides is derived from the relative intensities of the reporter ions. To identify proteins from a complex mixture, a 2D LC was used, where a SCX column was used as the first dimension, a RP column was used for the second, and two identical enrichment columns were used for trapping the peptides sequentially eluted from the SCX by injecting a series of KCL solutions of increasing concentrations.

The focus of the project is to simultaneously detect and quantify the differentially expressed proteins in vascular smooth muscle cells (A7r5) incubated with single enantiomers of propranolol (atenolol) as well as those in untreated cells by iTRAQ-coupled 2D LC-MS/MS. In addition, the secreted proteins by A7r5 cells in response to single enantiomers of propranolol (atenolol) were also examined to complement the results obtained for intracellular investigation of the pharmacologic action of enantiomers.

Propranolol is a nonselective β -blocker, exerting blocking effect on both β_1 -receptors

and β_2 ones, with the *S*-enantiomer demonstrated to be more active as compared with the *R*-enantiomer. In clinical practice it has been proved to be effective in the treatment of hypertension (Vlachakis & Aledort 1980, Witte *et al.* 2004), angina (Detry *et al.* 1994, Subramanian *et al.* 1982), and to prevent patients who have suffered from myocardial infarction from re-infarction (Norris *et al.* 1984, Shivkumar *et al.* 1998). However, the mechanism underlying those effects of propranolol has not been well addressed in the molecular level.

Of the 361 unique proteins identified in the three independent experiments, 20 proteins were differentially expressed between the propranolol-incubated and normal sample and further categorized into 6 metabolic enzymes, 3 signaling molecules, 7 cytoskeletal proteins and 4 proteins involved in DNA synthesis/protein translation based on cellular functions.

A number of enzymes involved in cellular anabolism and antioxidant activity in A7r5 cells treated with the *S*-enantiomer of propranolol showed higher protein levels as compared with those for control cells and *R*-enantiomer of propranolol-incubated ones, which was further validated by real-time PCR shown in Figure 3.6. Significantly, the increase in the anabolic activity resulted from the higher level of metabolic enzymes including L-lactate dehydrogenase and glutamate dehydrogenase 1, mitochondrial precursor was also supported by the higher concentration of the intracellular metabolic cofactor NAD^+ , the product of an increased oxidation of NADH. The higher expression level of thioredoxin in A7r5 cells treated with the *S*-enantiomer of propranolol may be of significance for increased antioxidant activity, as indicated by the ROS results shown in Figure 3.5, with the ROS level in *S*-enantiomer of propranolol-incubated cells being lower than other two types of cells. In addition,

deoxyribonuclease-1 precursor was significantly up-regulated in *S*-enantiomer of propranolol-incubated A7r5 cells, which remains to be investigated for its function in A7r5 cells.

The tabulated cytoskeletal proteins exhibited no specific trend in protein level changes and some of them displayed more dramatic changes induced by *R*-enantiomer of propranolol than those by *S*-enantiomer of propranolol, including plastin-3 and dynein heavy chain. Coincidentally 14-3-3 protein zeta/delta, the protein kinase C inhibitor protein 1 was also observed to show more intense change in protein expression level in response to *R*-enantiomer of propranolol than *S*-enantiomer of propranolol. In contrast to these findings discussed above, it is generally accepted that *S*-enantiomer of propranolol is more active than *R*-enantiomer of propranolol. The potentially underlying molecular mechanism involved in *R*-enantiomer of propranolol treatment has yet to be addressed.

To compare the potential mechanism of action of β_1 -selective blockers (exerting greater blocking activity on β_1 adrenergic receptors than on β_2 ones) with the non-selective ones, one of the commonly used β_1 -blockers, atenolol was examined. Similarly the protein profiles in A7r5 cells incubated with individual enantiomers of atenolol were analyzed on the platform of ESI LC/MS/MS coupled with iTRAQ quantitation. A total of 13 differentially expressed proteins were listed in Table 4.1 for further investigation and subsequently classified into calcium-binding proteins, cellular metabolism, cytoskeletal protein and proteins involved in signal transduction.

Our results indicated that several metabolic enzymes such as aspartate aminotransferase, glutathione *S*-transferase P, NADH-cytochrome *b*₅ reductase, and alpha-N-acetylgalactosaminidase precursor were up-regulated and displayed higher

protein levels in cells incubated with the *S*-enantiomer of atenolol as compared with no treatment or *R*-enantiomer of atenolol treatment. The involvement of NADH-cytochrome *b*₅ reductase in the intracellular anabolic activity was further supported by NAD⁺/NADH assay with a higher ratio of NAD⁺/NADH, indicative of a higher proportion of NAD⁺.

The listed calcium-binding proteins including calmodulin, protein S100-A11, protein S100-A4, and annexin A6 were all down-regulated and showed relatively lower protein levels in cells incubated with the *S*-enantiomer of atenolol than those incubated with the *R*-enantiomer of atenolol. The down-regulated calmodulin was able to increase GRK5 binding to the membrane, resulting in over-desensitization of G protein-coupled receptor, as illustrated in Figure 7.1. On the other hand the relatively lower intracellular Ca²⁺ concentration in *S*-enantiomer of atenolol-incubated cells would induce less interaction of S100-A11 with annexin A6, leading to less physical link between the plasma membrane and the cytoskeleton. The Ca²⁺ signals were transduced by calcium-binding proteins and acted on cytoskeletal proteins such as nestin and β -tropomyosin, which can play a complex role in regulation of the cytoskeletal modeling and cell contraction in conjunction with S100-A4. Additionally, a lower RhoA expression level in *S*-enantiomer of atenolol-incubated cells might result in less actin polymerization and thus induce less contractility with DRF proteins and ROCKs as compared with control and *R*-enantiomer of atenolol-incubated cells.

As mentioned above, a number of proteins in A7r5 cells incubated with individual enantiomers of propranolol (atenolol) showed differentially expressed levels compared with those in untreated ones. To expand the investigation of the mechanism of action of these two drugs, it is quite understandable to examine the extracellular

protein profiles for A7r5 cells in response to individual enantiomers of propranolol (atenolol). Our results indicated that the *S*-enantiomer of propranolol was able to trigger the secretion of T-kininogen by A7r5 cells more than control cells or *R*-enantiomer of propranolol-incubated ones as shown in Table 5.2, and the similar behavior was observed for *S*-enantiomer of atenolol that T-kininogen is up-regulated as shown in Table 6.2. Okamoto H. and co-workers reported that T-Kininogen secretion by rat vascular smooth muscle cells was markedly enhanced by the addition of angiotensin II (Okamoto *et al.* 1998), an oligopeptide in the blood that increases blood pressure. Our results therefore demonstrated that the secretion of T-kininogen by A7r5 cells stimulated by the addition of *S*-enantiomer of propranolol (atenolol) was in line with the results by Okamoto. However, it has not yet been addressed whether the generation of vasodilating peptides, kinins resulted from proteolytic activity of kallikreins on specific substrates kininogens (Bhoola *et al.* 1992), was due to cells' adaptation to the *S*-enantiomer of propranolol (atenolol), which might account for the potential regulation mechanism for propranolol or atenolol treatment in hypertension.

Although atenolol exerts different blocking effect from propranolol, it was observed that all of the listed metabolic enzymes in Table 4.1 were up-regulated in A7r5 cells incubated with *S*-enantiomer of atenolol, the phenomenon, which also occurred for most of the metabolic enzymes in Table 3.1 in response to the *S*-enantiomer of propranolol with the protein L-lactate dehydrogenase being the exception. As the small molecule metabolites within the cells could be a reflection of enzymatic activity to monitor both normal biochemical events and perturbations that lead to disease, the up-regulation of the metabolic enzymes is possible to result in the changes in the level of some metabolites in cells in response to single enantiomers of propranolol (atenolol) respectively, for example, NAD^+ , NADH and ROS, as illustrated in Figure 7.1 & 7.2.

More interestingly, the changes in level of metabolites in this study were seen more intense than those proteins associated with, providing additional evidence that metabolites could be more suitably used for monitoring the biochemical response than proteins.

Comparing the cytoskeletal proteins in Table 3.1 and Table 4.1, it was found that they exhibited quite different behaviors. Specifically, all of the cytoskeletal proteins in Table 4.1 displayed more intense changes induced by *S*-enantiomer of atenolol compared with *R*-enantiomer of atenolol or absence of atenolol, whereas for propranolol treatment some of the listed cytoskeletal proteins in Table 3.1 including plastin-3 and dynein heavy chain, showed more dramatic changes in *R*-enantiomer of propranolol-incubated cells as compared with other two types of cells, which should be further investigated to enlighten the potential mechanism of action of *R*-enantiomer of propranolol.

Additionally, 14-3-3 protein zeta/delta exhibited higher protein level in *S*-enantiomer of atenolol-incubated cells than *R*-enantiomer of atenolol-incubated and control ones, while for *S*-enantiomer of propranolol treatment, the 14-3-3 protein zeta/delta did not show any change compared with that in control cells, but with lower protein level being in *R*-enantiomer of propranolol-incubated ones. It was reported that the enhancement of this protein can lead to a novel therapeutic strategy for treatment of hyperglycemia-induced left ventricular dysfunction and limit the progression of diabetic cardiomyopathy (Thandavarayan *et al.* 2008). Consistently, there are quite a number of reports associated with atenolol treatment for hypertensive patients with type 2 diabetes (Fogari *et al.* 2008, Barrios *et al.* 2008, Flammer *et al.* 2007).

Our preliminary results demonstrated that the application of quantitative proteomics provided an effective approach to understand the mechanism of action of drug and built a better understanding of protein targets and their roles in disease. However, it must be noted that many proteins exert their functions through PTMs and protein interactions. It is clear that plenty of work is needed to enlighten the potential mechanism underlying the blocking effects in response to β -blockers, such as structure-based prediction of PTMs and narrowing the candidate interacting proteins. To date the area of proteomics, though expanding rapidly, still faces challenges. With the development of multi-separation technology, new algorithms and bioinformatics software packages, and newly invented quantitation methods introduced, it is undoubtedly that proteomics will ultimately drive the discovery process.

Figure 7.1: A schematic diagram depicting the possible role of metabolic enzymes associated with cellular anabolism and antioxidant activity and the stimulated secretion of T-kininogen in A7r5 cells incubated with *S*-enantiomer of propranolol. The oval represents the cell membrane, where the seven transmembrane β -adrenoceptors are located. The curved up arrows indicate the changes in increased direction, whereas the curved down arrows indicate the changes in decreased direction.

Figure 7.2: A schematic diagram illustrating the possible role of metabolic enzymes involved in cellular anabolism, the possible role of Ca²⁺ binding proteins and the enhanced secretion of T-kininogen in A7r5 cells incubated with *S*-enantiomer of atenolol. The oval represents the cell membrane, where the seven transmembrane β -adrenoceptors are located. The curved up arrows indicate the changes in increased direction, whereas the curved down arrows indicate the changes in decreased direction.

8. Future Directions

The differentially expressed proteins in A7r5 cells treated with individual enantiomers of propranolol (atenolol) were identified in the study, which were further validated with several molecular assays. However a great amount of work is needed to investigate the potential signal pathways in response to β -blockers such as knocking down particular gene expression and over-expressing some proteins of interest. It is likely that the expression change in one protein might alter the complete protein interaction network within the cells. In addition, PTMs and protein phosphorylation mediates many critical cellular responses, altering proteins' function and activity. Some techniques such as PhosphoScan and structure-based prediction of PTMs will provide more valuable information.

Systematic analysis of the metabolites in an organism can be used to assess the response of the biological system and provide a complementary picture with proteomic analyses. The change in the level of a particular metabolite can reflect a defective enzyme with impaired or even absent activity. In my study some metabolites such as NAD^+ and NADH showed more intense changes than those for the associated metabolic enzymes. The significant changes in metabolites might serve as potential indicators for monitoring metabolic pathways.

It is of great interest to expand the research into other β -blockers with proteomics. The differentially expressed proteins (protein profile) in response to each β -blocker can be identified, tabulated and compared. The similarities and dissimilarities between individual protein profiles can be summarized and compiled into a database, which will be helpful for drug screening to discover potential candidates with the similar mechanism of action as specific β -blocker in the database. This project, however, will

be greatly dependent on the development of proteomics techniques, which will be certainly pushed to new limits to address the increasing demands encountered in drug discovery.

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APPENDIX

Appendix I: Supplementary table 1 for the identified 361 proteins (score >2) in A7r5 cells incubated with propranolol

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unk PI:IP 00326433.10	Tax_Id=10116 10 kDa heat shock protein, mitochondrial	0.97±0.07	0.94±0.04
unk PI:IP 00566502.1	Tax_Id=10116 102 kDa protein	0.99±0.08	1.08±0.14
unk PI:IP 00559188.1	Tax_Id=10116 11 kDa protein	0.69 ± 0.04	1.07± 0.07
unk PI:IP 00388174.2	Tax_Id=10116 12 kDa protein	0.91±0.18	1.06±0.21
unk PI:IP 00777367.1	Tax_Id=10116 13 kDa protein	0.86±0.16	0.97±0.21
unk PI:IP 00325135.3	Tax_Id=10116 14-3-3 protein epsilon	1.07±0.11	1.11±0.16
unk PI:IP 00230835.4	Tax_Id=10116 14-3-3 protein gamma	0.99±0.10	0.99±0.06
unk PI:IP 00196661.1	Tax_Id=10116 14-3-3 protein theta	0.94±0.09	1.02±0.07
unk PI:IP 00324893.4	Tax_Id=10116 14-3-3 protein zeta/delta	1.04 ± 0.06	0.83 ± 0.05
unk PI:IP 00560461.1	Tax_Id=10116 15 kDa protein	0.94±0.15	0.95±0.16
unk PI:IP 00210975.1	Tax_Id=10116 150 kDa oxygen-regulated protein precursor	0.94	0.97
unk PI:IP 00778383.1	Tax_Id=10116 16 kDa protein	0.84 ± 0.12	0.92 ± 0.10
unk PI:IP 00565635.2	Tax_Id=10116 17 kDa protein	0.98±0.04	1.03±0.05
unk PI:IP 00421357.2	Tax_Id=10116 170 kDa protein	0.89±0.17	0.70±0.46
unk PI:IP 00370448.4	Tax_Id=10116 175 kDa protein	0.90±0.23	0.87±0.16
unk PI:IP 00781537.1	Tax_Id=10116 18 kDa protein	0.93±0.16	1.04±0.11
unk PI:IP 00569453.2	Tax_Id=10116 19 kDa protein	0.92±0.07	0.98±0.20
unk PI:IP 00476540.1	Tax_Id=10116 19 kDa protein	1.08±0.13	1.02±0.09
unk PI:IP 00365935.3	Tax_Id=10116 19 kDa protein	1.18±0.21	1.01±0.06
unk PI:IP 00476880.1	Tax_Id=10116 22 kDa protein	1.01±0.03	0.95±0.09
unk PI:IP 00391300.3	Tax_Id=10116 234 kDa protein	0.86±0.14	0.94±0.07
unk PI:IP 00778329.1	Tax_Id=10116 24 kDa protein	1.16±0.17	1.09±0.11
unk PI:IP 00779005.1	Tax_Id=10116 24 kDa protein	0.96±0.16	0.90±0.16
unk PI:IP 00421600.7	Tax_Id=10116 26S protease regulatory subunit 7	0.98±0.05	0.96±0.13
unk PI:IP 00212512.1	Tax_Id=10116 26S proteasome non-A TPase regulatory subunit 1	0.94±0.19	0.84±0.24
unk PI:IP 00362353.3	Tax_Id=10116 30 kDa protein	1.01	1.25
unk PI:IP 00777474.1	Tax_Id=10116 31 kDa protein	0.92±0.14	0.89±0.11
unk PI:IP 00566235.1	Tax_Id=10116 32 kDa protein	1.09±0.13	0.86±0.17
unk PI:IP 00782409.1	Tax_Id=10116 33 kDa protein	1.03±0.06	0.99±0.06

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unk IP1:IP 00195918.3	Tax_Id=10116 33 kDa protein	0.92±0.17	1.04±0.09
unk IP1:IP 00559028.1	Tax_Id=10116 34 kDa protein	0.90 ± 0.12	0.88 ± 0.05
unk IP1:IP 00782000.1	Tax_Id=10116 36 kDa protein	1.07±0.08	1.15±0.17
unk IP1:IP 00781944.1	Tax_Id=10116 39 kDa protein	1.02±0.07	0.92±0.13
unk IP1:IP 00373218.2	Tax_Id=10116 40 kDa peptidyl-prolyl cis-trans isomerase	0.97±0.24	1.00±0.18
unk IP1:IP 00191142.1	Tax_Id=10116 40S ribosomal protein S10	1.20 ± 0.11	0.88 ± 0.08
unk IP1:IP 00366014.2	Tax_Id=10116 40S ribosomal protein S13	1.09±0.06	1.04±0.03
unk IP1:IP 00201500.7	Tax_Id=10116 40S ribosomal protein S14	0.91±0.16	1.12±0.13
unk IP1:IP 00392390.2	Tax_Id=10116 40S ribosomal protein S2	0.99±0.02	0.91±0.03
unk IP1:IP 00212776.1	Tax_Id=10116 40S ribosomal protein S3	0.91±0.10	0.98±0.21
unk IP1:IP 00231693.4	Tax_Id=10116 40S ribosomal protein S3a	1.03 ± 0.08	0.89 ± 0.02
unk IP1:IP 00475474.2	Tax_Id=10116 40S ribosomal protein S4, X isoform	0.88 ± 0.10	0.84 ± 0.08
unk IP1:IP 00214582.1	Tax_Id=10116 40S ribosomal protein S7	1.44	1.05
unk IP1:IP 00215107.2	Tax_Id=10116 40S ribosomal protein SA	1.01±0.20	1.04±0.06
unk IP1:IP 00777179.1	Tax_Id=10116 44 kDa protein	0.97±0.20	0.96±0.21
unk IP1:IP 00189148.2	Tax_Id=10116 45 kDa protein	1.13±0.19	1.15±0.21
unk IP1:IP 00557715.2	Tax_Id=10116 46 kDa protein	1.16±0.17	1.16±0.21
unk IP1:IP 00204703.5	Tax_Id=10116 47 kDa heat shock protein precursor	0.97±0.10	1.03±0.15
unk IP1:IP 00564631.2	Tax_Id=10116 48 kDa protein	0.91±0.09	0.81±0.20
unk IP1:IP 00777007.1	Tax_Id=10116 49 kDa protein	0.99±0.13	1.07±0.20
unk IP1:IP 00203088.4	Tax_Id=10116 51 kDa protein	1.01±0.06	1.08±0.15
unk IP1:IP 00390801.2	Tax_Id=10116 56 kDa protein	1.14±0.24	1.05±0.07
unk IP1:IP 00390302.3	Tax_Id=10116 57 kDa protein	1.12±0.13	0.91±0.05
unk IP1:IP 00393333.2	Tax_Id=10116 5-aminimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	0.92±0.18	0.91±0.21
unk IP1:IP 00339148.2	Tax_Id=10116 60 kDa heat shock protein, mitochondrial precursor	0.95±0.09	0.96±0.07
unk IP1:IP 00200147.1	Tax_Id=10116 60S acidic ribosomal protein P0	0.88±0.14	0.94±0.09
unk IP1:IP 00192257.1	Tax_Id=10116 60S ribosomal protein L18a	1.06±0.05	1.04±0.13
unk IP1:IP 00395285.2	Tax_Id=10116 60S ribosomal protein L3	0.93±0.08	0.95±0.16
unk IP1:IP 00231346.4	Tax_Id=10116 60S ribosomal protein L30	1.07±0.06	0.94±0.06

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unk IP :PI00202512.6	Tax_ Id=10116 60S ribosomal protein L4	1.14 ± 0.07	1.19 ± 0.06
unk IP :PI00390343.5	Tax_ Id=10116 60S ribosomal protein L6	1.11±0.14	1.07±0.09
unk IP :PI00199543.2	Tax_ Id=10116 60S ribosomal protein L7	1.07± 013	1.19 ± 0.09
unk IP :PI00215208.2	Tax_ Id=10116 60S ribosomal protein L8	1.10±0.13	1.18±0.19
unk IP :PI00231293.6	Tax_ Id=10116 6-phosphofructokinase, muscle type	1.04±0.19	1.00±0.07
unk IP :PI00776871.1	Tax_ Id=10116 76 kDa protein	0.98±0.18	1.02±0.02
unk IP :PI00206624.1	Tax_ Id=10116 78 kDa glucose-regulated protein precursor	1.11 ± 0.03	1.10 ± 0.05
unk IP :PI00562672.1	Tax_ Id=10116 81 kDa protein	1.11±0.12	1.07±0.18
unk IP :PI00365985.4	Tax_ Id=10116 93 kDa protein	0.92±0.07	1.05±0.12
unk IP :PI00364504.4	Tax_ Id=10116 96 kDa protein	0.93±0.21	1.03±0.17
unk IP :PI00421539.3	Tax_ Id=10116 Aconitate hydratase, mitochondrial precursor	1.00±0.05	1.04±0.09
unk IP :PI00197129.1	Tax_ Id=10116 Actin, aortic smooth muscle	0.86 ± 0.07	0.91 ± 0.08
unk IP :PI00211512.6	Tax_ Id=10116 Actin-related protein 2/3 complex subunit 1B	0.99±0.14	0.94±0.06
unk IP :PI00231359.3	Tax_ Id=10116 Acyl-coenzyme A dehydrogenase, short chain	0.88±0.13	0.92±0.16
unk IP :PI00214246.1	Tax_ Id=10116 Acyl-protein thioesterase 2	0.94±0.06	0.81±0.22
unk IP :PI00555297.2	Tax_ Id=10116 Adenine phosphoribosyltransferase	1.19±0.23	1.19±0.19
unk IP :PI00231927.10	Tax_ Id=10116 ADP/A TP translocase 1	1.14±0.16	0.91±0.18
unk IP :PI00200466.2	Tax_ Id=10116 ADP/A TP translocase 2	0.99±0.12	0.96±0.16
unk IP :PI00231965.4	Tax_ Id=10116 ADP-ribosylation factor 5	0.86±0.26	0.89±0.14
unk IP :PI00197770.1	Tax_ Id=10116 Aldehyde dehydrogenase, mitochondrial precursor	1.05±0.09	1.00±0.06
unk IP :PI00231737.4	Tax_ Id=10116 Aldose reductase	0.97±0.13	1.06±0.06
unk IP :PI00421560.1	Tax_ Id=10116 alpha actinin 4	0.97±0.10	0.95±0.06
unk IP :PI00215465.1	Tax_ Id=10116 Alpha crystallin B chain	1.02±0.05	1.01±0.06
unk IP :PI00365423.1	Tax_ Id=10116 Alpha isoform of regulatory subunit A, protein phosphatase 2	0.90±0.14	0.91±0.16
unk IP :PI00212767.1	Tax_ Id=10116 Anionic trypsin-1 precursor	1.02±0.10	1.00±0.19
unk IP :PI00325146.6	Tax_ Id=10116 annexin A2	1.03±0.107	1.01±0.08
unk IP :PI00207390.7	Tax_ Id=10116 Annexin A3	0.97±0.07	1.01±0.13
unk IP :PI00471889.6	Tax_ Id=10116 Annexin A5	0.88±0.07	0.98±0.06
unk IP :PI00421888.2	Tax_ Id=10116 Annexin A6	0.75 ± 0.11	0.81 ± 0.08

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unk PI:PI00210920.1	Tax_Id=10116 Aspartate aminotransferase, mitochondrial precursor	0.90±0.15	0.97±0.16
unk PI:PI00206224.1	Tax_Id=10116 Aspartyl-tRNA synthetase	1.07±0.08	0.98±0.07
unk PI:PI00196107.1	Tax_Id=10116 A TP synthase B chain, mitochondrial precursor	1.07±0.19	1.08±0.21
unk PI:PI00195123.1	Tax_Id=10116 A TP synthase O subunit, mitochondrial precursor	1.03±0.18	1.01±0.24
unk PI:PI00396910.1	Tax_Id=10116 A TP synthase subunit alpha, mitochondrial precursor	1.00±0.04	1.23±0.29
unk PI:PI00551812.1	Tax_Id=10116 A TP synthase subunit beta, mitochondrial precursor	0.97±0.03	1.03±0.09
unk PI:PI00214665.2	Tax_Id=10116 A TP-citrate synthase	0.94±0.17	1.00±0.08
unk PI:PI00325144.4	Tax_Id=10116 A TP-dependent RNA helicase DDX39	0.58	0.47
unk PI:PI00203526.1	Tax_Id=10116 Barnotin	0.96±0.09	0.97±0.18
unk PI:PI00325912.1	Tax_Id=10116 Beta-catenin	0.93±0.08	0.95±0.09
unk PI:PI00231631.11	Tax_Id=10116 Beta-enolase	0.96±0.12	0.97±0.10
unk PI:PI00454431.1	Tax_Id=10116 Brain-specific alpha actinin 1 isoform	0.92±0.06	0.91±0.09
unk PI:PI00199636.1	Tax_Id=10116 Calnexin precursor	0.97±0.04	0.99±0.06
unk PI:PI00191728.1	Tax_Id=10116 Calreticulin precursor	0.99±0.21	0.92±0.24
unk PI:PI00197616.4	Tax_Id=10116 CAP, adenylate cyclase-associated protein 1	1.11±0.14	1.07±0.15
unk PI:PI00464670.1	Tax_Id=10116 Capping protein	0.98±0.19	0.96±0.18
unk PI:PI00230788.5	Tax_Id=10116 Carbonic anhydrase 3	0.86±0.22	0.90±0.16
unk PI:PI00190390.1	Tax_Id=10116 Cell division control protein 2 homolog	0.97±0.06	1.09±0.13
unk PI:PI00285606.4	Tax_Id=10116 Cell division cycle 42	1.13±0.13	0.96±0.10
unk PI:PI00366218.1	Tax_Id=10116 Chaperonin containing TCP1, subunit 2	0.96±0.16	0.95±0.17
unk PI:PI00470301.1	Tax_Id=10116 Chaperonin containing TCP1, subunit 5	0.95±0.06	0.98±0.18
unk PI:PI00188111.1	Tax_Id=10116 Chaperonin subunit 6a	0.91±0.08	0.86±0.15
unk PI:PI00421995.1	Tax_Id=10116 Chloride intracellular channel 1	1.02±0.17	0.99±0.24
unk PI:PI00206977.1	Tax_Id=10116 Citrate synthase	0.95±0.18	0.98±0.20
unk PI:PI00193983.1	Tax_Id=10116 Clathrin heavy chain	1.07±0.18	1.12±0.13
unk PI:PI00327144.6	Tax_Id=10116 Cofilin-1	0.84 ± 0.09	0.87 ± 0.10
unk PI:PI00361686.5	Tax_Id=10116 Complement component 1, q subcomponent binding protein	1.04±0.21	1.03±0.16
unk PI:PI00209397.1	Tax_Id=10116 Coronin-1B	0.81±0.19	0.97±0.06
unk PI:PI00205466.1	Tax_Id=10116 Cullin-associated NEDD8-dissociated protein 1	1.11±0.21	0.98±0.03

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unk PI:PI00200352.1	Tax_Id=10116 Cysteine-rich protein 2	0.56	0.66
unk PI:PI00231864.4	Tax_Id=10116 Cytochrome c, somatic	0.96±0.07	0.98±0.09
unk PI:PI00475835.2	Tax_Id=10116 D-3-phosphoglycerate dehydrogenase	1.07±0.08	1.19±0.24
unk PI:PI00464718.1	Tax_Id=10116 Ddx5	0.91±0.12	0.98±0.16
unk PI:PI00230876.4	Tax_Id=10116 Deoxyribonuclease-1 precursor	1.63 ± 0.30	1.16 ± 0.18
unk PI:PI00421517.6	Tax_Id=10116 Desmin	0.79 ± 0.11	0.83 ± 0.13
unk PI:PI00231662.6	Tax_Id=10116 diaphorase 1	1.21 ± 0.11	1.25 ± 0.18
unk PI:PI00193944.1	Tax_Id=10116 DNA topoisomerase 2-alpha	1.12±0.14	1.14±0.16
unk PI:PI00189212.4	Tax_Id=10116 DnaJ (Hsp40) homolog, subfamily A, member 4	1.05±0.21	1.03±0.05
unk PI:PI00327630.1	Tax_Id=10116 Dynein heavy chain, cytosolic	1.09 ± 0.11	0.80 ± 0.10
unk PI:PI00195372.1	Tax_Id=10116 Elongation factor 1-alpha 1	0.92±0.10	0.96±0.12
unk PI:PI00470317.4	Tax_Id=10116 Elongation factor 1-gamma	0.89±0.14	1.01±0.06
unk PI:PI00203214.5	Tax_Id=10116 Elongation factor 2	0.94±0.07	0.98±0.03
unk PI:PI00768951.1	Tax_Id=10116 enolase 1, alpha	0.93±0.06	0.94±0.08
unk PI:PI00207217.1	Tax_Id=10116 Enoyl-CoA hydratase, mitochondrial precursor	1.12±0.14	1.05±0.09
unk PI:PI00366398.2	Tax_Id=10116 Eukaryotic translation initiation factor 3, subunit 6	0.88 ± 0.08	0.76 ± 0.16
unk PI:PI00366436.1	Tax_Id=10116 Eukaryotic translation initiation factor 3, subunit 6 interacting protein	0.91±0.09	0.94±0.18
unk PI:PI00211216.3	Tax_Id=10116 Eukaryotic translation initiation factor 5A-1	1.06±0.05	0.90±0.07
unk PI:PI00287338.2	Tax_Id=10116 Exportin-1	0.95±0.12	0.95±0.06
unk PI:PI00200661.1	Tax_Id=10116 Fatty acid synthase	1.13 ± 0.09	1.18 ± 0.12
unk PI:PI00208091.2	Tax_Id=10116 Fibrillarin	1.06±0.07	0.89±0.15
unk PI:PI00231734.4	Tax_Id=10116 Fructose-bisphosphate aldolase A	1.23 ± 0.16	1.01 ± 0.15
unk PI:PI00368457.1	Tax_Id=10116 G1 to S phase transition 1	0.98±0.03	0.95±0.12
unk PI:PI00231275.6	Tax_Id=10116 Galectin-1	1.01±0.06	0.95±0.05
unk PI:PI00194341.4	Tax_Id=10116 Galectin-3	1.09±0.12	1.05±0.06
unk PI:PI00363974.3	Tax_Id=10116 Gelsolin precursor	0.78 ± 0.21	0.89 ± 0.09
unk PI:PI00231637.4	Tax_Id=10116 Glucose-6-phosphate 1-dehydrogenase	0.93±0.06	0.98±0.10
unk PI:PI00324633.2	Tax_Id=10116 Glutamate dehydrogenase 1, mitochondrial precursor	1.29 ± 0.11	1.02 ± 0.13
unk PI:PI00192301.1	Tax_Id=10116 Glutathione peroxidase 1	1.01±0.04	1.05±0.09

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unk PI:PI00411230.2	Tax_Id=10116 Glutathione S-trans ferase Mu 2	1.00±0.03	1.04±0.05
unk PI:PI00231229.8	Tax_Id=10116 Glutathione S-trans ferase P	1.07±0.19	0.99±0.09
unk PI:PI00569016.1	Tax_Id=10116 Gnl1 protein	0.89±0.17	0.95±0.18
unk PI:PI00212796.2	Tax_Id=10116 GTP-binding nuclear protein Ran	0.91±0.16	1.00±0.04
unk PI:PI00372214.3	Tax_Id=10116 Guanine monphosphate synthetase	0.88±0.20	0.84±0.18
unk PI:PI00231134.4	Tax_Id=10116 Guanine nucleotide-binding protein subunit beta 2-like 1	0.94±0.08	0.98±0.20
unk PI:PI00208205.1	Tax_Id=10116 Heat shock cognate 71 kDa protein	0.99±0.04	0.97±0.05
unk PI:PI00208256.1	Tax_Id=10116 Heat shock protein 86	0.99±0.08	0.98±0.10
unk PI:PI00471584.5	Tax_Id=10116 Heat shock protein HSP 90-beta	1.00±0.03	1.01±0.04
unk PI:PI00656385.1	Tax_Id=10116 Hypothetical protein	0.93±0.24	1.07±0.13
unk PI:PI00368347.2	Tax_Id=10116 Hypothetical protein LOC314432	0.91±0.21	0.94±0.08
unk PI:PI00204261.2	Tax_Id=10116 Importin beta-1 subunit	0.99±0.13	1.02±0.20
unk PI:PI00206712.2	Tax_Id=10116 Inositol monophosphatase	1.01±0.09	0.82±0.24
unk PI:PI00357924.1	Tax_Id=10116 Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial precursor	1.27±0.30	1.23±0.36
unk PI:PI00213571.1	Tax_Id=10116 Isoform 1 of Cytosolic acyl coenzyme A thioester hydrolase	1.21±0.35	1.06±0.11
unk PI:PI00208217.3	Tax_Id=10116 Isoform 1 of DNA-binding protein A	0.93±0.07	0.97±0.19
unk PI:PI00197888.2	Tax_Id=10116 Isoform 1 of Tropomyosin 1 alpha chain	0.93±0.07	0.97±0.05
unk PI:PI00230775.4	Tax_Id=10116 Isoform 1 of Tropomyosin beta chain	0.85 ± 0.05	0.79± 0.04
unk PI:PI00187731.4	Tax_Id=10116 Isoform 2 of Tropomyosin beta chain	0.88 ± 0.05	0.92 ± 0.06
unk PI:PI00760152.1	Tax_Id=10116 Isoform 3 of NADH-cytochrome b5 reductase	1.07±0.14	1.18±0.19
unk PI:PI00197553.1	Tax_Id=10116 Isoform B23.1 of Nucleophosmin	0.92±0.09	0.96±0.05
unk PI:PI00205745.3	Tax_Id=10116 Isoform Mitochondrial of Peroxiredoxin-5, mitochondrial precursor	0.83±0.25	0.91±0.10
unk PI:PI00201060.4	Tax_Id=10116 Lamin-A	0.95±0.07	0.96±0.06
unk PI:PI00372818.3	Tax_Id=10116 LEM domain containing 2	0.91±0.14	0.95±0.16
unk PI:PI00206020.1	Tax_Id=10116 Leucine-rich repeat-containing protein 59	0.85±0.20	0.87±0.17
unk PI:PI00363236.2	Tax_Id=10116 Leucyl-tRNA synthetase	1.12±0.24	1.17±0.21
unk PI:PI00207947.6	Tax_Id=10116 Leukotriene A 4 hydrolase	1.01±0.14	1.20±0.26
unk PI:PI00197711.1	Tax_Id=10116 L-lactate dehydrogenase A chain	1.03 ± 0.07	0.81 ± 0.06
unk PI:PI00776502.1	Tax_Id=10116 LUC7-like	0.89±0.12	0.95±0.08

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unk IP IP100230907.4	Tax_Id=10116 Macrophage migration inhibitory factor	0.79 ± 0.11	1.06 ± 0.14
unk IP IP100566583.1	Tax_Id=10116 Malate dehydrogenase, mitochondrial	1.07±0.06	0.96±0.10
unk IP IP100212015.1	Tax_Id=10116 Medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	1.37 ± 0.16	1.18 ± 0.14
unk IP IP100212314.4	Tax_Id=10116 Moesin	0.95±0.05	0.95±0.24
unk IP IP100567436.1	Tax_Id=10116 Muscleblind-like protein BLES04	0.82 ± 0.13	0.85 ± 0.22
unk IP IP100197585.1	Tax_Id=10116 MYG1 protein	0.83±0.21	0.91±0.16
unk IP IP100393867.3	Tax_Id=10116 Myosin I heavy chain	1.11±0.14	0.94±0.18
unk IP IP100365944.4	Tax_Id=10116 Myosin light polypeptide 6	0.96±0.13	0.95±0.07
unk IP IP100564409.2	Tax_Id=10116 Myosin regulatory light chain 2-A, smooth muscle isoform	0.91±0.09	0.96±0.06
unk IP IP100209113.2	Tax_Id=10116 Myosin-9	0.99±0.08	1.00±0.06
unk IP IP100230848.6	Tax_Id=10116 Myotrophin	0.99±0.21	1.07±0.19
unk IP IP100196202.1	Tax_Id=10116 NEDD8 precursor	0.91±0.16	0.94±0.18
unk IP IP100201261.1	Tax_Id=10116 Nestin	1.07±0.16	1.10±0.11
unk IP IP100211813.1	Tax_Id=10116 Neuronal myosin heavy chain	1.46	1.30
unk IP IP100208118.1	Tax_Id=10116 Non-muscle caldesmon	0.97±0.16	1.21±0.22
unk IP IP100205912.3	Tax_Id=10116 Non-POU domain-containing octamer-binding protein	1.02	0.97
unk IP IP100364994.1	Tax_Id=10116 Nuclear cap-binding protein subunit 1	1.05±0.18	1.03±0.15
unk IP IP100210009.1	Tax_Id=10116 Nuclear migration protein nudC	0.87±0.16	1.11±0.12
unk IP IP100768949.1	Tax_Id=10116 Nuclease sensitive element-binding protein 1	0.41	0.23
unk IP IP100208193.1	Tax_Id=10116 Nucleic acid binding factor pRM10	0.92±0.11	0.91±0.08
unk IP IP100215384.1	Tax_Id=10116 Nucleolin-related protein NRP	0.94	0.92
unk IP IP100194404.5	Tax_Id=10116 Nucleoside diphosphate kinase A	0.94±0.15	0.96±0.17
unk IP IP100327185.3	Tax_Id=10116 nucleosome assembly protein 1-like 1	0.97±0.09	0.98±0.18
unk IP IP100193279.1	Tax_Id=10116 Ornithine aminotransferase, mitochondrial precursor	1.04±0.16	1.00±0.14
unk IP IP100203753.1	Tax_Id=10116 Paraspeckle protein 1	0.90±0.17	1.00±0.10
unk IP IP100231107.4	Tax_Id=10116 Parathymosin	1.13±0.19	1.13±0.19
unk IP IP100387771.5	Tax_Id=10116 Peptidyl-prolyl cis-trans isomerase A	0.94±0.10	0.90±0.07
unk IP IP100201561.2	Tax_Id=10116 Peroxiredoxin-2	1.16 ± 0.18	0.86 ± 0.07
unk IP IP100231260.4	Tax_Id=10116 Peroxiredoxin-6	1.06±0.06	1.04±0.05

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unq PI:IP 00230937.4	Tax_Id=10116 Phosphatidylethanolamine-binding protein 1	0.89±0.13	1.05±0.24
unq PI:IP 00421428.6	Tax_Id=10116 Phosphoglycerate mutase 1	1.04±0.11	1.04±0.19
unq PI:IP 00231506.4	Tax_Id=10116 Phosphoglycerate mutase 2	1.11	1.10
unq PI:IP 00331919.4	Tax_Id=10116 Phosphoserine aminotransferase 1	0.99±0.04	0.99±0.07
unq PI:IP 00188112.1	Tax_Id=10116 Phosphoserine phosphatase	0.85 ± 0.17	0.97 ± 0.02
unq PI:IP 00210234.4	Tax_Id=10116 Plastin-3	1.04± 0.04	0.88 ± 0.04
unq PI:IP 00207333.1	Tax_Id=10116 Platelet-activating factor acetylhydrolase IB subunit gamma	1.05	1.12
unq PI:IP 00209000.2	Tax_Id=10116 Plectin 6	1.01±0.08	1.03±0.04
unq PI:IP 00464805.2	Tax_Id=10116 Poly(RC) binding protein 2	0.99±0.07	0.93±0.19
unq PI:IP 00189074.3	Tax_Id=10116 Polyadenylate-binding protein 1	1.13 ± 0.05	1.08 ± 0.17
unq PI:IP 00370382.2	Tax_Id=10116 PREDICTED: similar to 26S proteasome non-A TPase regulatory subunit 11	0.85±0.19	0.90±0.13
unq PI:IP 00202283.1	Tax_Id=10116 PREDICTED: similar to 26S proteasome non-A TPase regulatory subunit 13	1.01±0.15	1.06±0.15
unq PI:IP 00194471.1	Tax_Id=10116 PREDICTED: similar to 26S proteasome non-A TPase regulatory subunit 5	0.90±0.21	1.12±0.12
unq PI:IP 00359611.2	Tax_Id=10116 PREDICTED: similar to 26S proteasome non-A TPase regulatory subunit 7	0.95±0.09	0.98±0.04
unq PI:IP 00393643.4	Tax_Id=10116 PREDICTED: similar to 40S ribosomal protein S16	0.89±0.13	1.07±0.18
unq PI:IP 00211963.1	Tax_Id=10116 PREDICTED: similar to 60S acidic ribosomal protein P1	1.07±0.20	1.05±0.09
unq PI:IP 00563632.2	Tax_Id=10116 PREDICTED: similar to 60S ribosomal protein L21	1.23±0.26	1.09±0.10
unq PI:IP 00561668.2	Tax_Id=10116 PREDICTED: similar to 60S ribosomal protein L23a	1.06±0.21	1.08±0.07
unq PI:IP 00767436.1	Tax_Id=10116 PREDICTED: similar to 60S ribosomal protein L29	0.89±0.14	1.02±0.08
unq PI:IP 00358566.3	Tax_Id=10116 PREDICTED: similar to 60S ribosomal protein L29	1.16±0.13	1.00±0.05
unq PI:IP 00764150.1	Tax_Id=10116 PREDICTED: similar to 60S ribosomal protein L5	0.92±0.10	0.75±0.36
unq PI:IP 00764461.1	Tax_Id=10116 PREDICTED: similar to Actin, cytoplasmic 2	0.94±0.04	0.96±0.07
unq PI:IP 00763240.1	Tax_Id=10116 PREDICTED: similar to Acyl carrier protein, mitochondrial precursor	0.70	0.91
unq PI:IP 00769072.2	Tax_Id=10116 PREDICTED: similar to AHNK nucleoprotein isoform 1 isoform 2	0.95±0.08	0.94±0.10
unq PI:IP 00363563.3	Tax_Id=10116 PREDICTED: similar to alanyl-tRNA synthetase	1.07±0.18	1.07±0.13
unq PI:IP 00767147.1	Tax_Id=10116 PREDICTED: similar to Alpha-enolase	1.33	1.14
unq PI:IP 00765430.1	Tax_Id=10116 PREDICTED: similar to AP-2 complex subunit alpha-1	1.17±0.20	0.99±0.09
unq PI:IP 00765725.1	Tax_Id=10116 PREDICTED: similar to arsA (bacterial) arsenite transporter, A TP-binding, homolog 1	1.02±0.19	0.98±0.03
unq PI:IP 00768966.1	Tax_Id=10116 PREDICTED: similar to A TP synthase, H+ transporting, mitochondrial F0 complex, subunit	0.92±0.19	0.97±0.14

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unk PI:PI00213162.1	Tax_Id=10116 PREDICTED: similar to ATP-binding cassette, sub-family F (GCN20), member 2	0.91±0.07	1.06±0.12
unk PI:PI00202900.2	Tax_Id=10116 PREDICTED: similar to bcl2-associated X protein isoform 2	1.15±0.24	1.20±0.26
unk PI:PI00197362.2	Tax_Id=10116 PREDICTED: similar to calmodulin 1	0.95	0.86
unk PI:PI00769261.1	Tax_Id=10116 PREDICTED: similar to carbamoyl-phosphate synthetase 2, aspartate transcarbamylase	0.90±0.12	0.96±0.06
unk PI:PI00764098.1	Tax_Id=10116 PREDICTED: similar to CGI8769-PB, isoform B	0.92±0.05	0.91±0.21
unk PI:PI00210938.3	Tax_Id=10116 PREDICTED: similar to CTP synthase	0.96±0.16	0.91±0.09
unk PI:PI00767474.1	Tax_Id=10116 PREDICTED: similar to DEAD box polypeptide 17 isoform p82	0.93±0.16	0.94±0.16
unk PI:PI00210326.4	Tax_Id=10116 PREDICTED: similar to DNA replication licensing factor MCM2	0.97±0.20	1.08±0.07
unk PI:PI00358911.2	Tax_Id=10116 PREDICTED: similar to DNA replication licensing factor MCM6	0.88±0.16	0.95±0.20
unk PI:PI00364607.2	Tax_Id=10116 PREDICTED: similar to Embryonal Fyn-associated substrate	0.89	0.94
unk PI:PI00373011.3	Tax_Id=10116 PREDICTED: similar to Epithelial protein lost in neoplasm	1.16±0.21	1.07±0.17
unk PI:PI00476899.1	Tax_Id=10116 PREDICTED: similar to eukaryotic translation elongation factor 1 beta 2	0.89±0.22	0.95±0.15
unk PI:PI00188016.1	Tax_Id=10116 PREDICTED: similar to eukaryotic translation initiation factor 2, subunit 3, structural	1.04±0.21	1.10±0.12
unk PI:PI00765015.1	Tax_Id=10116 PREDICTED: similar to eukaryotic translation initiation factor 3, subunit 10 theta, 150	1.07±0.13	0.97±0.06
unk PI:PI00763549.1	Tax_Id=10116 PREDICTED: similar to exportin 5	1.05±0.07	0.98±0.24
unk PI:PI00767873.1	Tax_Id=10116 PREDICTED: similar to Fascin (Singed-like protein) isoform 2	1.00±0.19	0.99±0.24
unk PI:PI00409539.3	Tax_Id=10116 PREDICTED: similar to Filamin-A	0.97±0.04	0.98±0.06
unk PI:PI00373752.3	Tax_Id=10116 PREDICTED: similar to Filamin-B	0.95±0.06	0.99±0.05
unk PI:PI00767268.1	Tax_Id=10116 PREDICTED: similar to Filamin-C	0.85 ± 0.16	0.99± 0.06
unk PI:PI00767393.1	Tax_Id=10116 PREDICTED: similar to FK506-binding protein 4	0.87±0.19	0.89±0.15
unk PI:PI00769218.1	Tax_Id=10116 PREDICTED: similar to glyceraldehyde-3-phosphate dehydrogenase	1.01±0.11	1.07±0.09
unk PI:PI00364262.3	Tax_Id=10116 PREDICTED: similar to glycyl-tRNA synthetase	0.93±0.15	1.18±0.36
unk PI:PI00768214.1	Tax_Id=10116 PREDICTED: similar to H2A histone family, member V isoform 1	0.83 ± 0.25	0.94 ± 0.07
unk PI:PI00358211.3	Tax_Id=10116 PREDICTED: similar to Heterogeneous nuclear ribonucleoproteins A2/B1	1.05±0.12	1.00±0.07
unk PI:PI00769230.1	Tax_Id=10116 PREDICTED: similar to High mobility group protein 1	0.97±0.08	1.06±0.07
unk PI:PI00566481.2	Tax_Id=10116 PREDICTED: similar to histone 2, H2ac	1.00±0.19	0.95±0.14
unk PI:PI00559107.2	Tax_Id=10116 PREDICTED: similar to Histone H2A type 1	0.99±0.04	1.02±0.10
unk PI:PI00372796.5	Tax_Id=10116 PREDICTED: similar to Mitochondrial carrier homolog 2	1.10±0.17	1.19±0.23
unk PI:PI00388232.4	Tax_Id=10116 PREDICTED: similar to mitochondrial phosphoenolpyruvate carboxykinase 2	1.04±0.06	1.18±0.24

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg. S:C (±SD)	Avg. R:C (±SD)
unk PI :PI00198711.1	Tax_ Id=10116 PREDICTED: similar to mitochondrial ribosomal protein L53	0.92±0.13	0.77±0.27
unk PI :PI00368180.3	Tax_ Id=10116 PREDICTED: similar to myoferlin isoform b	0.89±0.18	0.94±0.05
unk PI :PI00766113.1	Tax_ Id=10116 PREDICTED: similar to Nascent polypeptide-associated complex alpha subunit	0.97±0.04	1.15±0.16
unk PI :PI00767277.1	Tax_ Id=10116 PREDICTED: similar to NonO/p54nrb homolog	1.45 ± 0.24	1.06 ± 0.13
unk PI :PI00372910.2	Tax_ Id=10116 PREDICTED: similar to Phosphoglycerate kinase 1	1.11±0.13	0.99±0.06
unk PI :PI00201300.1	Tax_ Id=10116 PREDICTED: similar to Polymerase I and transcript release factor	0.89±0.19	0.89±0.17
unk PI :PI00369683.2	Tax_ Id=10116 PREDICTED: similar to Pre-mRNA-processing-splicing factor 8	1.14±0.13	1.09±0.18
unk PI :PI00230992.5	Tax_ Id=10116 PREDICTED: similar to proteasome (prosome, macropain) subunit, beta type 5	1.18±0.20	1.09±0.12
unk PI :PI00189203.3	Tax_ Id=10116 PREDICTED: similar to Protein C10orf70	1.01±0.09	0.95±0.15
unk PI :PI00767073.1	Tax_ Id=10116 PREDICTED: similar to Protein KIAA0690	0.95±0.13	0.92±0.18
unk PI :PI00196210.3	Tax_ Id=10116 PREDICTED: similar to Putative ATP-dependent RNA helicase P110	1.07±0.08	1.13±0.17
unk PI :PI00372524.3	Tax_ Id=10116 PREDICTED: similar to pyrroline-5-carboxylate synthetase isoform 2	0.85±0.19	0.86±0.16
unk PI :PI00764193.1	Tax_ Id=10116 PREDICTED: similar to pyruvate kinase 3	0.95±0.05	0.97±0.04
unk PI :PI00373722.3	Tax_ Id=10116 PREDICTED: similar to RAN binding protein 5	0.98±0.16	1.03±0.09
unk PI :PI00365279.3	Tax_ Id=10116 PREDICTED: similar to RCC1-like	0.98±0.24	0.89±0.16
unk PI :PI00368073.6	Tax_ Id=10116 PREDICTED: similar to retinoblastoma binding protein 4	1.00±0.13	0.99±0.09
unk PI :PI00769113.1	Tax_ Id=10116 PREDICTED: similar to Rho-related GTP-binding protein RhoC precursor	1.06±0.13	1.01±0.07
unk PI :PI00211767.1	Tax_ Id=10116 PREDICTED: similar to ribosomal protein L10	0.95±0.20	1.06±0.10
unk PI :PI00368863.4	Tax_ Id=10116 PREDICTED: similar to ribosomal protein L19	1.02±0.12	1.14±0.15
unk PI :PI00765635.1	Tax_ Id=10116 PREDICTED: similar to ribosomal protein L27	0.89±0.13	1.08±0.15
unk PI :PI00361677.1	Tax_ Id=10116 PREDICTED: similar to ribosomal protein L31	0.95±0.07	0.99±0.03
unk PI :PI00367641.2	Tax_ Id=10116 PREDICTED: similar to ribosomal protein S17	0.94±0.15	0.92±0.07
unk PI :PI00210964.5	Tax_ Id=10116 PREDICTED: similar to ribosomal protein S5	1.05±0.07	1.01±0.04
unk PI :PI00767907.1	Tax_ Id=10116 PREDICTED: similar to RNA binding motif protein 25	1.16±0.17	1.12±0.14
unk PI :PI00367437.4	Tax_ Id=10116 PREDICTED: similar to RNA binding motif protein 3	0.91±0.12	0.91±0.07
unk PI :PI00372727.3	Tax_ Id=10116 PREDICTED: similar to SEC24 related gene family, member B	1.24±0.27	1.17±0.13
unk PI :PI00369060.2	Tax_ Id=10116 PREDICTED: similar to small nuclear ribonucleoparticle-associated protein	1.15±0.18	1.24±0.27
unk PI :PI00366952.3	Tax_ Id=10116 PREDICTED: similar to splicing factor 3b, subunit 1 isoform 1	0.98±0.10	1.04±0.13
unk PI :PI00553921.2	Tax_ Id=10116 PREDICTED: similar to Splicing factor, arginine/serine-rich 3	1.03±0.16	0.98±0.10

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unk IP IP 00370629.3	Tax_Id=10116 PREDICTED: similar to TATA-binding protein-associated factor 172	1.06±0.18	1.04±0.24
unk IP IP 00364286.3	Tax_Id=10116 PREDICTED: similar to T-complex protein 1 subunit eta	0.96±0.06	1.00±0.07
unk IP IP 00763263.2	Tax_Id=10116 PREDICTED: similar to THO complex subunit 4 (Tho4)	1.25	9.24
unk IP IP 00768412.1	Tax_Id=10116 PREDICTED: similar to transcription elongation factor B (SII), polypeptide 1	0.98±0.22	1.00±0.18
unk IP IP 00763269.1	Tax_Id=10116 PREDICTED: similar to Transcription intermediary factor 1-beta	0.93±0.08	1.03±0.24
unk IP IP 00363210.4	Tax_Id=10116 PREDICTED: similar to Treacle protein	1.04±0.19	1.08±0.10
unk IP IP 00371236.3	Tax_Id=10116 PREDICTED: similar to Tu translation elongation factor, mitochondrial	0.95±0.21	0.95±0.16
unk IP IP 00339167.3	Tax_Id=10116 PREDICTED: similar to Tubulin alpha-2 chain	0.93±0.04	1.08±0.05
unk IP IP 00557167.2	Tax_Id=10116 PREDICTED: similar to Ubiquitin-conjugating enzyme E2 L3	0.86	0.84
unk IP IP 00371106.2	Tax_Id=10116 PREDICTED: similar to vacuolar protein sorting 29 isoform 2	1.04±0.16	0.94±0.06
unk IP IP 00365286.3	Tax_Id=10116 PREDICTED: similar to Vinculin	1.16±0.16	1.03±0.05
unk IP IP 00768849.1	Tax_Id=10116 PREDICTED: similar to voltage-dependent anion channel 1	0.94±0.24	0.95±0.16
unk IP IP 00371656.3	Tax_Id=10116 PREDICTED: similar to Zinc finger SWIM domain containing protein 4	1.04±0.05	1.06±0.16
unk IP IP 00231358.5	Tax_Id=10116 Profilin-1	0.96±0.12	0.90±0.06
unk IP IP 00190557.2	Tax_Id=10116 Prohibitin-2	1.07±0.16	0.87±0.20
unk IP IP 00200861.1	Tax_Id=10116 Proliferating cell nuclear antigen	0.98±0.07	1.03±0.12
unk IP IP 00371270.1	Tax_Id=10116 Proliferation-associated 2G4, 38kDa	0.98±0.04	0.90±0.12
unk IP IP 00191748.3	Tax_Id=10116 Proteasome subunit alpha type 1	1.04±0.18	1.14±0.15
unk IP IP 00188584.1	Tax_Id=10116 Proteasome subunit beta type 2	0.75	0.97
unk IP IP 00781454.1	Tax_Id=10116 Protein	0.92±0.16	0.86±0.16
unk IP IP 00365999.1	Tax_Id=10116 Protein DEK	0.93±0.18	1.10±0.06
unk IP IP 00324741.2	Tax_Id=10116 Protein disulfide-isomerase A3 precursor	0.93±0.13	0.97±0.09
unk IP IP 00198887.1	Tax_Id=10116 Protein disulfide-isomerase precursor	0.94±0.10	1.00±0.08
unk IP IP 00230921.8	Tax_Id=10116 Protein S100-A10	0.95±0.18	0.91±0.07
unk IP IP 00554148.1	Tax_Id=10116 Protein S100-A11	0.85 ± 0.10	0.91 ± 0.06
unk IP IP 00204818.2	Tax_Id=10116 Protein S100-A6	0.92±0.13	0.93±0.10
unk IP IP 00358313.1	Tax_Id=10116 Proteolipid protein 2	0.99±0.09	0.90±0.11
unk IP IP 00209095.2	Tax_Id=10116 Prpsap1 protein	0.90±0.14	0.94±0.17
unk IP IP 00366221.1	Tax_Id=10116 RAB21, member RAS oncogene family	0.75	0.86

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S.C (±SD)	Avg.R.C (±SD)
unk PI:PI00422092.1	Tax_Id=10116 Ras-related C3 botulinum toxin substrate 1 precursor	1.02±0.09	1.01±0.16
unk PI:PI00363395.2	Tax_Id=10116 Ras-related protein Rap-1b precursor	1.07±0.10	1.10±0.16
unk PI:PI00196994.1	Tax_Id=10116 Rho GDP dissociation inhibitor (GDI) alpha	0.94±0.04	1.00±0.09
unk PI:PI00231303.4	Tax_Id=10116 Ribose-phosphate pyrophosphokinase I-like	1.25	1.34
unk PI:PI00779353.1	Tax_Id=10116 Ribosomal protein L11	0.87±0.16	1.01±0.08
unk PI:PI00195798.1	Tax_Id=10116 RPOP	0.95±0.11	0.93±0.15
unk PI:PI00421904.1	Tax_Id=10116 RTN3-A	1.14±0.16	1.12±0.14
unk PI:PI00195109.3	Tax_Id=10116 Serine hydroxymethyl transferase 2	0.93±0.10	0.95±0.12
unk PI:PI00360511.2	Tax_Id=10116 Short coiled-coil protein	0.93±0.15	0.83±0.25
unk PI:PI00763796.1	Tax_Id=10116 Similar to plastin-3	1.06±0.06	0.63 ± 0.15
unk PI:PI00209115.2	Tax_Id=10116 Skc25a3 protein	1.18 ± 0.11	1.12 ± 0.04
unk PI:PI00325260.4	Tax_Id=10116 Small glutamine-rich tetratricopeptide repeat-containing protein A	0.92±0.10	0.92±0.09
unk PI:PI00198369.2	Tax_Id=10116 Sorting nexin-1	0.95±0.12	1.16±0.21
unk PI:PI00210090.3	Tax_Id=10116 SP120	0.97±0.03	0.95±0.06
unk PI:PI00363265.2	Tax_Id=10116 Stress-70 protein, mitochondrial precursor	1.06±0.10	0.99±0.07
unk PI:PI00213013.1	Tax_Id=10116 Stress-induced-phosphoprotein 1	0.89±0.10	0.88±0.13
unk PI:PI00197555.5	Tax_Id=10116 Succinyl-CoA ligase [GDP-forming] subunit alpha, mitochondrial precursor	1.06±0.18	1.08±0.07
unk PI:PI00195160.1	Tax_Id=10116 Sulfated glycoprotein 1 precursor	0.93±0.07	0.95±0.09
unk PI:PI00200847.1	Tax_Id=10116 T-complex protein 1 subunit alpha	0.90±0.21	0.90±0.09
unk PI:PI00337168.4	Tax_Id=10116 T-complex protein 1 subunit delta	0.91±0.07	0.93±0.07
unk PI:PI00231368.4	Tax_Id=10116 Thioredoxin	1.25 ± 0.12	0.92 ± 0.11
unk PI:PI00365929.1	Tax_Id=10116 thioredoxin domain containing 7	1.07±0.20	1.07±0.07
unk PI:PI00362014.5	Tax_Id=10116 Thn1 protein	0.99±0.09	1.04±0.12
unk PI:PI00555171.2	Tax_Id=10116 Transgelin 2	0.96±0.08	0.96±0.11
unk PI:PI00212014.2	Tax_Id=10116 Transitional endoplasmic reticulum ATPase	1.01±0.11	0.94±0.13
unk PI:PI00231139.6	Tax_Id=10116 transketolase	0.89±0.15	0.76±0.32
unk PI:PI00358374.1	Tax_Id=10116 Transmembrane protein 43	0.92±0.19	1.08±0.15
unk PI:PI00231767.4	Tax_Id=10116 Triosephosphate isomerase	0.99±0.06	0.91±0.21
unk PI:PI00210941.1	Tax_Id=10116 Tropomyosin	0.91±0.07	0.97±0.17

Continued

Supplementary Table 1 Continued

Accession	Protein Name	Avg.S:C (±SD)	Avg.R:C (±SD)
unk PI:PI00214905.2	Tax_Id=10116 Tropomyosin alpha-4 chain	1.10±0.16	0.94±0.14
unk PI:PI00189795.1	Tax_Id=10116 Tubulin alpha-1 chain	0.90±0.15	1.13±0.16
unk PI:PI00362160.1	Tax_Id=10116 Tubulin beta-3 chain	0.86 ± 0.11	0.83 ± 0.08
unk PI:PI00197579.1	Tax_Id=10116 Tubulin beta-5 chain	0.90±0.06	0.97±0.05
unk PI:PI00361693.1	Tax_Id=10116 Twinfilin-1	0.88±0.16	0.95±0.10
unk PI:PI00454559.2	Tax_Id=10116 Txnrd1 protein	1.12±0.23	1.04±0.19
unk PI:PI00369093.1	Tax_Id=10116 Ubiquinol-cytochrome c reductase complex 11 kDa protein, mitochondrial precursor	0.96±0.10	0.92±0.21
unk PI:PI00188924.3	Tax_Id=10116 Ubiquinol-cytochrome-c reductase complex core protein 2, mitochondrial precursor	1.02±0.17	0.97±0.05
unk PI:PI00734588.1	Tax_Id=10116 Ubiquitin carboxyl-terminal hydrolase L5	1.15±0.13	0.98±0.12
unk PI:PI00190559.3	Tax_Id=10116 Ubiquitin-conjugating enzyme E2 N	1.10±0.11	0.91±0.10
unk PI:PI00230941.4	Tax_Id=10116 Vimentin	0.96±0.05	0.97±0.07
unk PI:PI00366523.3	Tax_Id=10116 WD repeat protein 61	1.02±0.06	1.07±0.17
unk PI:PI00421591.1	Tax_Id=10116 X-ray repair complementing defective repair in Chinese hamster cells 5	0.92±0.06	0.97±0.17

Appendix II: Supplementary table 2 for the identified 323 proteins (score>2) in A7r5 cells incubated with atenolol

Accession	Name	Avg.S:C (±SD)	Avg.R:C (±SD)
P26772 CH10_RAT	10 kDa heat shock protein, mitochondrial	0.99±0.06	1.00±0.07
P62260 1433E_RAT	14-3-3 protein epsilon	0.93±0.12	0.98±0.06
P68511 1433F_RAT	14-3-3 protein eta	1.17±0.15	1.02±0.11
P61983 1433G_RAT	14-3-3 protein gamma	0.92±0.23	1.02±0.11
P68255 1433T_RAT	14-3-3 protein theta	0.99±0.11	1.13±0.08
P63102 1433Z_RAT	14-3-3 protein zeta/delta	1.21±0.11	1.02±0.06
Q63617 OXRP_RAT	150 kDa oxygen-regulated protein precursor	0.95±0.15	1.00±0.17
P62193 PRS4_RAT	26S protease regulatory subunit 4	1.19±0.15	1.03±0.09
Q63347 PRS7_RAT	26S protease regulatory subunit 7	1.19±0.26	1.03±0.07
P62198 PRS8_RAT	26S protease regulatory subunit 8	1.08±0.10	0.98±0.25
O88761 PSMD1_RAT	26S proteasome non-ATPase regulatory subunit 1	1.06±0.10	0.92±0.06
P13437 THIM_RAT	3-ketoacyl-CoA thiolase, mitochondrial	0.99±0.11	0.93±0.09
Q6DGG0 PPID_RAT	40 kDa peptidyl-prolyl cis-trans isomerase	1.06±0.19	1.09±0.12
P62278 RS13_RAT	40S ribosomal protein S13	0.89±0.14	0.92±0.16
P13471 RS14_RAT	40S ribosomal protein S14	0.97±0.09	1.44±0.61
P62845 RS15_RAT	40S ribosomal protein S15	0.89±0.07	0.94±0.11
P04644 RS17_RAT	40S ribosomal protein S17	0.96±0.07	1.00±0.08
P62271 RS18_RAT	40S ribosomal protein S18	0.97±0.09	0.91±0.10
P17074 RS19_RAT	40S ribosomal protein S19	1.08±0.07	1.06±0.10
P27952 RS2_RAT	40S ribosomal protein S2	0.96±0.12	1.00±0.10
P60868 RS20_RAT	40S ribosomal protein S20	0.92±0.10	1.00±0.09
P05765 RS21_RAT	40S ribosomal protein S21	0.97±0.14	0.96±0.08
P62909 RS3_RAT	40S ribosomal protein S3	1.29±0.31	1.01±0.08
P62755 RS6_RAT	40S ribosomal protein S6	1.06±0.17	0.87±0.08
P62083 RS7_RAT	40S ribosomal protein S7	1.17±0.15	0.98±0.06
P62243 RS8_RAT	40S ribosomal protein S8	0.85±0.09	0.97±0.10
P29314 RS9_RAT	40S ribosomal protein S9	0.92±0.09	1.09±0.09
Q794F9 4F2_RAT	4F2 cell-surface antigen heavy chain	0.85±0.23	0.98±0.19

Continued

Supplementary Table 2 Continued

Accession	Name	Avg. S:C (±SD)	Avg. R:C (±SD)
P63039 CH60_RAT	60 kDa heat shock protein, mitochondrial precursor	0.95±0.09	0.96±0.07
P19945 RLA0_RAT	60S acidic ribosomal protein P0	1.06±0.13	0.99±0.07
P02401 RLA2_RAT	60S acidic ribosomal protein P2	1.21±0.20	1.00±0.07
Q6PDV7 RL10_RAT	60S ribosomal protein L10	0.93±0.07	1.02±0.06
P62914 RL11_RAT	60S ribosomal protein L11	0.94±0.14	0.96±0.21
P35427 RL13A_RAT	60S ribosomal protein L13a	0.95±0.19	1.13±0.14
Q63507 RL14_RAT	60S ribosomal protein L14	1.25±0.42	1.55±0.60
P61314 RL15_RAT	60S ribosomal protein L15	1.16±0.07	1.17±0.13
P12001 RL18_RAT	60S ribosomal protein L18	1.05±0.15	1.15±0.17
P83732 RL24_RAT	60S ribosomal protein L24	0.94±0.15	1.03±0.11
P61354 RL27_RAT	60S ribosomal protein L27	1.06±0.15	1.07±0.11
P21531 RL3_RAT	60S ribosomal protein L3	0.96±0.12	1.10±0.08
P50878 RL4_RAT	60S ribosomal protein L4	1.14±0.06	1.16±0.05
P09895 RL5_RAT	60S ribosomal protein L5	0.97±0.17	1.11±0.13
P21533 RL6_RAT	60S ribosomal protein L6	1.12±0.13	1.37±0.44
P05426 RL7_RAT	60S ribosomal protein L7	1.05±0.13	1.18±0.10
P62919 RL8_RAT	60S ribosomal protein L8	1.11±0.14	1.18±0.11
P17077 RL9_RAT	60S ribosomal protein L9	1.07±0.09	0.94±0.35
P47858 K6PF_RAT	6-phosphofructokinase, muscle type	1.02±0.16	1.01±0.33
P06761 GRP78_RAT	78 kDa glucose-regulated protein precursor	0.96±0.07	0.99±0.10
Q5X122 THIC_RAT	Acetyl-CoA acetyltransferase, cytosolic	1.11±0.07	1.02±0.27
Q9EST6 AN32B_RAT	Acidic leucine-rich nuclear phosphoprotein 32 family member B	1.11±0.43	1.07±0.12
Q5XIE0 AN32E_RAT	Acidic leucine-rich nuclear phosphoprotein 32 family member E	1.12±0.10	0.97±0.12
Q9ER34 ACON_RAT	Aconitate hydratase, mitochondrial precursor	1.01±0.11	1.02±0.09
P62738 ACTA_RAT	Actin, aortic smooth muscle	0.90±0.05	1.07±0.09
P63259 ACTG_RAT	Actin, cytoplasmic 2	1.03±0.11	1.04±0.05
Q63396 TCP4_RAT	Activated RNA polymerase II transcriptional coactivator p15 precursor	0.88±0.16	0.94±0.07
P11030 ACBP_RAT	Acyl-CoA-binding protein	1.07±0.12	1.07±0.19

Continued

Supplementary Table 2 Continued

Accession	Name	Avg S:C (±SD)	Avg R:C (±SD)
P10760 SAHH_RAT	Adenosylhomocysteinase	0.92±0.11	0.99±0.29
Q08163 CAPI_RAT	Adenyl cyclase-associated protein 1	0.95±0.11	0.96±0.05
Q05962 ADT1_RAT	ADP/ATP translocase 1	0.89±0.10	1.00±0.07
Q09073 ADT2_RAT	ADP/ATP translocase 2	1.01±0.21	1.02±0.14
P51635 AK1A1_RAT	Alcohol dehydrogenase	0.91±0.18	0.94±0.10
P11884 ALDH2_RAT	Aldehyde dehydrogenase, mitochondrial precursor	1.02±0.06	1.02±0.04
P07943 ALDR_RAT	Aldose reductase	1.00±0.06	1.05±0.07
P23928 CRYAB_RAT	Alpha crystallin B chain	1.22±0.31	1.06±0.11
Q9Z1P2 ACTN1_RAT	Alpha-actinin-1	0.90±0.15	0.97±0.07
Q9QXQ0 ACTN4_RAT	Alpha-actinin-4	1.09±0.09	1.01±0.05
P04764 ENOA_RAT	Alpha-enolase	1.03±0.06	1.02±0.04
Q66H12 NAGAB_RAT	Alpha-N-acetylgalactosaminidase precursor	1.71±0.43	1.08±0.09
P21396 AOFA_RAT	Amine oxidase [flavin-containing] A	1.05±0.22	1.00±0.10
P00762 TRY1_RAT	Anionic trypsin-1 precursor	1.05±0.04	0.89±0.07
P07150 ANXA1_RAT	Annexin A1	0.96±0.09	1.00±0.13
Q07936 ANXA2_RAT	Annexin A2	0.97±0.13	0.98±0.07
P14669 ANXA3_RAT	Annexin A3	0.94±0.14	0.99±0.10
P55260 ANXA4_RAT	Annexin A4	1.04±0.11	1.07±0.12
P14668 ANXA5_RAT	Annexin A5	0.99±0.12	0.98±0.08
P48037 ANXA6_RAT	Annexin A6	0.71±0.11	0.96±0.13
Q63690 BAXA_RAT	Apoptosis regulator BAX, membrane isoform alpha	1.14±0.24	0.86±0.07
P09034 ASSY_RAT	Argininosuccinate synthase	1.02±0.13	1.02±0.09
P49088 ASNS_RAT	Asparagine synthetase	0.89±0.09	0.90±0.06
P13221 AATC_RAT	Aspartate aminotransferase, cytoplasmic	1.17±0.28	0.93±0.10
P00507 AATM_RAT	Aspartate aminotransferase, mitochondrial precursor	1.25±0.13	1.07±0.07
P15178 SYDC_RAT	Aspartyl-tRNA synthetase, cytoplasmic	1.07±0.18	0.99±0.09
P19511 AT5F1_RAT	ATP synthase B chain, mitochondrial precursor	1.03±0.11	1.06±0.06
P35434 ATPD_RAT	ATP synthase delta chain, mitochondrial precursor	1.09±0.07	1.01±0.12

Continued

Supplementary Table 2 Continued

Accession	Name	Avg.S.C (±SD)	Avg.R.C (±SD)
Q06647 A_TPO_RAT	ATP synthase O subunit, mitochondrial precursor	1.07±0.11	1.05±0.07
P15999 A_TPA_RAT	ATP synthase subunit alpha, mitochondrial precursor	0.94±0.11	1.03±0.07
P10719 A_TPB_RAT	ATP synthase subunit beta, mitochondrial precursor	0.97±0.05	1.03±0.05
P16638 A_CLY_RAT	ATP-citrate synthase	1.06±0.11	1.02±0.05
Q5U216 DDX39_RAT	ATP-dependent RNA helicase DDX39	1.09±0.11	1.02±0.08
P15429 ENOB_RAT	Beta-enolase	1.02±0.06	1.00±0.05
O35567 PUR9_RAT	Bifunctional purine biosynthesis protein PURH	0.98±0.09	0.98±0.06
Q05175 BASP_RAT	Brain acid soluble protein 1	1.33±0.54	0.91±0.20
Q9WU49 CHSP1_RAT	Calcium-regulated heat stable protein 1	1.09±0.11	1.15±0.14
P62161 CALM_RAT	Calmodulin	0.68±0.16	0.84±0.17
Q64537 CPNS1_RAT	Calpain small subunit 1	1.12±0.26	0.88±0.15
P37397 CNN3_RAT	Calponin-3	0.98±0.06	1.00±0.07
P18418 CALR_RAT	Calreticulin precursor	0.99±0.06	1.00±0.09
P14141 CAH3_RAT	Carbonic anhydrase 3	1.02±0.27	1.13±0.18
Q9WU82 CTNBI_RAT	Catenin beta-1	0.90±0.10	1.00±0.06
P39951 CDC2_RAT	Cell division control protein 2 homolog	0.93±0.06	0.97±0.05
Q8CFN2 CDC42_RAT	Cell division control protein 42 homolog precursor	0.98±0.09	1.03±0.06
P62634 CNBP_RAT	Cellular nucleic acid-binding protein	0.91±0.15	0.86±0.11
Q8VHF5 CISY_RAT	Citrate synthase, mitochondrial precursor	1.00±0.12	1.00±0.05
P11442 CLH_RAT	Clathrin heavy chain	0.99±0.09	1.04±0.18
O35820 RCL_RAT	c-Myc-responsive protein Rcl	1.12±0.17	1.14±0.10
P45592 COF1_RAT	Cofilin-1	0.89±0.07	0.93±0.06
O35796 CIQBP_RAT	Complement component 1 Q subcomponent-binding protein, mitochondrial precursor	1.02±0.08	1.00±0.08
O89046 COORB_RAT	Coronin-1B (Coronin-2)	0.86±0.36	0.92±0.25
P97536 CAND1_RAT	Cullin-associated NEDD8-dissociated protein 1	1.23±0.31	1.08±0.11
P18757 CGL_RAT	Cystathionine gamma-lyase	0.94±0.35	1.10±0.22
Q62908 CSRP2_RAT	Cysteine and glycine-rich protein 2	1.00±0.08	0.96±0.13
P36201 CRIP2_RAT	Cysteine-rich protein 2	0.77±0.41	1.17±0.15

Continued

Supplementary Table 2 Continued

Accession	Name	Avg S:C (±SD)	Avg R:C (±SD)
P11240 COX5A_RAT	Cytochrome c oxidase subunit 5A, mitochondrial precursor	1.12±0.07	1.07±0.15
P62898 CYC_RAT	Cytochrome c, somatic	0.99±0.07	0.99±0.08
Q68FS4 AMPL_RAT	Cytosol aminopeptidase	0.93±0.16	1.01±0.06
O08651 SERA_RAT	D-3-phosphoglycerate dehydrogenase	0.99±0.16	0.98±0.09
P48675 DESM_RAT	Desmin	1.03±0.11	1.05±0.08
Q6P6R2 DLDH_RAT	Dihydrolipoyl dehydrogenase, mitochondrial precursor	0.96±0.41	0.99±0.15
Q62724 MCM6_RAT	DNA replication licensing factor MCM6	1.05±0.12	1.03±0.09
P41516 TOP2A_RAT	DNA topoisomerase 2-alpha	0.97±0.18	0.91±0.10
O35824 DNJA2_RAT	DnaJ homolog subfamily A member 2	1.07±0.10	1.02±0.08
P25235 RIB2_RAT	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 63 kDa subunit precursor	0.99±0.14	0.95±0.09
P07153 RIB1_RAT	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 67 kDa subunit precursor	1.13±0.19	0.97±0.08
P28023 DYNA_RAT	Dynactin-1	0.96±0.21	0.96±0.07
O35303 DNM1L_RAT	Dynammin-1-like protein	0.87±0.13	0.93±0.15
P39052 DYN2_RAT	Dynammin-2	1.14±0.09	1.03±0.07
P38650 DYHC_RAT	Dynein heavy chain, cytosolic	1.04±0.12	0.99±0.11
P13803 ETFA_RAT	Electron transfer flavoprotein subunit alpha, mitochondrial precursor	0.92±0.09	0.99±0.21
P62630 EF1A1_RAT	Elongation factor 1-alpha 1	0.97±0.06	0.98±0.08
Q68FR6 EF1G_RAT	Elongation factor 1-gamma	0.93±0.06	0.98±0.04
P05197 EF2_RAT	Elongation factor 2	0.97±0.06	0.98±0.04
P14604 ECHM_RAT	Enoyl-CoA hydratase, mitochondrial precursor	0.96±0.17	0.93±0.19
P07687 HYEP_RAT	Epoxide hydrolase 1	1.14±0.36	1.17±0.13
Q5RK11 IF4A2_RAT	Eukaryotic initiation factor 4A-II	0.98±0.08	1.04±0.06
P68101 IF2A_RAT	Eukaryotic translation initiation factor 2 subunit 1	0.93±0.19	1.04±0.10
Q4C061 IF39_RAT	Eukaryotic translation initiation factor 3 subunit 9	1.06±0.09	0.91±0.22
Q3T1J1 IF5A1_RAT	Eukaryotic translation initiation factor 5A-1	0.88±0.06	0.94±0.04
Q80U96 XPO1_RAT	Exportin-1	0.96±0.08	0.98±0.09
P31977 EZRI_RAT	Ezrin	1.12±0.15	0.89±0.14
Q04931 SSRP1_RAT	FACT complex subunit SSRP1	1.06±0.16	0.95±0.08

Continued

Supplementary Table 2 Continued

Accession	Name	Avg.S:C (±SD)	Avg.R:C (±SD)
Q99PF5 FUBP2_RAT	Far upstream element-binding protein 2	1.04±0.21	1.11±0.12
P12785 FAS_RAT	Fatty acid synthase	1.05±0.08	1.00±0.15
Q9WUH4 FHL1_RAT	Four and a half LIM domains protein 1	0.96±0.14	1.00±0.07
P05065 ALDOA_RAT	Fructose-bisphosphate aldolase A	1.11±0.10	1.09±0.08
P11762 LEGI_RAT	Galectin-1	0.98±0.08	0.93±0.05
P08699 LEG3_RAT	Galectin-3	1.08±0.06	1.15±0.11
P05370 G6PD_RAT	Glucose-6-phosphate 1-dehydrogenase	0.86±0.17	0.93±0.09
P10860 DHE3_RAT	Glutamate dehydrogenase 1, mitochondrial precursor	1.09±0.10	1.05±0.06
P04041 GPX1_RAT	Glutathione peroxidase 1	0.93±0.13	1.03±0.13
P04906 GSTP1_RAT	Glutathione S-transferase P	1.35±0.15	1.16±0.10
Q9Z339 GSTO1_RAT	Glutathione transferase omega-1	0.92±0.13	0.96±0.07
P04797 G3P_RAT	Glyceraldehyde-3-phosphate dehydrogenase	0.92±0.06	0.97±0.08
Q8K1Q0 NMT1_RAT	Glycylpeptide N-tetradecanoyl transferase 1	0.97±0.26	0.99±0.14
P62828 RAN_RAT	GTP-binding nuclear protein Ran	1.07±0.14	1.00±0.09
P63245 GBLP_RAT	Guanine nucleotide-binding protein subunit beta 2-like 1	1.01±0.09	1.09±0.12
O88600 HSP74_RAT	Heat shock 70 kDa protein 4	0.96±0.20	1.02±0.11
P63018 HSP7C_RAT	Heat shock cognate 71 kDa protein	0.99±0.04	0.98±0.06
P82995 HS90A_RAT	Heat shock protein HSP 90-alpha	0.99±0.08	0.98±0.06
P34058 HS90B_RAT	Heat shock protein HSP 90-beta	1.02±0.05	0.99±0.08
P42930 HSPB1_RAT	Heat-shock protein beta-1	1.00±0.14	0.96±0.07
P04256 ROA1_RAT	Heterogeneous nuclear ribonucleoprotein A1	0.96±0.08	0.95±0.04
Q6URK4 ROA3_RAT	Heterogeneous nuclear ribonucleoprotein A3	0.96±0.14	0.98±0.08
Q9J154 HNRPD_RAT	Heterogeneous nuclear ribonucleoprotein D0	0.91±0.13	0.98±0.06
Q794E4 HNRPF_RAT	Heterogeneous nuclear ribonucleoprotein F	1.11±0.10	0.99±0.05
P61980 HNRPK_RAT	Heterogeneous nuclear ribonucleoprotein K	1.04±0.07	0.99±0.05
Q62826 HNRPM_RAT	Heterogeneous nuclear ribonucleoprotein M	1.19±0.23	1.05±0.11
Q77P47 HNRPQ_RAT	Heterogeneous nuclear ribonucleoprotein Q	1.01±0.23	0.98±0.12
P05708 HXXK1_RAT	Hexokinase-1	1.06±0.23	1.00±0.07

Continued

Supplementary Table 2 Continued

Accession	Name	Avg.S:C (±SD)	Avg.R:C (±SD)
P63159 HMG1_RAT	High mobility group protein B1	1.08±0.07	0.99±0.07
P62959 HINT1_RAT	Histidine triad nucleotide-binding protein 1	1.09±0.14	1.02±0.08
P0C170 H2A1E_RAT	Histone H2A type 1-E	1.06±0.10	0.89±0.06
Q00728 H2A4_RAT	Histone H2A type 4	1.03±0.07	0.84±0.12
P0C0S7 H2AZ_RAT	Histone H2A.Z	0.98±0.07	1.00±0.09
Q00715 H2B1_RAT	Histone H2B type 1	0.91±0.04	0.95±0.03
Q6LED0 H31_RAT	Histone H3.1	0.86±0.12	0.96±0.31
P62804 H4_RAT	Histone H4	0.85±0.05	0.96±0.11
Q71UF4 RBBP7_RAT	Histone-binding protein RBBP7	1.09±0.05	1.05±0.09
P50503 F10A1_RAT	Hsc70-interacting protein	0.89±0.14	0.95±0.10
P27605 HPRT_RAT	Hypoxanthine-guanine phosphoribosyltransferase	1.02±0.08	1.03±0.14
P52296 IMB1_RAT	Importin beta-1 subunit	1.07±0.11	1.06±0.09
P97697 IMPA1_RAT	Inositol monophosphatase	1.09±0.31	1.01±0.24
P56574 IDHP_RAT	Isocitrate dehydrogenase	1.07±0.18	1.02±0.19
Q2PQA9 KINH_RAT	Kinesin heavy chain	1.16±0.26	0.97±0.18
Q6P7Q4 LGJL_RAT	Lactoylglutathione lyase	1.01±0.06	1.08±0.14
P48679 LMNA_RAT	Lamin-A	0.97±0.07	1.00±0.04
P15800 LAMB2_RAT	Laminin subunit beta-2 precursor	1.15±0.22	0.81±0.30
Q5RJR8 LRC59_RAT	Leucine-rich repeat-containing protein 59	0.99±0.10	1.01±0.07
P30349 LKHA4_RAT	Leukotriene A-4 hydrolase	1.15±0.10	1.19±0.16
Q99MZ8 LASPI_RAT	LIM and SH3 domain protein 1	1.00±0.16	1.04±0.12
P04642 LDHA_RAT	L-lactate dehydrogenase A chain	1.12±0.07	0.90±0.08
Q924S5 LONM_RAT	Lon protease homolog, mitochondrial precursor	1.01±0.07	0.99±0.11
Q6AYC4 CAPG_RAT	Macrophage capping protein	0.91±0.08	0.89±0.08
P30904 MIF_RAT	Macrophage migration inhibitory factor	1.15±0.08	1.09±0.06
O88989 MDHC_RAT	Malate dehydrogenase, cytoplasmic	1.05±0.15	1.01±0.08
P04636 MDHM_RAT	Malate dehydrogenase, mitochondrial precursor	1.03±0.10	0.99±0.07
P43244 MATR3_RAT	Matrin-3	1.03±0.18	0.97±0.10

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Supplementary Table 2 Continued

Accession	Name	Avg.S:C (±SD)	Avg.R:C (±SD)
P08503 ACADM_RAT	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	1.18±0.15	1.06±0.11
P38062 AMPM2_RAT	Methionine aminopeptidase 2	0.81±0.24	0.91±0.18
Q62760 TOM20_RAT	Mitochondrial import receptor subunit TOM20 homolog	0.98±0.16	0.98±0.10
P63086 MK01_RAT	Mitogen-activated protein kinase 1	0.92±0.10	0.97±0.11
O35763 MOES_RAT	Moesin	0.86±0.13	0.96±0.10
Q08201 MDR2_RAT	Multidrug resistance protein 2	1.13±0.15	0.94±0.16
O35821 MBB1A_RAT	Myb-binding protein 1A	1.08±0.20	1.02±0.09
Q64119 MYL6_RAT	Myosin light polypeptide 6	0.95±0.04	1.01±0.14
P13832 MLRA_RAT	Myosin regulatory light chain 2-A, smooth muscle isoform	1.02±0.08	0.98±0.11
Q9JLT0 MYH10_RAT	Myosin-10	0.96±0.09	0.99±0.08
Q62812 MYH9_RAT	Myosin-9	0.97±0.03	1.01±0.05
P62775 MTPN_RAT	Myotrophin	1.00±0.25	1.03±0.19
P20070 NCBSR_RAT	NADH-cytochrome b5 reductase	1.65±0.29	1.16±0.19
P21263 NEST_RAT	Nestin	0.80±0.14	0.96±0.15
P55770 NH2L1_RAT	NHP2-like protein 1	1.10±0.19	1.03±0.08
Q62736 CALD1_RAT	Non-muscle caldesmon	1.03±0.13	1.02±0.09
Q5FVM4 NONO_RAT	Non-POU domain-containing octamer-binding protein	1.03±0.12	1.01±0.07
Q63525 NUDC_RAT	Nuclear migration protein nudC	0.86±0.15	0.92±0.07
P62961 YBOX1_RAT	Nuclease sensitive element-binding protein 1	1.23±0.35	1.06±0.09
P13383 NUCL_RAT	Nucleolin	0.94±0.07	0.98±0.12
P13084 NPM_RAT	Nucleophosmin	0.98±0.07	0.99±0.06
Q05982 NDKA_RAT	Nucleoside diphosphate kinase A	1.06±0.13	0.98±0.19
P19804 NDKB_RAT	Nucleoside diphosphate kinase B	1.01±0.10	1.02±0.05
Q9Z2G8 NP1L1_RAT	Nucleosome assembly protein 1-like 1	0.98±0.07	0.99±0.10
Q6P686 OSTF1_RAT	Osteoclast-stimulating factor 1	0.85±0.18	0.92±0.11
Q62920 PDLI5_RAT	PDZ and LIM domain protein 5	0.98±0.08	0.96±0.11
P10111 PP1A_RAT	Peptidyl-l-prolyl cis-trans isomerase A	0.97±0.05	0.98±0.10
Q63716 PRDX1_RAT	Peroxiredoxin-1	1.00±0.07	1.00±0.08

Continued

Supplementary Table 2 Continued

Accession	Name	Avg.S:C (±SD)	Avg.R:C (±SD)
P35704 PRDX2_RAT	Peroxiredoxin-2	1.00±0.08	0.99±0.09
Q9R063 PRDX5_RAT	Peroxiredoxin-5, mitochondrial precursor	0.98±0.19	1.05±0.10
O35244 PRDX6_RAT	Peroxiredoxin-6	1.03±0.13	1.03±0.18
P16036 MPCP_RAT	Phosphate carrier protein, mitochondrial precursor	1.07±0.13	1.03±0.08
P31044 PEBP1_RAT	Phosphatidylethanolamine-binding protein 1	1.03±0.09	0.97±0.06
O55012 PICA_RAT	Phosphatidylinositol-binding clathrin assembly protein	1.04±0.08	0.95±0.15
P07379 PPCKC_RAT	Phosphoenolpyruvate carboxykinase, cytosolic	0.99±0.20	1.11±0.09
P16617 PGK1_RAT	Phosphoglycerate kinase 1	0.98±0.09	0.94±0.06
P25113 PGAM1_RAT	Phosphoglycerate mutase 1	1.01±0.10	1.00±0.13
O08618 KPRB_RAT	Phosphoribosyl pyrophosphate synthetase-associated protein 2	0.94±0.04	0.98±0.23
Q5M819 SERB_RAT	Phosphoserine phosphatase	0.91±0.18	0.96±0.11
Q6AXS5 PAIRB_RAT	Plasminogen activator inhibitor 1 RNA-binding protein	0.96±0.09	0.98±0.14
Q63598 PLST_RAT	Plastin-3	1.01±0.07	1.04±0.15
P30427 PLEC1_RAT	Plectin-1	1.01±0.03	0.98±0.07
Q9EPH8 PABP1_RAT	Polyadenylate-binding protein 1	1.15±0.17	1.05±0.06
Q00438 PTBP1_RAT	Poly pyrimidine tract-binding protein 1	1.10±0.09	0.99±0.06
Q9J141 PRP19_RAT	Pre-mRNA-processing factor 19	1.04±0.04	1.05±0.05
P62963 PROF1_RAT	Profilin-1	0.98±0.07	0.97±0.04
P67779 PHB_RAT	Prohibitin	1.04±0.07	0.96±0.09
Q5X1H7 PHB2_RAT	Prohibitin-2	0.96±0.10	0.95±0.07
P04961 PCNA_RAT	Proliferating cell nuclear antigen	1.07±0.15	1.23±0.33
P18420 PSA1_RAT	Proteasome subunit alpha type 1	1.04±0.16	0.96±0.11
P11598 PDIA3_RAT	Protein disulfide-isomerase A3 precursor	1.01±0.16	0.97±0.10
P38659 PDIA4_RAT	Protein disulfide-isomerase A4 precursor	0.82±0.15	0.89±0.29
Q63081 PDIA6_RAT	Protein disulfide-isomerase A6 precursor	1.11±0.21	1.07±0.11
P04785 PDIA1_RAT	Protein disulfide-isomerase precursor	1.00±0.21	1.02±0.12
O88767 PARK7_RAT	Protein DJ-1	1.05±0.19	1.02±0.13
Q6JE36 NDRG1_RAT	Protein NDRG1	0.89±0.16	0.97±0.13

Continued

Supplementary Table 2 Continued

Accession	Name	Avg.S:C (±SD)	Avg.R:C (±SD)
P05943 S10AA_RAT	Protein S100-A10	0.98±0.10	0.99±0.06
Q6B345 S10AB_RAT	Protein S100-A11	0.81±0.11	0.97±0.09
P05942 S10A4_RAT	Protein S100-A4	0.76±0.10	0.91±0.16
P05964 S10A6_RAT	Protein S100-A6	0.97±0.11	0.98±0.09
Q63945 SET_RAT	Protein SET	0.97±0.09	0.98±0.07
Q6P742 PLP2_RAT	Proteolipid protein 2	0.90±0.11	0.96±0.09
P11980 KPYM_RAT	Pyruvate kinase isozymes M1/M2	0.95±0.07	0.97±0.05
P12928 KPYP_RAT	Pyruvate kinase isozymes R/L	1.26±0.38	1.12±0.16
P50398 GDIA_RAT	Rab GDP dissociation inhibitor alpha	1.20±0.36	1.10±0.11
P50399 GDIB_RAT	Rab GDP dissociation inhibitor beta	0.85±0.15	0.89±0.18
P97680 RIN1_RAT	Ras and Rab interactor 1	1.06±0.08	1.02±0.09
Q6NYB7 RAB1A_RAT	Ras-related protein Rab-1A	0.82±0.37	1.01±0.19
P09527 RAB7_RAT	Ras-related protein Rab-7	0.93±0.17	0.91±0.06
Q62636 RAP1B_RAT	Ras-related protein Rap-1b precursor	1.02±0.06	1.03±0.11
Q9JK11 RTN4_RAT	Reticulon-4	1.05±0.16	1.08±0.13
P09330 PRPS2_RAT	Ribose-phosphate pyrophosphokinase II	0.90±0.10	0.98±0.19
P22509 FBRL_RAT	rRNA 2'-O-methyltransferase fibrillarin	1.16±0.17	1.02±0.07
Q4QQT4 2AAB_RAT	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform	0.85±0.18	1.08±0.07
P29457 SERPH_RAT	Serpin H1 precursor	0.95±0.13	0.96±0.07
P02770 ALBU_RAT	Serum albumin precursor	1.01±0.09	1.07±0.18
Q6P799 SYSC_RAT	Seryl-tRNA synthetase, cytoplasmic	0.97±0.10	1.01±0.05
P28042 SSB_RAT	Single-stranded DNA-binding protein, mitochondrial precursor	1.02±0.09	0.92±0.12
O70593 SGTA_RAT	Small glutamine-rich tetratricopeptide repeat-containing protein A	0.83±0.14	0.91±0.11
Q99N27 SNX1_RAT	Sorting nexin-1	1.17±0.12	1.02±0.09
P16086 SPTA2_RAT	Spectrin alpha chain, brain	1.02±0.12	0.94±0.09
Q6A YL6 SPT18_RAT	Spermatogenesis-associated protein 18	1.03±0.22	1.02±0.09
Q6PDU1 SFRS2_RAT	Splicing factor, arginine/serine-rich 2	0.97±0.21	0.99±0.11
Q09167 SFRS5_RAT	Splicing factor, arginine/serine-rich 5	1.02±0.09	1.02±0.08

Continued

Supplementary Table 2 Continued

Accession	Name	Avg.S.C (±SD)	Avg.R.C (±SD)
Q66X93 SND1_RAT	Staphylococcal nuclease domain-containing protein 1	1.04±0.11	1.09±0.12
P13668 STMN1_RAT	Stathmin	0.91±0.18	0.97±0.10
P48721 GRP75_RAT	Stress-70 protein, mitochondrial precursor	0.97±0.08	0.96±0.09
O35814 STPI1_RAT	Stress-induced-phosphoprotein 1	0.96±0.04	1.00±0.09
Q92012 DHSA_RAT	Succinate dehydrogenase	0.96±0.11	0.95±0.10
P13086 SUCA_RAT	Succinyl-CoA ligase	0.96±0.17	0.96±0.09
P10960 SAP_RAT	Sulfated glycoprotein 1 precursor	1.05±0.17	1.12±0.26
Q07116 SUOX_RAT	Sulfite oxidase, mitochondrial precursor	1.07±0.46	1.10±0.13
P07632 SODC_RAT	Superoxide dismutase	0.99±0.13	1.01±0.08
P28480 TCPA_RAT	T-complex protein 1 subunit alpha	0.97±0.17	1.02±0.20
Q5XIM9 TCPB_RAT	T-complex protein 1 subunit beta	1.05±0.11	1.04±0.07
Q7TPB1 TCPD_RAT	T-complex protein 1 subunit delta	1.03±0.11	0.96±0.19
Q68FQ0 TCPE_RAT	T-complex protein 1 subunit epsilon	0.98±0.10	0.98±0.07
Q6P502 TCPG_RAT	T-complex protein 1 subunit gamma	1.10±0.14	0.97±0.09
P11232 THIO_RAT	Thioredoxin (Trx)	1.14±0.07	1.02±0.04
O89049 TRXR1_RAT	Thioredoxin reductase 1, cytoplasmic	1.17±0.20	1.13±0.08
P62329 TYB4_RAT	Thymosin beta-4	1.09±0.09	1.21±0.08
P61589 RHOA_RAT	Transforming protein RhoA precursor	0.80±0.19	0.92±0.17
P46462 TERA_RAT	Transitional endoplasmic reticulum ATPase	0.98±0.09	0.98±0.05
P63029 TCTP_RAT	Translationally-controlled tumor protein	1.05±0.15	0.97±0.10
Q63584 TMEDA_RAT	Transmembrane emp24 domain-containing protein 10 precursor	1.15±0.34	1.03±0.17
Q64428 ECHA_RAT	Trifunctional enzyme subunit alpha, mitochondrial precursor	0.95±0.14	0.95±0.09
P48500 TPIS_RAT	Triosephosphate isomerase	0.93±0.08	0.97±0.09
P04692 TPM1_RAT	Troponin 1 alpha chain	0.96±0.08	1.03±0.08
P09495 TPM4_RAT	Troponin alpha-4 chain	1.01±0.20	0.99±0.06
P58775 TPM2_RAT	Troponin beta chain	0.67±0.28	0.89±0.25
P12788 TRY4_RAT	Trypsin-4 precursor	1.04±0.09	0.87±0.12
P68370 TBA1_RAT	Tubulin alpha-1 chain	0.95±0.08	0.94±0.11

Continued

Supplementary Table 2 Continued

Accession	Name	Avg.S.C (±SD)	Avg.R.C (±SD)
Q6P9V9 TBA2_RAT	Tubulin alpha-2 chain	1.30±0.17	1.13±0.11
Q4QRB4 TBB3_RAT	Tubulin beta-3 chain	0.91±0.08	0.95±0.07
P69897 TBB5_RAT	Tubulin beta-5 chain	0.92±0.11	1.04±0.15
Q5RJR2 TWF1_RAT	Twinfilin-1	0.93±0.06	1.06±0.11
Q4KM49 SYYC_RAT	Tyrosyl-tRNA synthetase, cytoplasmic	0.98±0.21	0.96±0.10
P32551 UQCR2_RAT	Ubiquinol-cytochrome-c reductase complex core protein 2, mitochondrial precursor	1.05±0.14	0.96±0.23
P62989 UBIQ_RAT	Ubiquitin	0.93±0.07	0.97±0.06
Q9EQX9 UBE2N_RAT	Ubiquitin-conjugating enzyme E2 N	1.17±0.39	0.94±0.21
Q6AYT3 CV028_RAT	UPF0027 protein	1.03±0.09	1.04±0.05
Q04462 SYV_RAT	Valyl-tRNA synthetase	1.06±0.15	1.02±0.13
Q9Z270 VAPA_RAT	Vesicle-associated membrane protein-associated protein A	1.04±0.15	1.05±0.23
Q9Z1A6 VIGLN_RAT	Vigilin	0.95±0.12	1.02±0.07
P31000 VIME_RAT	Vimentin	1.00±0.02	1.00±0.03
Q9Z2L0 VDAC1_RAT	Voltage-dependent anion-selective channel protein 1	0.99±0.09	1.03±0.04
Q5RKJ0 WDR1_RAT	WD repeat protein 1	1.12±0.22	1.03±0.11

Continued

Appendix III: MATLAB program for plot of cumulative distribution

1) The following program is used for plot of cumulative distribution of values of ratios of protein expression level in the *S*-enantiomer of propranolol-incubated cells relative to the control cells. (Adapted from: codes by W.R. Wilcox, Clarkson University, 2006). Paste the following script into your MATLAB Editor, save it as SOVERC.m and type in SOVERC in the MATLAB Command window.

```
% SOVERC.m
% plotting cumulative distribution
% for random numbers
% on normal distribution probability scale
% me = mean
% si = population standard deviation
% n1 = number of samples from population
% X1 = the vector of sample x values
% F= the fraction of values < x (for cumulative distribution)
% z1= erfinv(2F-1)
% For normal distribution give rise to straight line for z1 versus x1
% Note that F =(1 + erf z1)/2
clear, clc
% Input values of n1, me, si
n1 = input('Number of values to be generated: ');
me = input('Desired population mean: ');
sisq = input('Desired population variance: ');
si = sqrt(sisq);
% Generate the array of values of S:C from the supplemental table 1
X1 =[ 0.97
0.99
0.68
0.91
0.86
.
.
.
1.02
1.15
1.1
0.96
1.02
0.92
];
% Sort the numbers
X1 = sort(X1);
% Generate z1
j1=(1:n1); F=(j1-1/2)/n1; z1=erfinv(2*F-1);
% To calculation the normal distribution line:
Xn(1)=me-2*sqrt(2)*si; Xn(2)=me+2*sqrt(2)*si;
zn=[-5,5];
```

```

plot (X1,z1,'o',Xn,zn);
xlabel('x'); ylabel('z = erfinv(2F-1)');
title ('Cumulative distribution using a normal probability scale')
legend ('samples (S:C)', 'normal distribution', 'Location', 'SouthEast')

```

2) The following program is used for plot of cumulative distribution of values of ratios of protein expression level in the *R*-enantiomer of propranolol-incubated cells relative to the control cells. (Adapted from: codes by W.R. Wilcox, Clarkson University, 2006). Paste the following script into your MATLAB Editor, save it as ROVERC.m and type in ROVERC in the MATLAB Command window.

```

% ROVERC.m
% Plotting cumulative distribution
% for random numbers
% on normal distribution probability scale
% me = mean
% si = population standard deviation
% n2 = number of samples from population
% X2 = the vector of sample x values
% F = the fraction of values < x (for cumulative distribution)
% z2 = erfinv(2F2-1)
% For normal distribution give rise to straight line for z versus x
% Note that F =(1 + erf z2)/2
clear, clc
% Input the values of n2, me, si
n2 = input('Number of values to be generated: ');
me = input('Desired population mean: ');
sisq = input('Desired population variance: ');
si = sqrt(sisq);
% Generate the array of the values of S:C from the supplemental table 1
X2 =[0.94
1.08
1.07
1.06
0.97
1.11
.
.
.
0.92
0.97
0.98
0.91
1.07
0.97
];
% Sort the numbers

```



```
X2 = sort(X);  
% Generate z2  
j2=(1:n2)'; F=(j2-1/2)/n2; z2=erfinv(2*F-1);  
% To calculate of the normal distribution line:  
Xn(1)=me-2*sqrt(2)*si; Xn(2)=me+2*sqrt(2)*si;  
zn=[-5.0,5.0];  
plot(X2,z2,'o',Xn,zn);  
xlabel('x'); ylabel('z = erfinv(2F-1)');  
title('Cumulative distribution using a normal probability scale')  
legend('samples(R:C)','normal distribution','Location','SouthEast')
```