Proteomic investigation of the molecular targets of mycophenolic acid in human cells

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Date of oral examination: 20/01/2012
DECLARATION

I hereby declare that the Ph.D. thesis entitled “Proteomic investigation of the molecular targets of mycophenolic acid in human cells” has been written independently, with no other sources than quoted, and no portion of the work referred to in the thesis has been submitted in support of an application for another degree.

Muhammad Qasim
# Table of contents

List of abbreviations ........................................................................................................................................... 1

List of Figure .......................................................................................................................................................... 4

List of Tables .......................................................................................................................................................... 5

1. General introduction ........................................................................................................................................... 6

   1.1 Mycophenolic acid ......................................................................................................................................... 6
       1.1.1 Metabolism .............................................................................................................................................. 7
       1.1.2 Cellular and adverse effects of MPA ...................................................................................................... 10

   1.2 Intestinal epithelial barrier ........................................................................................................................... 13
       1.2.1 Tight junctions ......................................................................................................................................... 15
       1.2.2 Factors modulating intestinal permeability ............................................................................................ 18
       1.2.3 Regulation of TJ structure and function ................................................................................................. 20
       1.2.4 Caco-2 cells as an in vitro model for intestinal epithelial integrity ....................................................... 21

   1.3 Rationale for proposed research .................................................................................................................. 23

2. Differential proteome analysis of human embryonic kidney cell line (HEK-293) following mycophenolic acid treatment .......................................................................................................................... 24

   2.1 Abstract .......................................................................................................................................................... 25

   2.2 Introduction ................................................................................................................................................... 26

   2.3 Materials and Methods ................................................................................................................................ 27
       2.3.1 Reagents ................................................................................................................................................ 27
       2.3.2 Cell culture ............................................................................................................................................. 27
       2.3.3 Proliferation assay .................................................................................................................................... 28
       2.3.4 Sample preparation for proteome analysis ............................................................................................. 28
       2.3.5 2-DE ........................................................................................................................................................ 29
       2.3.6 Protein visualization, densitometric analysis and in-gel digestion .......................................................... 29
       2.3.7 Q-TOF LC-MS/MS analysis of protein identification .............................................................................. 30
       2.3.8 Functional classification ........................................................................................................................ 31
       2.3.9 Western blotting ...................................................................................................................................... 31
       2.3.10 RNA isolation and cDNA synthesis ........................................................................................................ 32
2.3.11 Real-time PCR .......................................................... 32
2.3.12 Apoptosis assay ......................................................... 33

2.4 Results ............................................................................. 34
2.5 Discussion ....................................................................... 40
2.6 Conclusion ....................................................................... 43

3. Mycophenolic acid mediated disruption of the intestinal epithelial tight junctions ......................................................... 44

3.1 Abstract ............................................................................ 45
3.2 Introduction ....................................................................... 46
3.3 Materials and methods ....................................................... 47
  3.3.1 Reagents ....................................................................... 47
  3.3.2 Cell culture ................................................................. 48
  3.3.3 Lactate dehydrogenase (LDH) measurement ................... 48
  3.3.4 Determination of caspase 3 activity ............................... 48
  3.3.5 Determination of Trans-epithelial resistance (TER) .......... 49
  3.3.6 FITC-dextran paracellular permeability .......................... 49
  3.3.7 RNA isolation, cDNA synthesis and real-time PCR .......... 50
  3.3.8 Immunoblotting ........................................................... 51
  3.3.9 Immunofluorescence microscopy of TJs proteins ............ 52
  3.3.10 Statistics ..................................................................... 53

3.4 Results .............................................................................. 53
  3.4.1 MPA altered TER and TJs permeability in a concentration and time dependant manner .. 53
  3.4.2 AcMPAG modulation of TER and TJs permeability .......... 54
  3.4.3 MPA and AcMPAG mediated increase in permeability was not due to cell death/apoptosis .......................................................... 55
  3.4.4 MPA and AcMPAG increased the expression of MLC2 and MLCK in Caco-2 cells ....... 56
  3.4.5 MPA and AcMPAG increased MLC2 phosphorylation in Caco-2 cells ............................. 58
  3.4.6 MPA and AcMPAG altered TJ proteins expression and distribution ............................................. 58
  3.4.7 MPA and AcMPAG modulation of Caco-2 F-actin .................. 60
  3.4.8 MPA-mediated increase in MLC phosphorylation through MLCK ................................. 62
  3.4.9 MLCK inhibition partially prevented MPA effects on TER and permeability ................. 64
3.4.10 Inhibition of MLCK prevented MPA mediated alteration of TJ proteins

3.5 Discussion

3.6 Conclusion

4. Summary

5. References

6. Appendix

7. Acknowledgements

8. Curriculum Vitae
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AcMPAG</td>
<td>Acyl glucuronide of mycophenolic acid</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Cytochalasin D</td>
</tr>
<tr>
<td>cdc42</td>
<td>Cell division control protein 42 homolog</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Cmax</td>
<td>Maximum plasma concentrations</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotrophin-releasing factor</td>
</tr>
<tr>
<td>CT</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P</td>
</tr>
<tr>
<td>DEVD-pNA</td>
<td>Asp-Glu-Val-Asp p-nitroanilide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxyribonucleotides</td>
</tr>
<tr>
<td>DSMZ</td>
<td>German collection of microorganisms and cell cultures</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EC-MPS</td>
<td>Enteric-coated mycophenolate sodium</td>
</tr>
<tr>
<td>EF-2</td>
<td>Elongation factor 2</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent essay</td>
</tr>
<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinases</td>
</tr>
<tr>
<td>ESI-QTOF-MS</td>
<td>Electro spray ionization time of flight mass spectrometry</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous polymers actin</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FD 4</td>
<td>Fluorescein isocyanate-dextran</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational (unit of centrifugation)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MUPP</td>
<td>Multi-PDZ Domain Protein</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>p</td>
<td>Probability</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMR</td>
<td>Perijunctional actomyosin ring</td>
</tr>
<tr>
<td>P&lt;sub&gt;app&lt;/sub&gt;</td>
<td>Apparent permeability</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycols</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>pl</td>
<td>Peak list</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PML</td>
<td>Multifocal leukoencephalopathy</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>Prdx1</td>
<td>Peroxiredoxin-1</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>ROCKs</td>
<td>Rho-associated, coiled-coil containing protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris boric acid-tween</td>
</tr>
<tr>
<td>TER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine diphosphate-glucuronosyltransferase</td>
</tr>
<tr>
<td>VEGF-α</td>
<td>Vascular endothelial growth factor alpha</td>
</tr>
<tr>
<td>ZONAB</td>
<td>ZO-1-associated nucleic acid-binding protein</td>
</tr>
</tbody>
</table>
List of Figure

Figure 1.1 Chemical structure of MMF, EC-MPS, MPA, and their metabolites, AcMPAG, MPA 7-O-glucoside and 6-o-desmethyl MPA..................................................................................................................7
Figure 1.2 Metabolism of MPA ........................................................................................................................................8
Figure 1.3 Adverse effects of MPA ..................................................................................................................................11
Figure 1.4 Intestinal epithelial barrier................................................................................................................................17
Figure 2.1 Inhibition of HEK-293 cells proliferation by MPA treatment....................................................................................36
Figure 2.2 Differential protein expression after incubation of HEK-293 cells with MPA.......................................................................36
Figure 2.3 Functional classification of regulated proteins .........................................................................................................37
Figure 2.4 Differential expression of Prdx1 and MLC2 by MPA treatment......................................................................................38
Figure 2.5 Expression of MLC2 in MMF treated rat kidney lysate and HT-29 cells.................................................................39
Figure 2.6 Measurement of MPA induced caspase-3 activity .......................................................................................................39
Figure 3.1 MPA treatment decreased TER and increased FD4 permeability of Caco-2 cell monolayers...54
Figure 3.2 AcMPAG treatment caused a time dependant decrease in TER and increase in FD4 permeability in Caco-2 cell monolayers..........................................................................................................................55
Figure 3.3 Effect of MPA and AcMPAG on cell viability and apoptosis in Caco-2 cells...............................................................56
Figure 3.4 Effect of MPA and AcMPAG on MLC2, MLCK and ROCK expression in Caco-2 cells................................................57
Figure 3.5 Effect of MPA and AcMPAG on the phosphorylation of MLC2 in Caco-2 cells.........................................................58
Figure 3.6 Effects of MPA and AcMPAG on ZO-1 and occludin distribution................................................................................59
Figure 3.7 Effect of MPA and AcMPAG on occludin protein expression in Caco-2 cells .........................................................60
Figure 3.8 MPA and AcMPAG-induced remodelling of the F-actin cytoskeleton........................................................................61
Figure 3.9 Effect of ML-7 on MPA-mediated increases in MLC2, MLCK and MLC phosphorylation.................................63
Figure 3.10 ML-7 co-treatment reversed the effect of MPA on TER and FD4 permeability .....................................................64
Figure 3.11 ML-7 co-treatment reversed the effect of MPA on distribution of proteins...............................................................65
Figure 3.12 Effect of ML-7 co-treatment with MPA on occludin protein expression in Caco-2 cells.................................66
Figure 4.1 A proteomic approach for identification of novel MPA molecular targets .................................................................73
Figure 4.2 Proposed model of MPA mediated TJ disruption .......................................................................................................74
Figure 5.1 A graphical representation of relative abundance (%volume) of all differentially regulated proteins ......................................................................................................................................................100
List of Tables

Table 2.1 Differentially regulated proteins by MPA in HEK-293 cells identified by mass spectrometry ........35

Table 5.1 MS/MS analysis table of all differentially regulated proteins ..................................................101
1. General introduction

1.1 Mycophenolic acid

Mycophenolic acid (MPA) is an active fungal agent derived from *Pencillium Brevicopactum* and related fungi. MPA was discovered in 1893 by an Italian physician, Bartolomeo Gosio as an antibiotic against *Bacillus anthracis* (reviewed in [1]) and was named by Alsberg and Black in 1913 [2]. MPA selectively and competitively inhibits inosine monophosphosphate dehydrogenase (IMPDH), which is a key regulatory enzyme in the *de novo* pathway of purine biosynthesis. IMPDH converts inosine monophosphate (IMP) to guanosine monophosphate (GMP), an important intermediate in the synthesis of DNA, RNA, proteins, and glycoproteins. Inhibition of IMPDH leads to cell cycle arrest in synthesis (S) phase due to the blocking of *de novo* guanosine nucleotide synthesis. MPA exhibits cytotoxic effects on all cell types including its main target, lymphocytic cells [3][4,5]. Lymphocytes presumably utilize a *de novo* pathway for purine biosynthesis while non-lymphocytic cells depend only partially on this pathway, and can utilize a salvage pathway [4]. In the salvage pathway, guanine obtained from the breakdown of nucleic acids is directly converted to guanosine monophosphate and used for purine synthesis [6]. Additionally, MPA has five fold more potent inhibitory action on IMPDH II, an isoform mainly expressed in B & T lymphocytes, than on IMPDH I, which is expressed in all body cells [4]. Consequently, the cytostatic effects of MPA on lymphocytes are greater than on other cell types, which contributes to the prevention of graft rejection, making MPA an especially useful immunosuppressant in transplantation medicine [3,4].

MPA is marketed in two forms: the ester pro-drug mycophenolate mofetil (MMF; CellCept, Roche, Grenzach-Wyhlen, Germany) and enteric-coated mycophenolate sodium (EC-MPS; myfortic®; Novartis Pharma AG, Basel, Switzerland) [7]. MMF gained approval by the United States Food and Drug Administration (FDA) in 1995 for the prevention of renal, cardiac, and hepatic allograft rejection [8,9]. MPA is now the drug of choice in transplantation medicine for the prevention of acute rejection in patients undergoing allogenic renal, cardiac and liver transplantation [4,10]. Furthermore, MPA has proved to be effective in the treatment of autoimmune
disorders of the eyes and skin as well as in Wegener's granulomatosis and lupus nephritis [11-14], hypertension [15,16] and neuromuscular autoimmune diseases [17,18]. MPA has also been reported to possess anti-viral [19], anti-fungal [20], antibacterial [1], anti-tumor [21], and anti-psoriasis [22,23] activities.

![Chemical structure of MMF, EC-MPS, MPA, and their metabolites AcMPAG, MPA 7-O-glucoside, and 6-o-desmethyl MPA.](image)

Figure 1.1: Chemical structure of MMF, EC-MPS, MPA, and their metabolites AcMPAG, MPA 7-O-glucoside, and 6-o-desmethyl MPA.

### 1.1.1 Metabolism

MMF and EC-MPS is rapidly hydrolyzed by esterases in the gut, blood, liver, and kidney [24]. Maximal MPA plasma concentrations (C\text{max}) are generally reached within 1-1.5 hr and 1.5-2.5 hr after oral administration of MMF and EC-MPS respectively [7]. EC-MPS is insoluble in the acidic pH of the stomach but highly soluble in the neutral pH of the intestine. This effect is responsible for later peak concentrations seen after EC-MPS administration when compared to MMF [7,25]. Following intravenous administration, MMF is also rapidly hydrolyzed to MPA with C\text{max} achieved within approximately 1.58 hr and with an absorption half life of only a
few minutes [26]. The mean bioavailability of MPA is 81%-94% and 72% following administration of MMF and EC-MPS respectively [7,27], while the mean bioavailability of MPA after oral administration of MMF is estimated to be 94.1% relative to the intravenous route [28]. Trough plasma MPA concentrations are in the range of 0.3-3.4 mg/L [261]. MPA binds extensively (97-99%) to plasma albumin producing free fractions of only <3%. The free fraction of MPA is mainly responsible for the pharmacological effects of MPA [7,24,29,30].

Figure 1.2: Metabolism of MPA.

MMF and EC-MPS are hydrolyzed to their active form MPA in the GI tract. MPA then absorbed and subsequently glucuronated by UGT to MPAG in the hepatocytes. In addition, other metabolites including AcMPAG, MPA 7-O-glucoside, and 6-o-desmethyl MPA are also formed. MPAG is largely excreted into urine by hOATs while some is secreted into bile by MRP, where it is then either excreted into feces or reconverted to MPA by glucuronidases present in gut bacteria and returned to the systemic circulation (enterohepatic recycling).
Like many other xenobiotics, MPA undergoes glucuronidation, which is the major pathway for phase II metabolism for xenobiotics in humans [36]. MPA is conjugated by uridine diphosphate-glucuronosyltransferase (UGT) enzymes to form MPA glucuronide (MPAG) in hepatocytes, kidneys and intestinal mucosa [30,31]. UGT 1A9, UGT1A7, 1A8 and 1A10 are mainly responsible for MPAG formation [32]. Beside MPAG, other minor metabolites of MPA, such as 7-O-glucoside and the active acyl glucuronide of MPA (AcMPAG) are formed by UGT2B7 and miscellaneous UGTs respectively [32,33]. In addition, an oxidation product 6-O-desmethyl MPA is formed by cytochrome P-450 (CYP3A4/5) [34]. The chemical structures of MPA, its pro-drugs, and metabolites are given in Figure 1.1.

MPAG does not exhibit pharmacological activity but is present in 20 to 100-fold higher concentrations than MPA in the blood [24] and achieves its C$_{\text{max}}$ in 1 hr after the MPA C$_{\text{max}}$ [251]. MPAG has a protein binding of 82% and has the capacity to interfere with the MPA-albumin binding. MPAG at high concentrations is known to displace MPA from its albumin binding sites, thus modulating the free fraction of MPA, which is important for the pharmacological activity of MPA [30,35].

AcMPAG, a pharmacologically active metabolite, is believed to be responsible for some of the adverse effects of MPA [33,36,37]. Acyl glucuronides are formed by esterification of carboxylic acid with glucuronic acid [36]. Such acyl glucuronides have been observed for several clinically useful therapeutic drugs including non steroidal anti-inflammatory drugs (NSAIDs) [36]. AcMPAG plasma concentrations are 10-20% of MPA concentrations [75,76]. Mean AcMPAG area under curve (AUC), over 12 hr is generally 10.3% of simultaneous MPA-AUC [75] and AcMPAG reaches its C$_{\text{max}}$ in 1-3 hr following the C$_{\text{max}}$ of MPA {Schutz, 2000 680 /id}.

MPA is eliminated from the body mainly through the kidneys. 93% of the orally administered dose of MMF is excreted in urine and 6% in the feces. MMF is predominantly (87%) excreted as MPAG in the urine and a small amount (<1%) as MPA. Like MMF, orally administered EC-MPS is also excreted maximally through urine, with 60% as MPAG and approximately 3% as MPA 1-7,4,8,9.
A proportion of MPAG is secreted into bile through multidrug resistant protein (MRP) transporters, specifically MRP2 [41]. MPAG then goes into the intestine where gut bacteria deglucuronidate MPAG to reform MPA, which is then reabsorbed back into systemic circulation [24,30]. This reabsorption is responsible for a second peak of MPA concentration detected in plasma 6 to 12 hr and 6 to 8 hr following oral administration of MMF and EC-MPS respectively [45]. This process is known as enterohepatic circulation and accounts for 10 to 60% of total MPA exposure [24,30,31,42]. The mean elimination half-life of MPA is 13 and 13–17 hr following oral administration of MMF and EC-MPS respectively [7,24]. The simplified overview of distribution and metabolism of MPA is shown in Figure 1.2.

### 1.1.2 Cellular and adverse effects of MPA

MPA causes the depletion of guanosine triphosphate (GTP) pools, which is assumed to be responsible for MPA associated anti-proliferative effects in vitro and in vivo [43,44]. Nucleotide inhibition leads to G1 cell cycle arrest and thus inhibits cell growth of immune (T and B lymphocytes) and non immune cells (smooth muscle cells, entothelial cells, renal tubular and mesangial cells) in a dose-dependant manner [43,44,46-47]. MPA is also a potent anti-inflammatory agent which inhibits proliferation of immune cells, inhibits pro-inflammatory cytokines such as tumor necrosis factor alpha, interleukin 1 beta, interleukin-17, vascular endothelial growth factor alpha (VEGF-α), and blocks the migration of leucocytes to inflammation sites [48,49]. MPA has promising effects in reducing myofibroblast infiltration, collagen III deposition and inhibition of the proliferation of both immune (lymphocytes) and non immune (fibroblasts, vascular smooth muscle and tubular) cells which are involved in the development of fibrosis [50-52]. MPA inhibits tumor growth and metastasis through G1-S cell cycle arrest, induction of differentiation in a variety of human tumor cell lines, induces apoptosis, as well as suppress the glycosylation and expression of several adhesion molecules (integrins, ICAM-1, VCAM-1, E-selectin and P-selectin) which promote tumor metastasis [21,53-56].
Figure 1.3: Adverse effects of MPA.
Various proposed mechanisms of MPA associated side effects are highlighted such as (a) alteration in gene expression making individuals more susceptible to stress [78], (b) immunosuppression leading to opportunistic infections [58], (c) AcMPAG adduct toxicity results in ultrastructural abnormalities, metabolic dysfunction, and oxidative damage [58][36].

MPA is generally a well tolerated immunosuppressive agent and produces less nephrotoxicity compared to other immunosuppressives drugs (reviewed in [57]). GI toxicity is the common adverse effect of MPA, occurs in 20% of renal patients on MMF therapy, and is dose dependant (reviewed in [57]). Symptoms of GI toxicity include diarrhea, abdominal pain, nausea, anorexia, vomiting, [58-60], gastritis, esophagitis, duodenal ulcers, colonic ulceration [60,61], and small intestinal villous atrophy [62,63]. MMF can cause enterocolitis and a Crohn’s disease-like colitis syndrome [37,64,65].

In addition to GI tract symptoms MPA can cause genitourinary symptoms such as frequency, urgency, dysuria, sterile pyuria, and hematuria. These symptoms have
reen reported to occur during the first year of MPA therapy (reviewed in [2,57]). In addition, MPA can also occasionally produce neurologic disturbances such as weakness, headache, tinnitus and insomnia (reviewed in [66]). Some cases of progressive multifocal leukoencephalopathy (PML) have been reported in patients on MMF therapy. These patients developed clinical features such as hemiparesis, apathy, confusion, cognitive deficiencies, and ataxia [67,68,68]. The occasional skin problems with MPA use include exanthematous eruptions, acne, pedal edema, urticaria, dishydrotic eczema, blistering hand dermatitis, and onycholysis (reviewed in [57,66]).

MPA can cause cardio-respiratory toxicity causing dyspnoea, cough, chest pain, palpitations, hypertension, acute respiratory failure, pulmonary edema, pulmonary fibrosis and pneumonitis [57,69]. Metabolic disturbances are also reported in MMF treated patients. Findings in these patients includes hypercholesterolemia, hypophosphatemia, hypokalemia, hyperkalemia, hyperglycemia (reviewed in [57,66]). There have been reports of mild, dose-related haematologic effects occurring in 5% of patients. Findings include anemia, leucopenia, and thrombocytopenia (reviewed in [2,57]). Pure red cell aplasia (PRCA) has been observed in some patients treated with MPA in combination with other immunosuppressive drugs [70]. An increased incidence of infectious complications occurs in 2% of renal and cardiac transplant patients and in 5% of hepatic transplant patients treated with MPA (reviewed in [57]). Like other immunosuppressive therapies, opportunistic infections occur in up to 40% of transplant patients given MMF (reviewed in [66,71]). Several viral, bacterial and fungal complications have been observed, including infection with herpes simplex virus, herpes zoster virus, human herpes virus type 6, papillomavirus, aspergillosis, encephalitis, streptococcus B septic shock, recurrent E.coli associated epididymitis, pediatric disseminated varicella, candidiasis, cryptococcosis, mucormycosis pneumocystis carinii pneumonia, and intestinal microsporidiosis (Reviewed in [3,56,74]).

Diarrhoea is the most common GI adverse effect caused by MPA but the exact mechanism responsible for this have not yet been clearly defined [72]. Several mechanisms have been suggested to be responsible for the adverse events associated with MPA therapy including direct cytotoxic effects on GI cells, release of pro-inflammatory cytokines by AcMPAG [73,74], and formation of AcMPAG protein
adducts [75]. Covalent AcMPAG-protein adducts are formed through two pathways; transacylation and glycation. Transacylation involves direct binding of an aglycone moiety to the proteins while the glycation mechanism involves intramolecular rearrangement resulting in a open-chain conjugate with a free aldehyde group which binds with the amino group on various proteins [36,77].

AcMPAG adducts may cause cellular toxicity through a number of proposed mechanisms. These adducts may modify protein structure and thus interfere with normal cell function, or they may activate the immune system resulting in hypersensitivity reactions or autoimmunity. Furthermore, AcMPAG adducts cause oxidative stress via glutathione depletion [58][36]. Previously, it was demonstrated that AcMPAG forms covalent protein adducts in the kidney, liver and intestine of rats treated with MMF [76,77]. The proteins involved are associated with diverse cellular functions. Another study revealed that MMF down-regulates mRNA expression of polymeric immunoglobulin receptor (resulting in decreased protection against invading pathogens and reactive drugs), catalase (cells were more prone to oxidative stress), and CCAAT/enhancer-binding proteins (interference with the defence system against free radicals) [58,78]. The adverse effects of MPA therapy are summarized in Figure 1.3.

1.2 Intestinal epithelial barrier

The mammalian intestine is lined with a single layer of specialized simple columnar epithelium that separates the intestinal lumen from the underlying lamina propria [79,80]. The intestinal lining consists of proliferative crypts, which contain intestinal stem cells, and villi, which contain differentiated specialized cell types such as the absorptive enterocytes, mucous-secreting goblet cells, and hormone-secreting enteroendocrine cells [81] (reviewed in [82]). In addition, there are Paneth cells which are differentiated cells at the bottom of crypts bottom that perform several functions including limiting gut microbial populations by secreting defensins, (antimicrobial peptides) and protecting the intestinal lining from bacterial toxins [80].

The intestinal epithelium represents the major contact between a person and their external environment and covers an extensive surface area of >300 m² [83].
Structural components of the intestinal barrier include the unstirred water layer, the hydrophobic mucosal surface, the surface mucous coat, epithelial factors (tight junctions), and endothelial factors [84]. The intestinal epithelium has two vital functions. It selectively filters, allowing the absorption of nutrients, electrolytes, and water from the intestinal lumen into the circulation while it serves as a barrier to prevent luminal pro-inflammatory factors, luminal pathogens and their antigens or toxins from invading the tissues [84-87]. In addition, the stirred water layer plays a role in transport of many nutrients and drugs, especially lipid-soluble compounds [84]. Mucus from goblet cells provides a protective layer against the physical friction, chemical digestion, and adhesion of bacteria. In addition, it also acts as a diffusion barrier [84,88]. The hydrophobicity of the mucosal surface acts as an important barrier to bacterial and other factors within the gut lumen. Many factors such as nonsteroidal anti-inflammatory drugs (NSAIDs), dextran sodium sulfate, trinitrobenzenesulfonic acid, lipopolysaccharide (LPS) and ammonium [89] can decrease this hydrophobicity.

The epithelial layer constitutes the key component of intestinal barrier. It acts as a selectively permeable filter allowing the transport of essential dietary nutrients, electrolytes, and water from the intestinal lumen into the circulation [84,90]. In addition, the intestinal epithelia controls chloride permeability which is responsible for secretion of protective fluids into the intestinal lumen which limits bacterial colonization and entry of toxins into the intestinal cells [91]. Another important function of the intestinal lining is its secretion of local immunoglobulins such as epithelial secretory immunoglobulin-A (IgA) which targets antigens at the mucosal surface, and constitutes a humoral component of the mucosal immune system [92-94].

Permeability of the intestinal epithelium is regulated via transepithelial/transcellular and paracellular pathways. Transcellular transport is an active process and involves transport of water, amino acids, electrolytes, short-chain fatty acids, and sugars across the plasma membrane by specific ion channels and transporters [15,16][95]. Paracellular transport is a passive process which involves the movement of solutes and water across the intercellular space and is regulated by intercellular complexes. Paracellular transport is determined by molecular size or the ionic charge or both and is mainly regulated by tight junctions (TJ) [95,96].
1.2.1 Tight junctions

Intestinal epithelial cells are connected to one another by adhesive junctional complexes which serve as a physiological and structural paracellular barrier. Components that constitute the multimolecular junctional complex include desmosomes, adherens junctions, and TJs [97,98]. TJ, the most apical component of the junctional complex are generally considered to be the major barrier to the passage of molecules between adjacent cells and through the intercellular space. The TJ barrier is not absolute but is selectively permeable and is able to discriminate between solutes on the basis of size and charge. TJ complex contains more than forty proteins, having various functions [99]. The structure of TJ was first described with the help of electron microscopy [100]. TJ consist of transmembrane proteins (JAMs, occludin and claudins), adaptors (ZO (type 1-3), MAGI (type 1-3), PAR 3/6, cingulin, PATJ and MUPP1), regulatory proteins (Rab 13, Rab 3b, G proteins, PKC, PP2A and PTEN), and both transcriptional and post-transcriptional regulators (symplekin, ZONAB, and huASH1). All these proteins interact with each other to form a complex protein network [101,102], responsible for TJ functions including their interaction with F-actin [103]. The basic architecture of TJ is shown in Figure 1.4. All of the TJ proteins listed above play an important role in the structure and function of TJ, but only a brief description of the function of some TJ proteins (ZO-1, occludin, and claudin), which have been extensively studied in the context of TJ disruption [104] are described below.

ZO, the first TJ-associated protein to be identified [105], belongs to the membrane associated guanylate kinase family (MAGUK) and contains three N-terminal PDZ repeats, an SH3 domain, and a C-terminal region homologous to guanylate kinases [106]. There are different isotypes of ZO including ZO-1, ZO-2 and ZO-3 with a variety of cellular functions. ZO-1 is a 210-225 KDa peripheral membrane protein and is a major constituent of the cytoplasmic domain of TJ. The C-terminal domain of ZO-1 interacts with other TJ proteins including claudins [107]. ZO-1 is also in close association with actin cytoskeleton responsible for linking transmembrane proteins of the TJ to the actin cytoskeleton that plays a regulatory role in TJ actions. The C-terminal portion of occludin, claudin, ZO-2, and ZO-3 interact closely with the N-terminus of ZO-1. Additionally, the C-terminal half of ZO-1
interacts with F-actin regulating cytoskeleton [107,108]. The expression and
distribution of ZO proteins are regulated by myosin light chain kinase (MLCK) and
their alteration can lead to defective function of epithelial barriers [109]. The down-
regulation and redistribution of ZO-1 has been observed in TJ disruption conditions,
such as those involving cytokines [110,111], ethanol [112] and oxidants [113].

Occludin is a 60 KDa protein and was identified as the first among
transmembrane TJ proteins in 1993 [114,115]. Occludin is a member of the Marvel
(MAL-related proteins for vesicle trafficking and membrane link) domain containing
protein family [116]. Occludin has a tetraspan structure that constitutes its
extracellular strand within TJ and amino- and carboxy-terminal chains projecting into
the cytoplasm [117]. The interactions of occludin with various intracellular TJ
proteins, including ZO-1, ZO-2, and ZO-3 have been well documented [118]. Occludin plays an important role in regulating TJ dynamics as demonstrated by the
fact that its depletion leads to increases in the permeability of larger-sized molecules
shown in both in vitro and in vivo intestinal models [119]. The down-regulation of
occludin proteins, associated with increased permeability has been observed in
several inflammatory bowel diseases such as Crohn's disease, ulcerative colitis, and
celiac disease [120-122], as well as in animal models of inflammatory bowel disease
[123,124]. It has been proposed that a decrease in intestinal occludin expression
may be an important mechanism responsible for increased intestinal epithelial TJ
permeability. Occludin in epithelial cells is highly phosphorylated on serine and
threonine residues and its phosphorylation plays a critical role in the regulation of TJ
integrity. Occludin phosphorylation is regulated by the balance between protein
kinases (eg. c-Src, PKCζ, and PKCλ/ι,) and protein phosphatases (eg. PP2A, PP1,
and PTP1B) [125-127]. Occludin has also been reported to be phosphorylated at
tyrosine which has been proposed to be implicated in disruption of TJs by various
toxins such as hydrogen peroxide and acetyldehyde [128,129].

Claudins are 20-27 KDa integral membrane TJ proteins that contains four
hydrophobic transmembrane domains which have a cytoplasmic N terminus, two
extracellular loops, and a C-terminal cytoplasmic domain. The claudin family is a
multigene family comprised of at least 24 members [130]. On the basis of their role in
controlling permeability, claudins has been divided into two sub-categories,
paracellular barrier forming claudins and paracellular, ion permeability forming claudins. Both are vital for proper and tissue-specific functioning of the TJs; making claudins a critical player in regulation of paracellular function [131,132]. The carboxy terminus of claudins binds to PDZ domains of proteins including those of the ZO proteins [107]. In addition, interaction of claudin-1 with ZO-1 is critical for ZO-1 integration into epithelial TJs [133]. Claudin isotypes 1 to 5 are present in the intestinal cells in various intestinal regions [134,135]. Down-regulation of claudin 1 is believed to be linked to the TJ disruption in inflammatory mucosa by Crohn's disease and ulcerative colitis and is believed to be associated with enhanced paracellular permeability [136]. Claudin 4 down-regulation has also been observed in collagenous colitis, characterized by barrier defects and associated with reduced net Na⁺ and Cl⁻ absorption [137].

**Figure 1.4: Intestinal epithelial barrier.**
Schematic diagram of intestinal epithelial cells showing tight junctions (TJ), adherens junctions (AJs), desmosomes and gap junctions. The TJs are positioned at the most apical parts of the plasma membranes of enterocytes, whereas AJs and desmosomes are present mainly at the basal parts of the lateral membranes. TJ and AJ are linked to actin and play an important role in regulation of intestinal permeability (Left panel). The molecular components of epithelial tight junctions (TJs) are outlined (right panel), and consist of transmembrane proteins (occludin, claudins and JAMs), adaptors (ZO (1-3), PAR 6, and PATJ), regulatory proteins (Rac, cdc42, RhoA, and PKC), and other associated proteins. Occludin, claudins and JAMs are linked to the zona occludens, and they are connected to actin forming the main TJ assembly. Adopted from (Aktories K and Barbieri JT 2005, Nat Rev Microbiol) [138].
TJ defects have been described in several patho-physiological conditions such as brain diseases [139,140], pulmonary inflammation, allergic rhinitis [141], obstructive jaundice [142], kidney diseases [143,144], diabetic retinopathy [145], cancers [146], blood-borne metastases [140,147] and bowel diseases [148-150]. Intestinal epithelial barrier dysfunction is a major factor contributing to the predisposition to inflammatory diseases, including food allergy, IBD, and celiac disease. The presence of environmental factors in the intestinal lumen and inappropriate host immune responses are key determinants of the development of IBD [87,148]. In IBD, epithelial barrier function is impaired leading to either diarrhoea because of a leaky flux mechanism or translocation of toxins and macromolecules into intestinal cells causing associated dysfunction [151].

1.2.2 Factors modulating intestinal permeability

Several endogenous molecules such as glucose [153], hormones [154], nucleotides [155], and growth factors [156-158] provide physiological modulation of TJ permeability. In addition, a growing list of pathological agents has been suggested as etiologic factors in several diseases as a result of causing increased mucosal permeability [159]. Dietary components are crucial in the regulation of barrier integrity (reviewed in [160]). Gliadin, a glycoprotein present in wheat, is the key factor in the pathogenesis of celiac disease and is responsible for TJ disruption leading to increased permeability [161]. Several dietary components have been found to increase TJ permeability including: cayenne pepper (*Capsicum frutescens*), paprika (*Capsicum anuum*), galangal (*Alpinia officinarum*), marigold (*Tagetes erecta*), Acer nikoense, and hops (*Humulus lupulus*) (reviewed in [160]). In contrast, black pepper (*Piper nigrum*), green pepper, nutmeg, bay leaf extracts, linden (*Tilia vulgaris*), star anise (*Illicium anisatum*), Arenga engleri, and black tea (*Camellia sinensis*) have been found to decrease paracellular flux and increase transepithelial resistance (TER) (reviewed in [160]).

Pro-inflammatory cytokines have also been proposed as pathophysiological stimuli which trigger several cellular pathways leading to pathological conditions including bowel diseases [110,111,162-166]. For example, TNFα modulates epithelial barrier properties and has a critical role in IBDs [167] and graft-versus-host disease [152]. TNFα up-regulates MLCK which acts as the central player in TNFα induced
barrier loss, both in vitro [168] and in vivo [150,169]. Additionally, TNFα has also been shown to be involved in the down regulation of apical Na⁺-H⁺ exchange, which then is linked to the development of diarrhoea [169,170]. Mast cells (MC) have been reported to regulate intestinal permeability, as suggested by the fact that their degranulation results in blood flow modulation, as well as increased epithelial and endothelial permeability, mucosal secretion, gastrointestinal tract motility, immunologic reactions, and angiogenesis (reviewed in [84]). Several physiological and pathological conditions have been reported to be associated with MC mediated intestinal permeability including food allergy, irritable bowel syndrome, and after stressful conditions [171,172]. Intracellular mediators including nitric oxide (NO) regulate barrier properties by altering the function of epithelial cells and the GI microcirculation [173]. The activity and synthesis of NO is increased by endotoxin (LPS), cytokines, and ethanol (EtOH), which results in barrier dysfunction via protein oxidation, nitration, S-nitrosylation, cGMP activation, and cellular energy depletion (reviewed in [84]).

Epithelial-microbe interactions are responsible for alterations in the structure and function of the epithelial barrier, regulation of fluid and electrolyte secretion, and modulation of inflammatory signalling (reviewed in [174]). More than 400 microbial species that have a profound impact on gut physiology reside in the gastrointestinal lumen [80]. Pathogenic bacteria, such as Escherichia coli, Klebsiella pneumoniae, Streptococcus viridans, Clostridium difficile, Bacteroides fragilis, Vibrio cholerae, and Helicobacter pylori, as well as viruses and parasites (giardia) can disrupt the intestinal barrier (reviewed in [84]). Beneficial bacteria: however, such as Lactobacillus brevis maintain TJ and reduce intestinal permeability [175]. Psychological stress is another factor responsible for alterations in epithelial barrier physiology (reviewed in [84]). Various parts of brain, brain stem, various CNS afferents, and the neuro-endocrinal system are proposed to be involved in the stress response. During psychological stress corticotrophin-releasing factor (CRF) is released, which triggers the enteric nervous system that causes alterations in gut motility, exocrine and endocrine functions, and the microcirculation (reviewed in [176]). Oxidative stress is caused largely by reactive oxygen (ROS) species such as hydrogen peroxide (H₂O₂), nitric oxide, peroxynitrite and hypochlorous acid which disrupt the epithelial and endothelial barrier function by destabilizing TJs [177].
1.2.3 Regulation of TJ structure and function

Regulation of the assembly, disassembly, and maintenance of TJ structure is highly dynamic and is influenced by diverse protein-protein interactions that respond to both extra-cellular and intra-cellular physiological, pharmacological, and pathophysiological stimuli. TJ regulation is controlled by several signalling proteins, including tyrosine kinase, Ca^{2+}, phospholipase C (PLC), protein kinase C (PKC), calmodulin, mitogen-activated protein kinase (MAPK), MLCK, the Rho family of small GTPases, adenosine, 3’,5’-cyclic monophosphate (cAMP), and heterotrimeric G proteins [178-182]. Actin has a vital role in the structure and function of TJ. Multiple TJ components interact with the actin cytoskeleton and regulate the permeability of TJs [183,184]. Reorganization of the actin cytoskeleton as a result of interactions between transmembrane proteins and the actomyosin ring [178,183,185,186] and as well as the phosphorylation state of TJ proteins are both critically involved in alterations in TJ physiology [187,188]. Members of the Rho family GTPase (Rac, Rho, and Cdc42) have also been shown to be able to reorganize the actin cytoskeleton and modulate TJ physiology [179,189-191]. Changes in the phosphorylation status of TJ proteins such as ZO, occludin, E-cadherin, β-catenin, and claudins act as a molecular switch that regulates TJ structure and function [125,187,188,192,193].

MLC phosphorylation is an important regulator of barrier function in health and disease [194]. Increased MLC phosphorylation leads to the rearrangement of TJ proteins (ZO-1, occludin, claudin-1 and claudin-4), disruption of perijunctional F-actin, and increases TJ permeability [103,184]. The main pathways associated with MLC phosphorylation are controlled either directly by MLCK activity or indirectly by Rho kinase mediated inhibition of phosphatase [182]. MLCK mediated MLC phosphorylation is sufficient to trigger downstream events necessary for barrier regulation and has a central role in many diseases that are characterized by intestinal barrier dysfunction (reviewed in [170]). Increased MLCK expression or activity has been observed in GI pathology following TNF α [169], interleukin 1β [195,196], lipopolysaccharide [197], and ethanol [112,198] exposure. Similar increased MLCK activity is observed after exposure to virulence factors associated with GI infections with Enteropathogenic Escherichia coli (EPEC) [199,200] and
Helicobacter pylori [201,202], as well as parasitic diseases like giardiasis [203]. The role of Rho family of small GTPases has been described in the regulation of TJ structure and function including the perijunctional actomyosin ring [194,204]. ROCKs regulate the phosphorylation of MLC by inactivating MLCP (myosin light chain phosphatase), which is involved in decreasing MLC phosphorylation [191]. ROCK inhibition causes the redistribution of F-actin structures and modulates TJ permeability. In addition, ROCK co-localizes with the ZO-1 and its inhibition prevents proper localization of TJ proteins during TJ assembly [204].

PKC is an important member of the serine-threonine kinases family which regulate epithelial barrier structure and function. PKC modulates the expression of subcellular localization and phosphorylation states of TJ proteins which alters barrier dynamics [206]. PKC proteins are also involved in various signal transduction pathways such as the Toll-like receptor 2 (TLR2) pathway. Activation by PKC isoforms results in increases in TER and redistribution of ZO-1 (reviewed in [160]). PKC also interacts with MLCK. PKC phosphorylates MLCK which leads to decreases in MLC phosphorylation, reduces tension on the perijunctional actomyosin ring (PAMR), and increases permeability [207]. The MAPK pathway is a major intracellular signalling pathway involved in cell growth, differentiation, and TJ regulation [208]. Several growth factors, cytokines, and oxidative stresses are involved in the stimulation of the MAPK pathway (reviewed in [160,209]). Members of MAPK have been implicated in modulation of TJ structure and function since extracellular signal regulated kinases (ERK) interact directly with the C-terminal region of occludin to prevent H₂O₂-induced disruption of TJ [208].

1.2.4 Caco-2 cells as an in vitro model for intestinal epithelial integrity

A number of both in vitro and in vivo experimental models are being used to study the integrity of TJs [210-213][214,215]. Caco-2 is one of the most widely used intestine cell models for in vitro studies of intestinal barrier functions [211,216,217], intestinal absorption, and toxicity of xenobiotics [216-218]. Caco-2 cells were first generated from the differentiated colon adenocarcinoma of a 72-year old patient
Caco-2 cells grown in culture usually reach confluency within 3-6 days, a stationary growth phase after 10 days [220], and complete their differentiation within 20 days [221]. Once differentiated these cells exhibit properties similar to enterocytes both structurally, bio-chemically, and functionally including having microvilli, intercellular junctions, nutrient transporters, efflux transporters, and enzymes (alkaline phosphatase, sucrase isomaltase and aminopeptidase) [222,223]. Caco-2 cells also express various transport and metabolizing enzymes such as cytochrome P450 isoforms and UDP-glucuronosyltransferases, sulfotransferases and glutathione-S-transferases [224,225]. Although, most Caco-2 properties resemble those of enterocytes, some differ. Caco-2 cells lack the crypt-villus axis (which is important for in vivo transport) and mucus producing goblet cells (leading to a lack of prominent mucus layers [226-228]).

Transepithelial electrical resistance (TER) and paracellular permeability to tracers molecules are two parameters that are commonly used to investigate the integrity and function of the TJ in in vitro models such as Caco-2 monolayers [213,229-232]. Usually, TJ barriers limit the ionic diffusion through cell monolayers, which creates a potential difference that is measured as transpithelial resistance (TER). TER has a direct relationship with TJ integrity. The greater the TER, the more intact the TJ [223,233,234]. Paracellular permeability of tracers in cell layers is measured by the diffusion rate of such tracers from apical to basal or vice versa. Paracellular flux is inversely related to TJ integrity (ie. increased paracellular flux suggests TJ disruption [213,233]). A variety of paracellular flux markers are used to investigate the effects of physiological and pathological agents on TJ integrity. The most frequently used paracellular markers include polyethylene glycols (PEG), fluorescein-5 and -6 sulfonic acid [235], inulin [119,236,237], fluorescein isothiocyanate dextrans (FITC-dextran), urea, mannitol, L-glucose [119,237], raffinose [238], atenolol [239] and lucifer yellow [240]. Size, shape, and charge of the solutes used control the permeability properties of any particular paracellular marker [233,241].
1.3 Rationale for the proposed research

Mycophenolic acid is a frequently used immunosuppressive agent and has a wide range of pharmacological actions. The present study was undertaken to identify novel molecular targets of MPA using a comprehensive 2-DE based expression proteomics approach. Whole cell lysates from HEK-293 cells which had been exposed to MPA were resolved by 2-DE, and differentially expressed proteins were identified by QTOF MS/MS analysis. In an attempt to examine effects with possible clinical relevance on a regulated protein, myosin light chain 2 (MLC2), we investigated the effects of MPA on TJ integrity using Caco-2 monolayers as a colonic cell culture model. After employing various physiological assays as well as immunoblotting and immunofluorescence analyses, we found that exposure to therapeutic concentrations of MPA modulated tight junction physiology via MLC2 phosphorylation. The current study may help to understand the etiology of MPA associated adverse intestinal effects.
2. Differential proteome analysis of human embryonic kidney cell line (HEK-293) following mycophenolic acid treatment

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2.1 Abstract

Mycophenolic acid (MPA) is widely used as a post transplantation medicine to prevent acute organ rejection. In the present study we used proteomics approach to identify proteome alterations in human embryonic kidney cells (HEK-293) after treatment with therapeutic dose of MPA. Following 72 hours MPA treatment, total protein lysates were prepared, resolved by two dimensional gel electrophoresis and differentially expressed proteins were identified by QTOF-MS/MS analysis. Expressional regulations of selected proteins were further validated by real time PCR and Western blotting. The proliferation assay demonstrated that therapeutic MPA concentration causes a dose dependent inhibition of HEK-293 cell proliferation. A significant apoptosis was observed after MPA treatment, as revealed by caspase 3 activity. Proteome analysis showed a total of 12 protein spots exhibiting differential expression after incubation with MPA, of which 7 proteins (complement component 1 Q subcomponent-binding protein, electron transfer flavoprotein subunit beta, cytochrome b-c1 complex subunit, peroxiredoxin 1, thioredoxin domain-containing protein 12, myosin regulatory light chain 2, and profilin 1) showed significant increase in their expression. The expression of 5 proteins (protein SET, stathmin, 40S ribosomal protein S12, histone H2B type 1 A, and histone H2B type 1-C/E/F/G/I) were down-regulated. MPA mainly altered the proteins associated with the cytoskeleton (26%), chromatin structure/dynamics (17%) and energy production/conversion (17%). Both real time PCR and Western blotting confirmed the regulation of myosin regulatory light chain 2 and peroxiredoxin 1 by MPA treatment. Furthermore, HT-29 cells treated with MPA and total kidney cell lysate from MMF treated rats showed similar increased expression of myosin regulatory light chain 2. The emerging use of MPA in diverse pathophysiological conditions demands in-depth studies to understand molecular basis of its therapeutic response. The present study identifies the myosin regulatory light chain 2 and peroxiredoxin 1 along with 10 other proteins showing significant regulation by MPA. Further characterization of these proteins may help to understand the diverse cellular effects of MPA in addition to its immunosuppressive activity.
2.2 Introduction

Mycophenolic acid (MPA) is a frequently used immunosuppressant for the prevention of acute rejection in patients undergoing allogenic renal, cardiac, lung, and liver transplantations [4,10]. MPA is a selective, reversible and uncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), a key regulatory enzyme in the de novo pathway of purine synthesis. It exhibits cytotoxic effects on most of the cell types, but exerts greater effects on T and B lymphocytes, thus preventing solid organ rejection [4]. IMPDH inhibition by clinically relevant concentration of MPA results in guanine nucleotide depletion which is associated with G1 cell cycle arrest. MPA also triggers apoptosis by up-regulating pro-apoptotic proteins (p53, p21 and bax) and down-regulating proteins that are important for cell cycle progression, such as bcl-2, survivin p27 and c-myc [242]. IMPDH type II is significantly over-expressed in several tumor cells, for this reason IMPDH could be considered as a potent target for anti-cancer therapy, as well as immunosuppressive chemotherapy [243].

MPA and its metabolites effect most of the cellular functions by influencing biological pathways, like apoptosis [244], immune associated signaling [245] and general cell signaling pathways involving mitogen-activated protein kinases, extracellular-signal regulated kinases, c-Jun N-terminal kinases, p53 and Rho-associated protein kinase [244,246,247]. Collectively, MPA possesses anti-microbial, anti-inflammatory, anti-fibrotic, pro-apoptotic [4], anti-angiogenic, anti-cancerous [248] and anti-oxidant activities [249]. Due to MPA diverse therapeutic activities in the cell, it is also used for the treatment of dermatological diseases, neuromuscular diseases and autoimmune disorders such as lupus [248,250]. Gastrointestinal tract (GIT) complications i.e., diarrhoea, nausea, abdominal pain, vomiting, anorexia, gastritis, intestinal ulceration and small intestinal villous atrophy are common complication for some transplant patients on MPA therapy. Other MPA associated adverse effects are anemia, myelosuppression and risk of opportunistic infections [251]. The exact molecular mechanism of MPA organ toxicity is unknown, but possible mechanisms include direct toxicity by its anti-proliferative effect, opportunistic infections due to myelosuppression and toxicity, and acyl MPA glucuronide (AcMPAG) proteins adduct formation [36,251].
Here we use HEK-293 cell line to uncover cellular protein response to the exposure of clinical dose of MPA. In the present study we used a proteomics based approach to resolve proteins of total cell lysates on two dimensional electrophoresis (2-DE) gels following treatment with DMSO and MPA. The differentially expressed proteins were in-gel tryptic digested and identified by QTOF-MS/MS analysis. Several proteins were identified with modified expression in response to MPA treatment which might be helpful to broaden our understanding regarding the cellular effects of MPA.

### 2.3 Materials and methods

#### 2.3.1 Reagents

Cell culture media (DMEM and MacCoy’s), fetal calf serum (FCS), phosphate buffer saline (PBS), penicillin and streptomycin were purchased from PAA Laboratories, Colbe, Germany. Urea, thiourea, dithiothreitol (DTT), trypsin, trifluoroacetic acid (TFA), sodium carbonate, ammonium bicarbonate, MPA and DMSO were purchased from Sigma-Aldrich, Steiheim, Germany. Acetonitril (ACN) was obtained from Promochem, Wasel, Germany. CHAPS was obtained from AppliChem, Darmstadt, Germany. Ampholytes, protein assay kit and immobilised pH gradient strips (IPG strips) were procured from Bio-Rad, Munich, Germany, while protease and phosphatase inhibitor cocktails were purchased from Roche, Mannheim, Germany. Bromophenol blue and trizma base were obtained from Carl Roth, Karlsruhe, Germany. Sodium dodecyl sulfate (SDS) was obtained from Serva, Heidelberg, Germany. Glycerin, potassium ferricyanide and sodium thiosulfate were purchased from Merck, Darmstadt, Germany and formic acid from BASF, Ludwigshafen, Germany.

#### 2.3.2 Cell culture

HEK-293 and HT-29 cell lines were purchased from German collection of microorganisms and cell cultures (DSMZ), Braunschweig, Germany. The cells were
grown in 75 cm² culture flasks (Sarstedt, Nuemberecht, Germany) and maintained in culture at 37°C in 95% humidity, 20% O₂ and 5% CO₂. DMEM and MacCoy’s media supplemented with L-glutamine, 10% fetal calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin was used to grow HEK-293 and HT-29 cells respectively.

2.3.3 Proliferation assay

Briefly, cells were grown in 96 well plates at a density of 3.5 X 10⁴ cells/well at least 24 h prior to the start of the experiment. The cells were then incubated with DMSO (control) or 0 to 100 µmol/L MPA for a period of 72 hr. After completion of incubation, proliferation was determined using ELISA based BrdU cell assay (Roche Diagnostics) according to manufacturer’s recommendations. Four independent experiments were performed. IC₅₀ values were calculated by a Grafit software package, version 5 (Erithacus Software, London, UK).

2.3.4 Sample preparation for proteome analysis

The HEK-293 and HT-29 cells were grown for 24 hr followed by treatment with DMSO or MPA (7.5 µmol/L and 10 µmol/L for HEK-293 and HT-29 respectively) for 72 h. Cells were harvested by scraping and were washed three times with ice cold PBS. After washing, cells were pelleted down at 250 x g for 10 min and lysed in a buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% w/v CHAPS, 2% ampholyte pH 3-10 and 1% DTT. The lysates were centrifuged and protein content was measured by Bradford assay [252] using Bio-Rad protein reagent (Bio-Rad, Munich, Germany) according to manufacturer’s instructions. Sample aliquots were kept at -80°C until further use. Protein lysate was prepared from 21 days MMF treated adult female Wistar rat’s kidney according to the previously reported protocol [253] and were used for Westernblotting.
2.3.5 2-DE

The 2-DE was performed as described by Gorg et al 2000 [254] with some minor modifications. Protein samples of HEK-293 cell (110 µg) were mixed with rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 0.2% ampholyte [pH 3-10], and 0.2% DTT) containing trace amount of bromophenol blue to a total volume of 350 µL. Samples were applied to linear IPG strips [pH 3-10], Bio- Rad) for 1 hr and then covered with mineral oil for passive rehydration overnight at room temperature. Iso-electric focusing (IEF) was performed in Protean IEF cell (Bio-Rad) with a program of 1 h at 100 volts, 1 h at 500 volts, 2 hr at 1000 volts and 8000 volts with a total of 32000 volts-hr. For the second dimension electrophoretic separation, focused strips were equilibrated for 30 min at room temperature in a buffer containing 50 mmol/L Tris-HCL [pH 8.8], 6 mol/L urea, 30% v/v glycerol, 2% SDS and 10 g/L DTT followed by an identical incubation but replacing DTT with 40 g/L iodoacetamide. The proteins in the equilibrated strips were then resolved on the 12.5% SDS-PAGE in a Protean II chamber (Bio-Rad) at 100 V /4°C.

2.3.6 Protein visualization, densitometric analysis and in-gel digestion

Gels were silver stained as described by Blum et al 1987 [255]. After fixation, gels were washed and sensitized. The gels were then incubated in freshly prepared silver nitrate solution (0.2% silver nitrate and 0.026% formaldehyde) for 20 min at room temperature followed by 3 times washes of 20 sec each in distilled water. Gels were placed in developing solution (6% sodium carbonate, 0.018526% formaldehyde and 6% sodium thiosulfate) until standard marker stained completely and adequate spots were visualized. Gels were scanned with a gel Cano scan 8400 (Canon, Tokyo, Japan). Densitrometric analysis was done by using Delta 2D software version 3.6 (Decodon GmbH, Gerifswald, Germany) [256]. Spot intensities were first normalized and the relative intensity of each spot was calculated by dividing the intensity of each spot by the sum of all spots intensities on the corresponding gel. Fold change, SD and Student’s t test probability were calculated using Microsoft excel software. Spots having at least 1.5 fold expressional changes (p < 0.05) were
considered statistically significant. Four independent 2-DE experiments were performed.

Differentially regulated protein spots were excised from the silver stained gel with a clean scalpel blade followed by in-gel digestion according to the method adopted and modified from Shevchenko et al [257]. Briefly, the gel pieces were washed twice in 100 mmol/L ammonium bicarbonate/acetonitrile (1:1, v/v) initially for 10 min and then until all visible dye was removed. The gel pieces were dried using vacuum centrifuge (UNIVAPO 150 H; uniEquip, Matinsried, Germany) followed by reconstitution in the trypsin digestion solution (10 ng/µL in 100 mmol/L ammonium bicarbonate) overnight at 37°C. After incubation the supernatant containing digested peptides was transferred to a tube and 50 µL of 0.1% TFA was added followed by sonication for 30 min. After sonication, the supernatant was pooled with the previous one. Two further extractions were collected in the same way using 0.1% TFA in 30% and then 60% ACN. The pooled extracts of peptides were dried in vacuum centrifuge and reconstituted in 0.1% formic acid.

## 2.3.7 Q-TOF LC-MS/MS analysis of protein identification

The reconstituted peptide samples (1 µL) were introduced onto µ-precolumn™ cartridge (C18 pepMap; 300 µm x 5 mm; 5 µm particle size) and further separated through a C18 pepMap 100 nano- Series™ (75 µm x 15 cm; 3 µm particle size) analytical column (LC Packings, Germering, Germany) using an CapLC autosampler (Waters, Eschborn, Germany). The mobile phase consisted of solution A (0.1% formic acid prepared in 5% ACN) and solution B (0.1% formic acid prepared in 95% ACN). The sample run time was set to 60 min and the flow rate of the pump to 5 µL/min. The exponential gradient was initiated at 5 min after loading from 10% to 95% for the period of 50 min. Tip flow rate of 250 nL/min was achieved through a flow splitter. The eluted peptides were injected into a Q-TOF Ultima Global (Micromass, Manchester, UK) mass spectrometer equipped with a nanoflow ESI Z-spray source in positive ion mode. Data was acquired by MassLynx (v 4.0) software and peak list (pkl file) was generated from acquired MS/MS raw data using ProteinLynx Global Server bioinformatics tool (PLGS; v 2.2; Waters, Manchester,
U.K.) under the following settings; Electrospray, centroid 80% with minimum peak width 4 channel, noise reduction 10%, Savitzky-Golay, MSMS, medium deisotoping with 3% threshold, no noise reduction and no smoothing.

The generated pkl files were searched using the online MASCOT \((\text{http://www.matrixscience.com})\) algorithm against the SwissProt data base release 15.5 (515203 sequence entries, 18134896 elements). The search criteria was set as follows: enzyme, trypsin; allowance of up to one missed cleavage peptide; mass tolerance ±0.5 Da and MS/MS tolerance ±0.5 Da; modifications of cysteine carboxamidomethylation and methionine oxidation. Proteins were finally identified on the basis of two or more peptides, whose ion scores exceeded the threshold, \(P < 0.05\), which indicated the 95% confidence level for these matched peptides. To ensure accurate identification, protein spots were digested from more than two gels and analyzed with MS. Proteins were considered as identified if the threshold was exceeded and the protein spot possessed the correct molecular weight and pl value of the corresponding spot on 2-DE.

### 2.3.8 Functional classification

Biological function annotations for all of the identified proteins were done by KOGnitor \((\text{http://www.ncbi.nlm.nih.gov/COG/grace/kognitor.html})\) [258].

### 2.3.9 Western blotting

Proteins were separated on 12.5% SDS-PAGE and blotted onto PVDF membrane (ImmobilonP, Millipore) using semidry Trans-Blot® SD cell system (Bio-Rad, Munich, Germany) for 30 min at 15 V in a blotting buffer (192 mmol/L glycine, 20% methanol, 25 mmol/L Tris [pH 8.3]). The membranes were blocked with 5% (w/v) skimmed milk repared in TBS-T buffer (50 mmol/L Tris–HCl [pH 7.5], 200 mmol/L NaCl, 0.05% Tween 20) for 1 hr at room temperature and washed twice with TBS-T buffer. The membranes were incubated with 1:1000 mouse anti Prdx1 antibody (Abcam, Cambridge, MA), 1:1000 rabbit anti MLC2 (Cell Signaling Technology, Inc., Danvers, MA) and 1:1000 mouse anti beta tubulin (Biovender,
Czech Republic) overnight at 4°C, followed by washes with TBS-T buffer. Membranes were further incubated with appropriate HRP-conjugated secondary antibodies for 1 hr at room temperature. The signals on the blots were detected by using ECL system (GE Healthcare) according to manufacturer’s instructions. Signal intensities from each Western blot were quantified by using Lab Image software, version 2.71 (Leipzig, Germany). β tubulin was used as a loading control and at least four independent experiments were performed.

2.3.10 RNA isolation and cDNA synthesis

RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s recommendations. Briefly, cells were scraped, washed and then homogenized in Trizol reagent. RNA was separated by chloroform/isopropanol precipitation method. The concentration of RNA was determined by the GeneQuant II RNA/DNA calculator (Pharmacia Biotech, Freiburg, Germany). The RNA quality was verified at OD$_{260}$/OD$_{280}$ nm ratios and subsequent electrophoretically on 1% agarose gels using ethidium bromide staining. The cDNAs were synthesized from 2 µg total RNA in a 30 µL reaction mix containing 1X reverse transcriptase (RT) PCR buffer (10 mmol/L Tris-HCL [pH 8.3], 15 mmol/L KCl, 0.6 mmol/L MgCl$_2$), 0.5 mmol/L of dNTPs mix, 1 U/µL RNase inhibitor and 13.3 U/µL M-MLV RT enzyme. The RT reaction was performed in a thermocycler (Biometra, Goettingen, Germany) at 42°C for 1 hr. cDNA was stored at -70°C until use.

2.3.11 Real-time PCR

Relative quantitative PCR were carried out using the LightCycler instrument (Roche Diagnostic Systems, NJ, USA). The primers for the human Prdx1 (forward 5’- TGGGGTCTTTAAGGCTGATG-3’ and reverse 5’-TCCCCATGTTTGTCAGTGAAG -3’), human MLC2 (forward 5’- CAGGAGTTCAAGAGCCTTCAAC -3’ and reverse 5’- CTGTACAGCTCATCCACTTCTCA -3’) and elongation factor 2 (forward 5’- GACATCACCAGGGTGTGCAG-3’ and reverse 5’-GGTGTCAGCACACTGGCATA-3) were designed by the Primer3 software (http://frodo.wi.mit.edu) [259]. The total volume of 20 µL PCR contained 1 µL of cDNA solution, 2 µL of 10X PCR buffer
(Invitrogen), 2 µL Syber green, 1 µL BSA, 1 µL DMSO, 0.25 µL of each primer (Eurofins MWG-Biotech, Ebersberg, Germany), 2.0 mmol/L MgCl₂, 0.2 mmol/L dNTPs mix and 0.15 U/µL PAN Script DNA polymerase (PAN Biotech, Aidenbach, Germany). The amplification conditions for Prdx1 and MLC2 were: initial denaturation 30 sec at 95°C and repeated cycles of denaturation (95°C for 1 sec), primer annealing (55°C for 5 sec), elongation (72°C for 10 sec), and fluorescence reading at 82 °C. For elongation factor 2 (EF-2) PCR conditions were similar to Prdx1 except for primer fluorescence reading which was measured at 88°C.

The relative expression of Prdx1 and MLC2 mRNA in the treated samples was determined as a fold increase compared with control samples using the comparative threshold cycle (C_T) method \(2^{-\Delta\Delta C_T}}\) (ΔΔC_T = ΔC target genes − ΔC reference gene) [260]. EF-2 was used as the internal control gene. Experiments were performed four times. Statistical difference (p value) in mRNA expression level between MPA and DMSO samples were calculated using the Mann-Whitney U test. The PCR product was run on a 1% ethidium bromide-agarose gel to confirm the presence of desired specific amplified product.

### 2.3.12 Apoptosis assay

The caspase 3 activity was measured using CaspACE™ Assay kit (Promega Corporation, WI, USA) according to the manufacturer's protocol. Cells were treated with DMSO and MPA for 72 hr, harvested and briefly suspended in lysis buffer. Proteins were extracted and quantified by Bradford method [252]. Briefly, 70 µg of protein lysate were mixed with reaction mixtures containing colorimetric substrate peptides specific for caspase 3 (DEVD-pNA) and then incubated at room temperature for overnight. The absorbance of the cleaved p-nitroanilide from the substrate DEVD-pNA was measured at 405 nm using EL808 microplate reader (Bio-Tek instruments, VT, USA). Five independent experiments were performed.
2.4 Results

In the present study the alteration in the cellular proteome by the MPA treatment was investigated using HEK-293 as cell culture model. Incubation of HEK-293 cells with MPA followed a dose dependent inhibition of cell proliferation (Figure 2.1). The IC$_{50}$ concentration (7.5 µmol/L or 2.4 mg/L) of MPA was selected as standard dose for further analysis, which is within the therapeutic range (0.3 to 3.4 mg/L) [261]. Cells were treated with MPA and DMSO (as vehicle) for 3 days, and total cell lysates were prepared. Total protein extracts of MPA and DMSO treated cells were separated by 2-DE using pH 3-10 linear IPG strips and visualized by silver stain. The protein spots which showed ≥±1.5 fold change (p < 0.05 using Student’s t test) as compared to DMSO treated controls were considered as differentially expressed proteins. Statistical analysis showed that a total of 12 proteins exhibited significantly altered expression due to MPA treatment (Table 2.1). The altered expression pattern of the HEK-293 proteins by MPA is shown in Figure 2.2.

Among 12 regulated proteins spot under MPA treatment, 7 proteins were significantly up-regulated and 5 proteins showed down-regulated expression. The up-regulated spots under MPA treatment were identified as complement component 1Q subcomponent binding protein (C1q), electron transfer flavoprotein subunit beta, cytochrome b-c1 complex subunit, thioredoxin domain-containing protein 12, myosin regulatory light chain 2 (MLC2), peroxiredoxin1 (Prdx1) and profilin 1. Five proteins, which showed down-regulated expression, were identified as protein SET, stathmin, 40S ribosomal protein S12, histone H2B type 1-A, and histone H2B type 1-C/E/F/G/I. A bar diagram, showing relative abundance (% Vol), SD and statistical significance of all the significantly regulated protein is provided as figure 5.1. Figure 2.2 shows an exemplary gel of DMSO (vehicle) and MPA with marked regulated proteins. The extent of regulation in protein expression with predicted and actual pl, as well as molecular masses with their SwissProt accession numbers are provided in Table 2.1 and MS/MS spectral information is provided in the figure 5.2.
Table 2.1. Differentially regulated proteins by MPA in HEK-293 cells identified by mass spectrometry

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<th>Spot No</th>
<th>Acc</th>
<th>M/M_o (kDa)</th>
<th>Score</th>
<th>pI_t/pI_o</th>
<th>Pep</th>
<th>Protein name</th>
<th>Function</th>
<th>Expression change (in folds)</th>
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<td>31.3/31.0</td>
<td>141</td>
<td>4.74/4.5</td>
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<td>8.27/8.14</td>
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<td>Cytoskeleton</td>
<td>1.51**↑</td>
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</table>

Acc: Accession number; Mt: theoretical molecular mass; Mo: observed molecular mass; pIt: theoretical isoelectric point; pIo: observed isoelectric point; pep: number of peptides sequenced for identification; Score: Peptide mass fingerprint probability score as defined by Mascot (www.matrixscience.com). Individual ions score >42 indicate identity or extensive homology (p < 0.05); ↓: down-regulated; ↑ up-regulated; *p < 0.05, **p < 0.005. Molecular function determined from the online protein reference database KOGnitor NCBI. (http://www.ncbi.nlm.nih.gov/COG/grace/kognitor.html).
Figure 2.1: Inhibition of HEK-293 cells proliferation by MPA treatment.
The cell proliferation was determined after 72 hr of treatment with different doses of MPA (0–100 µmol/L) using BrdU colorimetric based method. Results are shown as percentage of control (DMSO treated) and represent four independent experiments.

Figure 2.2: Differential protein expression after incubation of HEK-293 cells with MPA.
Total protein lysate from DMSO and MPA treated cells was separated by 2-D gel electrophoresis and silver stained. Encircled differentially regulated proteins spots were identified using Q-TOF MS/MS analysis. The figure shows exemplary 2-DE gels of DMSO and MPA treated HEK-293 cells.
Functional classification of differentially regulated proteins was done using KOGnitor, an online biological function annotation tool [258]. The proteins altered by MPA treatment belong to various categories i.e., cytoskeleton (26%), chromatin structure/dynamics and energy production/conversion (17% each) (Figure 2.3). Gels spot diagram of two selected protein spots (MLC2 and Prdx1) in 4 biological replicates are shown in Figure 2.4a.

To validate the 2-DE results, the expression of MLC2 and Prdx1 were confirmed by Western blotting and real time PCR analysis. Expression of Prdx1 and MLC2 were up-regulated at both transcriptional (Figure 2.4b) and protein level (Figure 2.4c). Specifically, MPA increased MLC2 protein (Mean fold: +1.78, p < 0.005, n = 4, Western blotting) and mRNA expression (Mean fold: +2.25, p < 0.05, n = 4, real time PCR). Prdx1 expression was also up-regulated, both at protein level (Mean fold: +2.73, p < 0.005, n = 4) and mRNA level (Mean fold: +1.93, p < 0.05, n = 4). To check whether over-expression of MLC2 following MPA treatment is only HEK-293 cells specific, we determined MLC2 expression in total protein lysate prepared from kidney of MMF (pro-drug of MPA) treated rats (Figure 2.5a) and MPA treated HT-29
cells (Figure 2.5b). MLC2 expression was increased both in kidney total protein lysate and HT-29 cells by (Mean fold: +2.57, p < 0.005, n = 4) and (Mean fold: +1.95, p < 0.005, n = 4) respectively.

Figure 2.4: Differential expression of Prdx1 and MLC2 by MPA treatment.
(a) Selected areas in the silver stained gels showing differential expression of Prdx1 and MLC2. Delta 2D software was used for densitometric analysis. The quantification of the level of expression (% volume) in MPA treated cells and control cells (DMSO) is illustrated as a bar chart with the mean and SD of four separate experiments (*p < 0.05). (b) Expression patterns of Prdx1 and MLC2 genes determined by real-time PCR. The relative expression of Prdx1 and MLC2 mRNA in the treated samples was determined as a fold change compared with control samples using the comparative threshold cycle (C_T) method (2^-ΔΔC_T) as described in materials and methods part. Results shown are representative of four independent experiments. EF-2 was used to normalize the values. The boxes represent range in variation statistics and the lines across the boxes represent the medians and the whiskers extend to the highest and lowest values. Significance was calculated using the Mann-Whitney-U test (*p < 0.05) (c) Effect of MPA treatment on Prdx1 and MLC2 protein expression. Protein extracts from MPA and DMSO treated cells were Western blotted using specific antibodies against Prdx1 and MLC2. Densitometric analysis was done using Lab image version 2.71 software. β tubulin signal was used to control the equal protein load. The experiments were repeated four times and error bars represent ± SD (**p < 0.005).
Figure 2.5: Expression of MLC2 in MMF treated rat kidney lysate and HT-29 cells.
Protein lysate was prepared and immunoblotted for MLC2 as described in method section. β tubulin was used to show equal protein load. Lab image software was used for quantification of protein bands. Four independent experiments were performed and results presented as mean ± SD (**p < 0.005).

Figure 2.6: Measurement of MPA induced caspase-3 activity.
Cells were treated with MPA and DMSO for 72 hr. Protein extracts from each was measured for caspase-3 activity. Five independent experiments were performed and results presented as mean absorbance ± SD (**p < 0.005).
To demonstrate the effect of MPA on cell apoptosis, caspase-3 activity (apoptosis marker) was determined using a commercially available colorimetric assay. There was a significant difference in caspase-3 activity between MPA and DMSO treatment groups. MPA increased mean absorbance by 2 fold (p < 0.005, n = 5) as compared to DMSO treated cells. The results from caspase-3 assay revealed that MPA treated cells exhibit more apoptosis than cells treated with DMSO alone (Figure 2.6).

2.5 Discussion

We have used a 2-DE and mass spectrometric based proteomics approach to develop a better understanding of the influence of MPA therapeutic dose on the proteome in HEK-293 cells. HEK-293 cells are widely used cell culture model to study the mechanisms of drug action, investigating drug targets and molecular aspects of xenobiotic toxicity [262-264]. The regulated proteins are found to be involved in diverse functions including apoptosis and cell signaling mechanism. Apoptosis assay showed that MPA has a pro-apoptotic role in HEK-293 cell line, a property which makes it a drug with potential anti-tumor activities. MLC2 is an important myosin regulatory subunit, which regulates smooth muscle and nonmuscle cells contractile activity [265]. MLC2 displayed an increased expression by MPA treatment. It is already reported that MPA influences the cellular cytoskeletal architecture via modulating mesangial actin reorganization by activating actin polymerization and inhibiting actin-depolymerization [266,267]. Phosphorylation of MLC2 causes significant changes in the physiological dynamics of actin cytoskeleton, leading to barrier defects in intestine [184], heart [268] and lungs [269]. However, it remains unclear if such cytoskeleton reorganization in different organs may lead to a completely different outcome, for example in intestine, diarrhoea is associated with MPA therapy in some patients [251]. In the present study, we observed that MLC2 over-expression is not limited to a specific cell type (i.e. HEK-293) but was reproducible in MMF treated rat kidney and in MPA treated HT-29 cells protein lysates.
We observed an increase Prdx1 expression by MPA treatment, both at gene and protein level. Prdx1 is a cytoplasmic stress-inducible anti-oxidant enzyme and a major member of peroxiredoxin family [270]. Cells deficient in Prdx1 have increased sensitivity to oxidative DNA damage [271]. Prdx1 along with its anti-oxidant activity also possesses anti-inflammatory and anti-atherogenic effects [272]. Oxidative stress contributes to the pathophysiology of diverse clinical conditions, including ischemia-reperfusion mediated post transplantation graft injuries [273]. Prdx1 expression was also reported to be up-regulated in human gingival fibroblasts by cyclosporine A (another commonly used immunosuppressive drug) treatment [274]. MPA has previously been reported to diminish oxidative injuries and induce anti-oxidant effects by preventing the production of reactive oxygen species [249]. Furthermore, MPA exerts lesser oxidative stress in renal transplant patients, as compared to everolimus, cyclosporine and other calcineurin inhibitors [275,276].

Prdx1 contribute to the inhibition of tumorigenesis through PTEN/Akt pathway [277] and its lower expression in the tumor indicated high tumor proliferation, increased metastasis and could be used as cancer biomarker [278]. Prdx1 is also involved in ageing process as Prdx1-deficient mice have a shortened lifespan and other malignancies [271]. Anti-tumor drugs like histone deacetylase inhibitors (HDACIs) activate Prdx1, a tumor suppressor, which leads to apoptosis [279]. Previously it was observed that MPA also inhibit histone deacetylases (HDACs) [21]. A further investigation is needed to gain a deeper insight into the Prdx1 regulation by MPA through HDACs inhibition interaction with Prdx1 and its role in anti-tumor activities.

Profilin 1, another cytoskeletal protein was up-regulated by MPA treatment. Profilins are widely distributed actin binding proteins [280], involved in actin filament dynamics and several signaling pathways [281]. Profilin 1 over-expression has been reported to cause cell proliferation inhibition, apoptosis induction and tumor suppression [282]. Whether MPA via profilin over-expression exerts extended anti-proliferative or anti-tumor activities requires further investigation. Stathmin was down regulated by MPA. Stathmin is a 19 kDa cytoplasmic protein, which plays an important role in the regulation of the microtubule cytoskeleton. Stathmin regulates microtubule turnover by promoting microtubules depolymerization and hydrolyze
guanosine triphosphate (GTP) from terminal tubulin, preventing polymerization of tubulin heterodimers [283]. Previously, our group demonstrated that AcMPAG alters tubulin polymerization in a concentration-dependent manner [284]. Furthermore, stathmin repression stabilizes microtubules, inhibits angiogenesis [285] and suppress tumors [286].

Thioredoxin domain-containing protein 12, also known as endoplasmic reticulum resident protein 18 (ERp18) is ubiquitous in mammalian cells and acts as a disulfide isomerase in the endoplasmic reticulum (ER). It provides defense against oxidative stress, refolds disulfide-containing proteins, and regulates transcription factors [287]. ERp18 expressional up-regulation might cause cell adoptivity in response to MPA induced ER stress. SET protein was down-expressed by MPA. SET, a major cellular serine threonine phosphatase is a potent inhibitor of protein phosphatase 2A (PP2A) activity [288] and a negative regulator of histone acetylation [289], thus involved in cell growth and signaling cascades [290]. PP2A expression induced by down-regulation of SET leads to the apoptosis and growth suppression [291].

MPA triggers nuclear stress and causes disruption of the nucleus, leading to the activation of p53, which may initiate cell cycle arrest and apoptosis [292]. In the present study histone H2B was down-regulated by MPA treatment, which is a major component of eukaryotic nucleosome core. Post translational modification such as methylation, acetylation, phosphorylation and ubiquitination of histone proteins alter transcription, DNA replication, and DNA repair [293,294]. Previous data showed that MPA mediated down-regulation of HDAC2 which might relate with potential epigenetic regulations [21]. The microrarray analysis of mononuclear cells treated with AcMPAG (a metabolite of MPA) showed down-regulation of histones in a previous study by our group [295].

MPA affects ribosomal machinery by decreasing intracellular guanine nucleotide level, depending on dosage and cell type, resulting in global reduction of RNA synthesis [292]. Other studies suggested that guanine nucleotide depletion by IMPDH leads to a decrease in pre-ribosomal RNA synthesis, nuclear disruption, and p53 activation [296]. Disorganization of nuclear and ribosomal biogenesis is suggested to be an effective therapeutic target in cancers [297]. We observed a
down-regulation of 40S ribosomal protein S12 by MPA, which might be due to the altered ribosome biogenesis. The proapoptotic stimuli including chemotherapeutic agents induced a dose-dependent increase in the expression of the cytochrome c proteins [298].

In the present study we also observed up-regulation of cytochrome b-c I complex by MPA which suggests a possible role of MPA in the regulation of energy metabolism. Complement component 1 Q subcomponent-binding protein (C1q), a component of complement system involved in the clearance of apoptotic cells was up-regulated by MPA. C1q binds to surface blebs of apoptotic cells, which follows subsequent phagocytosis [299]. C1q deficiency leads to a significant decline in the clearance of apoptotic cells in both C1q- and C4-deficient mice, causing glomerulonephritis [300]. MPA causes cellular apoptosis and cells might utilize C1q over-expression to clear the apoptotic cells.

2.6 Conclusion

This investigation identifies proteins related to diverse cellular functions which altered their expression by MPA treatment; many of which are reported for the first time in this context. The expression of Prdx1 (involved in apoptosis) and MLC2 (protein important for epithelial barrier integrity) were observed to be regulated at RNA and protein level. Further investigations of the regulated proteins will provide new insights into the cellular pathways influenced by MPA therapy and could help in more rational use of MPA in transplantation medicine.
3. Mycophenolic acid mediated disruption of the intestinal epithelial tight junctions

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Submitted
3.1 Abstract

Gastrointestinal toxicity is a common adverse effect of mycophenolic acid (MPA) treatment in solid organ transplantation patients, through poorly understood mechanisms. Phosphorylation of myosin light chain 2 (MLC2) is associated with epithelial tight junction modulation which leads to defective epithelial barrier function, and has been implicated in gastrointestinal diseases. The aim of this study was to investigate whether MPA could induce epithelial barrier permeability via MLC2 regulation. Human colonic cells (Caco-2) monolayers were exposed to therapeutic concentrations of MPA, and MLC2 and myosin light chain kinase (MLCK) expression were analysed using PCR and immunoblotting. Permeability was assessed by measuring transepithelial resistance (TER) and the flux of paracellular permeability marker FITC-dextran across the epithelial monolayers. MPA increased the expression of both MLC2 and MLCK at both the transcriptional and translational levels. In addition, the amount of phosphorylated MLC2 was increased after MPA treatment. Confocal immunofluorescence analysis showed disrupted distribution of tight junction proteins (ZO-1 and occludin) after MPA treatment. This MPA mediated tight junction disruption was not due to apoptosis or cell death. AcMPAG, a reactive metabolite of MPA, also showed similar effects on TER and TJ proteins expression and distribution. Additionally ML-7, a specific inhibitor of MLCK was able to reverse both the MPA mediated decrease in TER and the increase in FITC-dextran influx, suggesting a modulating role of MPA on intestinal epithelial barrier permeability via MLCK activity. These results suggest that MPA induced alterations in MLC phosphorylation may have a role in the patho-physiology of intestinal epithelial barrier disruption and may be responsible for the adverse effects of MPA on the intestine.
3.2 Introduction

The tight junctions (TJs) are intercellular, multifunctional complexes present in the epithelial and endothelial cells which form the paracellular diffusion barrier [97,100]. This barrier contributes to the regulation of epithelial permeability and intramembrane diffusion of ions and solutes through the paracellular space [301-303]. TJs are comprised of transmembrane (occludin, claudins and junctional adhesion molecules) and peripheral membrane proteins (zonula occludins [ZO-1], membrane-associated guanylate kinase, and the Ras-related protein Rab13). These proteins interact with each other to form a complex protein network [304]. Various intestinal and non-intestinal disorders including inflammatory bowel disease, celiac disease, and diarrhoeal infections are characterized by barrier dysfunction which is thought to play a crucial role in their pathogenesis [303].

Mycophenolic acid (MPA) is the active agent in the two currently commercially available formulations: the MPA ester mycophenolate mofetil (MMF) and the enteric-coated salt mycophenolate sodium (EC-MS) [7]. After oral ingestion, MPA is liberated in the gastrointestinal tract, absorbed and metabolized in the liver to form MPA glucuronide (MPAG) and two other metabolites, 7-O-glucoside and acyl glucuronide (AcMPAG). AcMPAG is pharmacologically active and believed to be responsible for some MPA associated GI tract adverse effects [30]. MPA is an immunosuppressant which is frequently used for the prevention of acute transplant rejection. MPA is also used for the treatment of non-transplant, autoimmune, renal, rheumatological, gastrointestinal, ophthalmological, dermatological and neurological diseases [250].

Several immunosuppressive drugs including MPA used in solid organ transplantation lead to diarrhoea [305]. Various possible aetiologies of this diarrhoea have been described including infectious agents, drug reactions, metabolic alterations, and surgical complications. MPA has been claimed to account for 50% of all drug induced post-transplantation diarrhoea [306], while 20% of total MPA complications involve the GI tract [307,308]. GI symptoms similar to those seen with Crohn’s disease and enterocolitis are also observed in patients receiving MPA therapy [65,309-312]. The underlying mechanisms of MPA induced GI toxicity remain unclear; however, several hypotheses exist including direct toxicity as a
result of its anti-proliferative effects, myelosuppression induced opportunistic infections, variations in local immune response, and AcMPAG adduct toxicity [36,251,305].

Several GI associated abnormalities, including inflammatory bowel disease (Crohn's disease and ulcerative colitis), and Graft verses host disease are characterized by epithelial barrier defects which contribute to increased intestinal permeability [313]. The effects of MPA or its metabolites on cell junction biophysical properties including paracellular permeability, and the regulation of TJ proteins, especially in relation to intestinal barrier defects, have not been well studied. Studies were conducted to explore the molecular effects of MPA and its active AcMPAG metabolite on gut integrity via possible effects on TJs. We used Caco-2 cell monolayers as in vitro model of intestinal epithelia [314] and incubated them with therapeutic concentrations (3.1 mg/L, or 10 µmol/L) of MPA and (10 µmol/L) AcMPAG. Trans-epithelial resistance (TER) measurements, paracellular influx assays, immunoblotting and immunofluorescence analyses were then conducted to evaluate integrity of the TJs complex. We hypothesized that MPA may modulate the TJs by altering expression and distribution of crucial TJs proteins.

3.3 Materials and methods

3.3.1 Reagents

Reagents (and their sources) included: agarose (Gibco BRL, Paisley, UK), Magnesium chloride (MgCl₂), M-MLV RT enzyme and 5X buffer (Invitrogen, Karlsruhe, Germany), deoxynucleotide triphosphate (dNTP) (Roche, Mannheim, Germany), Ribonuclease (RNAase) inhibitor (Promega, Mannheim, Germany), MPA, fluorescein isocyanate-dextran 4 KDa (FD4), 1-5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7) and cytochalasin (CD) (Sigma-Aldrich, Mannheim, Germany) and PCR primers (Eurofins, Ebersberg, Germany). AcMPAG was a kind gift from Roche (Roche, Mannheim, Germany).
3.3.2 Cell culture

The human colon adenocarcinoma cell line (Caco-2) was purchased from DSMZ (German collection of microorganisms and cell cultures, Braunschweig, Germany). Tissue culture media ingredients were obtained from PAA Laboratories (Pasching, Austria). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (4.5 g/L glucose) supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L glutamine, 50 IU/mL penicillin, 50 mg/mL streptomycin and non-essential amino acid supplement (1% v/v) under conditions of 37°C, 5% CO₂ and 90% relative humidity. The Caco-2 cells were allowed to grow for 21 days of post-confluence to form differentiated and polarised monolayer growth [315]. The culture medium was changed every second day.

3.3.3 Lactate dehydrogenase (LDH) measurement

LDH measurements were performed using a commercially available LDH measurement kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. This assay is based on the principle that LDH catalyzes the conversion of NADH (substrate) to NAD and the rate of this conversion is directly proportional to LDH activity. Briefly, cells were incubated in DMSO, 10 µmol/L MPA or 10 µmol/L AcMPAG for 72 hr. Following incubation, supernatant medium was collected, centrifuged for 5 min at 15,700 x g at 4°C and LDH was measured photometrically using a Hitachi analyzer (Roche, Mannheim, Germany). The experiments were repeated at least four times and values were represented as mean IU/L ± SEM.

3.3.4 Determination of caspase 3 activity

Cell were treated with DMSO, 10 µmol/L MPA or 10 µmol/L AcMPAG for 72 hr and the caspase specific activity was measured using CaspACE™ Assay kits (Promega, WI, USA) as previously described [316]. Briefly, cell proteins (70 µg) were mixed with reaction mixtures containing the colorimetric substrate Ac-DEVD-p-nitroanaline (Ac-DEVD-pNA). The pNA released from Ac-DEVD-pNA due to caspase
activity was measured at a wavelength of 405 nm using a EL808 microplate reader (Bio-Tek instruments, VT, USA). Caspase 3 specific activity (CSA) in the cell extract was measured using the standard formula (CSA = pmol pNA liberated per hour/ µg protein). Five independent experiments were performed and results were expressed as mean pmol pNA liberated per hour/ µg protein.

3.3.5 Determination of trans-epithelial resistance (TER)

TER was measured as previously described [317]. Briefly, cells were seeded on polyester transwell inserts (6.5 mm diameter, 0.4 µm pore size, 0.33 cm² growth area, Corning Costar Corporation, NY, USA) at 2.0 x 105 cells/well and grown for 21 days post-confluence. Cells were treated with DMSO, 10 µmol/L MPA, 10 µmol/L AcMPAG or CD (10 µmol/L) for 72 hr or pre-treated with ML-7 (10µmol/L) for 1 hr followed by 72 hr treatment of MPA (10 µmol/L) after cells developed into a differentiated and polarised monolayer. TER was measured using an EVOM voltohmmeter with a STX2 electrode (WPI, FL, USA). For epithelial resistance measurements, both the apical and basolateral sides of the epithelia were bathed in cell culture medium. Resistance (TER) = [RC - RE] X A; where RC is resistance of the cells (Ω); RE is resistance of the blank (Ω); and A is surface area of the membrane insert (cm²). TER was calculated as Ω.cm² for at least four consecutive measurements.

3.3.6 FITC-dextran paracellular permeability

Epithelial permeability was assessed using a previously reported method [318,319]. Briefly, Caco-2 cells were grown into monolayers and treated as described above. Following treatment, cells were rinsed with PBS and incubated in Hank’s balanced salt solution containing 1mg/mL FITC-dextran 4 kD (FD4) solution for 2 hr. Permeability marker flux was assessed by taking 100 µL from the basolateral chamber. Fluorescent signal was measured using a Lambda fluoro 320 fluorescence plate reader (MWG Biotech, Ebersberg, Germany) using 492 nm excitation and 520 nm emission filters. FD4 concentrations were determined using standard curves
generated by serial dilution of FD4. Fluxes were calculated using the apparent permeability coefficient ($P_{\text{app}}$) equation: $P_{\text{app}} = \frac{\Delta C_A / \Delta t}{V_A / A * C_L}$, where $P_{\text{app}}$ is the apparent permeability (cm/s), $\Delta C_A$ is the change of FD4 concentration, $A$ is the surface area of the membrane (cm$^2$), $\Delta t$ is the change of time, $V_A$ is the volume of the abluminal medium, and $C_L$ is the initial concentration in the luminal chamber.

3.3.7 RNA isolation, cDNA synthesis and real-time PCR

Total cellular RNA was extracted using the acid guanidinium-phenol-chloroform method (Trizol reagent; Invitrogen, CA) according to manufacturer’s recommendations. Briefly, Caco-2 monolayers were scraped into Trizol reagent, homogenized, and RNA was extracted using chloroform/isopropanol precipitation. The precipitated RNA was dissolved in sterile water and stored at -80°C until analysis. RNA concentration was determined with the GeneQuant II RNA/DNA calculator (Pharmacia Biotech, Freiburg, Germany) and quality was verified by OD$_{260}$/OD$_{280}$ nm ratios and subsequent electrophoresis in 1.5% agarose gels using ethidium bromide staining. cDNA was synthesized from 2 µg total RNA in a 30 µL reaction mix containing 1x RT-PCR buffer (10 mmol/L Tris-HCl [pH 8.3], 15 mmol/L KCl, 0.6 mmol/L MgCl$_2$), 0.5 µmol/L of each dNTP, 1 U/µL RNase inhibitor and 13.3 U/µL M-MLV RT enzyme. The RT reactions were performed in a thermocycler (Biometra, Goettingen, Germany) at 75°C for 5 min, and then 42°C for 1 hr. cDNA was stored at -80°C until use. Primers for real time PCR were selected using the online Primer 3 software [320]. The primers used in this study were as follows: MLC2 (forward 5’-CAGGAGTTCAAGAGGCCTTAAC-3’, reverse 5’-CTGTACAGCTCATCCACTTCTCA-3’); MLCK (forward 5’-CAACAGGGTCACCAACCAGC-3’, reverse 5’-GCCTTGCAGGTACTTGGC-3’); ROCK (forward 5’-GTGAAGGTGATTGGTAGAGGTGC-3’, reverse 5’-CCACCAGGCAATGTATTCCATC-3’) and elongation factor 2 (forward 5’-GACATCACAAGGGTGTGCAG-3’, reverse 5’-GCGGTCAGCACA CACTGGCAT-3). Relative quantitative PCR was carried out using the LightCycler instrument (Roche, Manheim, Germany). The total PCR volume of 20 µL contained 1 µL of cDNA solution, 2 µL of 10X PCR buffer (Invitrogen, Darmstadt, Germany), 2 µL syber
green, 1 µL BSA, 1 µL DMSO, 0.25 µL of each primer (Eurofins MWG-Biotech AG, Ebersberg, Germany), 2.0 mmol/L MgCl$_2$, 0.2 mmol/L of each dNTP, and 0.15 U/µL PAN Script DNA polymerase (PAN Biotech, Aidenbach, Germany). Amplification conditions were set to: MLC2 (initial denaturation 30 sec at 95°C, repeated cycles of denaturation at 95°C, for 1 sec, primer annealing at 55°C for 5 sec, elongation at 72°C for 10 sec, and fluorescence reading at 82°C), MLCK (initial denaturation for 30 sec at 95°C and repeated cycles of denaturation at 95°C for 1 sec, primer annealing at 60°C for 5 sec, elongation at 72°C for 10 sec, and fluorescence reading at 82°C). ROCK (initial denaturation for 30 sec at 95°C and repeated cycles of denaturation at 95°C for 1 sec, primer annealing at 60°C for 5 sec, elongation at 72°C for 10 sec, and fluorescence reading at 80°C), elongation factor 2 (EF-2) (initial denaturation for 30 sec at 95°C, repeated cycles of denaturation at 95°C, for 1 sec, primer annealing (55°C, 5 sec), elongation (72°C, 10 sec), and fluorescence reading at 88°C). For each sample, real-time PCR reactions were performed in quadruplicate. RNA relative expression was calculated as fold change using the comparative threshold cycle (C$_T$) method ($2^{-ΔΔC_{T}}$) [260] with EF-2 used as the internal control gene. The relative expression of mRNA in the treated samples was determined as a fold increase compared with control samples. The PCR product was run on 1.5% agarose gel electrophoresis to confirm the specificity of the amplified product.

### 3.3.8 Immunoblotting

Protein lysates were separated by SDS-PAGE and blotted onto PVDF (Immobilon, Millipore, MA, USA) using the Trans-Blot SD cell system (Bio-rad, Munich, Germany) for 30 min at 15 V in a blotting buffer (192 mmol/L glycine, 20% methanol, and 25 mmol/L tris [pH 8.3]). The membranes were blocked with 5% (w/v) milk in TBS-T buffer (50 mmol/L TrisHCl [pH 7.5], 200 mmol/L NaCl, 0.05% Tween 20) for 1 hr at room temperature followed by washing twice in TBS-T for 5 min. The membranes were incubated with a 1: 500 dilution of a mouse monoclonal anti-MLC antibody (Sigma, Mannheim, Germany), 1: 10000 dilution of mouse monoclonal anti-MLCK antibody (Sigma, Mannheim, Germany), 1: 1000 rabbit anti-phospho MLC antibody (Cell Signaling, Beverly, USA), 1 µg/mL rabbit anti-ZO-1, 0.5 µg/mL mouse anti-occludin (Zymed, CA, USA), or 1: 5000 anti-β actin (Sigma, Mannheim,
Germany) in 5% BSA in TBS-T overnight at 4°C. Following washing in TBS-T, membranes were then incubated with appropriate HRP-conjugated secondary antibodies (Bio-rad, Munich, Germany). The membranes were washed with PBS and prepared for enhanced chemiluminescence (GE, Buckinghamshire, UK) according to the manufacturer's instructions. Developed membranes were then exposed to hyperfilm-ECL (GE, Buckinghamshire, UK). The films were scanned and protein band densities were quantified with the Lab Image software, version 2.71 (Kapelan, Leipzig, Germany).

3.3.9 Immunofluorescence microscopy of TJs proteins

Cell monolayers were grown on Lab-Tek™ eight chamber slides (Nunc, Naperville, IL, USA) and treated as indicated above. Cells were immunolabelled as previously described [321] with some modifications. Briefly, cells were rinsed with PBS and fixed in 3.7% formaldehyde at room temperature for 20 min. Cell monolayers were then rinsed in PBS and permeabilized in 0.2% Triton X-100 for 7 min at room temperature. Cells were rinsed in PBS followed by blocking with 1% bovine serum albumin (BSA) for 30 min at room temperature. Cells were incubated with 3 µg/mL anti-rabbit ZO-1 and 2 µg/mL anti-mouse occludin (Zymed, San Francisco, USA) overnight at 4°C. After washing with PBS, cells were incubated with anti-rabbit IgG conjugated to Alexa 488 and anti-mouse IgG conjugated to cydye 3 (Molecular Probes, Eugene, OR, USA) in 1% BSA for 1 hr at room temperature. For F actin localization cells were incubated in 0.33 µg/mL of FITC-conjugated phalloidin (Sigma-Aldrich, St. Louis, USA) in PBS for 30 min as described previously [322]. Cells were also incubated with Hoechst dye (10 µg/mL in PBS) (Molecular Probes, Eugene, USA) for 10 minutes to stain nuclei. After washing with PBS, cells were mounted using the Dako fluorescence mounting medium (Dako, Carpintera, USA) and stored at 4°C in the dark until analyzed. The fluorescence was visualized using Axiovert 200M confocal microscope (Carl Zeiss, Jena, Germany). All of the fluorescent labelling experiments were repeated four times to ensure reproducibility.
3.3.10 Statistics

The data are presented as sample means with error bars indicating the standard error of the mean. The p value was calculated using a Student's t test and a p value <0.05 was considered statistically significant.

3.4 Results

3.4.1 MPA altered TER and TJs permeability in a concentration and time dependant manner

In the present study, the effect of MPA on Caco-2 TJ integrity was determined by measuring TER and epithelial permeability to the paracellular marker FD4. To assess the influence of MPA treatment on TER, cells were incubated with different concentrations of MPA (5-100 µmol/L) for up to 72 hr. DMSO did not have any significant effect on TER of polarised Caco-2 cell monolayers. Increasing concentrations of MPA exhibited concentration- and time-dependant decreases in Caco-2 TER (Figure 3.1a). The mean cell monolayer TER decreased from 190.4 to 181.5, 190.8 to 147.5 and 193.1 to 120.4 Ω.cm² after 5, 10 and 50 µmol/L MPA treatment respectively. The maximal decrease in Caco-2 TER was observed at 100 µmol/L MPA concentration (86 ± 0.7 Ω.cm²). The decrease in Caco-2 TER increased with time between 12 hr and 72 hr (Figure 3.1a).

Similarly, MPA was associated with a concentration-dependent increase in Caco-2 paracellular permeability to FD4 (Figure 3.1b). FD4 permeability analysis following 72 hr MPA treatment showed a concentration-dependant increase in FD4 influx. The FD4 influx from the apical to the basolateral chamber was increased 1.5, 2.7, 4.6 and 7.9 fold after incubation with 5, 10, 50 and 100 µmol/L MPA concentrations respectively (Figure 3.1b).
Figure 3.1: MPA treatment decreased TER and increased FD4 permeability of Caco-2 cell monolayers.

Caco-2 cells were cultured on filter inserts and grown for 21 days post-confluence to form differentiated monolayers. (a) Caco-2 cells were treated with MPA (5-100 µM) for 0-72 hr. MPA concentration and time dependent decrease in TER were observed. Graph shows TER (Ω.cm²) vs. time (hr) with means ± SEM from four independent experiments. (b) Paracellular flux of FD4. Values are means of apparent permeability for FD4 (cm/sec) which is the amount of apical FD4 crossing the insert membrane per cm² per sec. Bars show SEM and ***=p< 0.0005.

3.4.2 AcMPAG modulation of TER and TJs permeability

To determine whether AcMPAG, a reactive metabolite of MPA, influenced the TER and FD4 influx, we incubated Caco-2 cells with AcMPAG (10 µmol/L). A time dependant decrease in TER (0 hr: 101.1%, 12 hr: 94.2%, 24 hr: 86.5%, 48 hr: 74.4%, 72 hr: 67.1% relative to the DMSO control) (Figure 3.2a) was observed after incubation with AcMPAG. FD4 influx analysis from apical to basal chamber showed that AcMPAG exhibited a mean 2.78 fold increase in FD4 permeability (Figure 3.2b).
3.4.3 MPA and AcMPAG mediated increase in permeability was not due to cell death/apoptosis

To determine whether MPA or AcMPAG induced decreases in TER and increased FD4 permeability were due to TJs regulation and not due to the cell death, the LDH release from the treated cells was determined. LDH measurement has previously been used as an indicator of cell death [112]. Exposure to 10 µmol/L MPA and 10 µmol/L AcMPAG for up to 72 hr did not result in any significant increase in LDH release from the Caco-2 cells (Figure 3.3a). Furthermore, caspase 3 activity was measured to check the effect of MPA or AcMPAG on cell apoptosis. Neither 10 µmol/L MPA nor 10 µmol/L AcMPAG exposure for 72 hr caused any significant apoptosis as compared to DMSO (vehicle) (Figure 3.3b). These findings suggest that the TJs disruption caused by MPA/AcMPAG was not associated with cell death or apoptosis.
Figure 3.3: Effect of MPA and AcMPAG on apoptosis and cell viability in Caco-2 cells.

Caco-2 were grown for 21 days post-confluence and treated with MPA or AcMPAG for 72 hr. (a) Apoptosis was determined by measuring the caspase-3 activity in cell lysates using CaspACE™ Assay kits. Caspase 3 activity is expressed as pmol/hr/µg (b) Cell viability was assessed by measuring the lactate dehydrogenase (LDH) release in the culture media. Data represent IU/L LDH released into the media. Values are presented as the mean ± SEM; of four independent experiments and the significance was determined by Student's t-test.

3.4.4 MPA and AcMPAG increased the expression of MLC2 and MLCK in Caco-2 cells

In a previous study we reported that MPA increased the total MLC2 in HEK-293 cells [323]. Additionally we observed up-regulation of MLCK and ROCK expression by MPA in HEK-293 and HT-29 cells (data not shown). In view of these findings, we investigated regulation of MLC2 expression and MLCK in Caco-2 cells. MLCK is involved in the regulation of barrier function through the phosphorylation of MLC2 in response to diverse stimuli [153,324]. In line with the previous findings [323], MPA treatment increased the expression of MLC2 at both the mRNA (1.5 fold increase) and protein level (1.47 fold increase) in Caco-2 cells (Figure 3.4a and Figure 3.4b). AcMPAG (10 µmol/L) exposure for 72 hr, however, increased MLC2 protein expression (1.68 fold increase) without any significant change in mRNA expression. MLCK expression was also up-regulated by MPA (10 µmol/L) at the mRNA (1.9 fold) and protein level (2.1 fold). AcMPAG also increased the expression of MLCK at the mRNA (1.3 fold increase) and protein (1.7 fold increase) level. ROCK expression was significantly regulated by MPA (1.53 fold increase) while AcMPAG had no significant effect (Figure 3.4 a).
Figure 3.4: Effect of MPA and AcMPAG on MLC2, MLCK and ROCK expression in Caco-2 cells.

Caco-2 monolayers (21 days post-confluence) were incubated with DMSO (vehicle), MPA (10 µmol/L) or AcMPAG (10 µmol/L) for 72 hr. (a) mRNA expression analysis for MLC2, MLCK and ROCK. Total RNA was extracted, reverse transcribed and subjected to real time PCR analysis. EF-2 was used as a house keeping gene and the relative mRNA expression of MLC2, MLCK, and ROCK in the MPA, AcMPAG and DMSO (vehicle) treated samples was determined using the comparative threshold cycle (C_T) method (2^ΔΔC_T) as described in material and methods. Data indicate the mean of four independent experiments ± SEM. (b) Immunoblot analyses for MLC2 and MLCK. Whole cell lysates were resolved on 1DE and immunoblotted using MLC2 and MLCK specific antibodies. β actin was used as a control for an equal amount of protein load. Densitrometric analysis was done using the Lab image software. The data represent mean relative intensities ± SEM from four independent immunoblots. *p< 0.05 and **p< 0.005 significance relative to DMSO.
3.4.5 MPA and AcMPAG increased MLC2 phosphorylation in Caco-2 cells

MLC phosphorylation has been extensively studied with regard to tight junction regulation and has been reported to be required for increased paracellular permeability [153,184]. To determine whether MPA and AcMPAG caused any defect in the epithelial barrier through phosphorylation of MLC2, we checked the phosphorylation of MLC2 using specific phospho-MLC2 antibody. MPA and AcMPAG treatment (10 µmol/L each) for 72 hr increased the expression of phospho-MLC2 by 2.8 and 2.3 fold respectively (Figure 3.5).

Figure 3.5: Effect of MPA and AcMPAG on the phosphorylation of MLC2 in Caco-2 cells.

Caco-2 monolayers (21 days post-confluent) were incubated with either vehicle (DMSO), MPA or AcMPAG (10 µmol/L each) for 72 hr. Whole cell lysates were resolved on 1DE and immunoblotting was performed using a specific phospho-MLC2 antibody. β actin was used as a loading control for equal amount of protein load. Densitrometric analyses were done using the Lab image software. The representative data are average of relative intensities ± SEM from four independent immunoblot. **=p< 0.005.

3.4.6 MPA and AcMPAG altered TJ proteins expression and distribution

The modulatory effect of MPA on TJs proteins, ZO-1 and occludin was investigated by immunofluorescent labelling. Changes in the distribution and expression of occludin and ZO-1 can be used as the markers for determination of TJs disruption which has been implicated in several GI tract diseases [147,325].
Figure 3.6: Effects of MPA and AcMPAG on ZO-1 and occludin distribution.

Caco-2 cells were grown for 21 days post-confluence and treated with DMSO (vehicle), MPA or AcMPAG for 72 hr. Cells were fixed, permeated, and stained for ZO-1 and occludin, as described in materials and methods section. Figure shows the distribution of ZO-1 and occludin in Caco-2 cells exposed to DMSO (vehicle) (a, b, and c), 10 µmol/L MPA (d, e, and f) alone, 10 µmol/L AcMPAG (g, h, and i). Cells were doubled stained for ZO-1 (a, d, g) and occludin (b, e, h). An overlay (ZO-1, occludin, DAPI) is shown in the right panel (c, f, i). Corresponding proteins were detected with secondary antibodies conjugated with either FITC 488 (green; ZO-1) or cydye 3 (red; occludin). DAPI (blue; nuclei) was used to stain nuclei. Images were examined using confocal microscopy. Images presented are representative images of 5 independent experiments.

Confocal analyses of ZO-1 and occludin distribution showed uniform and continuous staining at the plasma membrane in control cells (DMSO) (Fig 3.6 (a-c)). MPA and AcMPAG treatment (10 µmol/L) for 72 hr led to redistribution of ZO-1 and occludin proteins. The most prominent features were disappearance of staining at the cellular periphery, with aggregation and paracellular openings between the adjacent cells (MPA: Figure 3.6 (d-f), AcMPAG: Figure 3.6 (g-i)). These microscopic alterations at the apical cellular borders correlated with the amount of increased TJs permeability (Figure 3.1 and Figure 3.2) observed.
We further investigated whether MPA or AcMPAG quantitatively altered the expression of TJs proteins in Caco-2 cells. Immunoblot analysis showed that 10 µmol/L of both MPA and AcMPAG decreased the expression of occludin by 2.1 and 2.7 fold respectively (Figure 3.7). These expressional changes are consistent with the immunostaining of occludin protein which also revealed disappearance and redistribution of occludin protein from the membranes (Figure 3.6).

Figure 3.7: Effect of MPA and AcMPAG on occludin protein expression in Caco-2 cells.
Cell were grown to 21 days post-confluency and then treated for 72 hr with DMSO (vehicle), MPA or AcMPAG (10 µmol/L each). Whole cell lysates were extracted, separated on 1-DE and occludin detected using specific antibody as mentioned in the methods section. Four independent experiments were carried out and results represent mean ± SEM. *=P< 0.05, **=P< 0.05.

3.4.7 MPA and AcMPAG modulation of Caco-2 F-actin

The perijunctional ring of F-actin is the fundamental unit of the actin cytoskeleton that supports the tight junction and thus plays an important role in barrier regulation [326]. Structural alterations of the F-actin-based cytoskeleton are used to detect changes in actin and tight junctions [327,328].
Figure 3.8: MPA and AcMPAG-induced remodelling of the F-actin cytoskeleton.

Caco-2 cells grown to 21 days post-confluence followed by 72 hr treatment with DMSO (a, b), 10 µmol/L MPA (c, d) and 10 µmol/L AcMPAG (e, f). Cells were fixed, permeated, and F-actin was stained with FITC-phalloidin (red) and nuclei were stained with DAPI (blue), as described in methods section. Fluorescence images were obtained using Axiovert 200M confocal microscope. Images are representative of 4 independent experiments.

To investigate whether MPA or AcMPAG mediated colonic epithelial barrier disruption was associated with structural modulation of the F-actin cytoskeleton, we stained Caco-2 cells with FITC-labelled phalloidin, a commonly used fluorescent marker for F-actin [322,328]. In the vehicle control (DMSO) cell monolayers, the F-actin cytoskeleton was uniformly organized as shown in figure 3.8 (a-c). Following 72 hr exposure to 10 µmol/L of either MPA (Figure 3.8 d-f) or AcMPAG (Figure 3.8 g-i), the uniform distribution of actin staining in epithelial cells appeared disrupted and was marked by randomly distributed dense patches of staining, which suggest disruption of the actin cytoskeleton as a possible mechanism for the alterations in the TJs by MPA and AcMPAG.
3.4.8 MPA-mediated increase in MLC phosphorylation through MLCK

To analyze for the possible involvement of MLCK in the MPA mediated TJs disruption, we determined the effect of MPA on total protein expression of MLC2, MLCK, and phospho-expression of MLC2 in the presence of ML-7 (Figure 3.9). ML-7 acts as a selective antagonist of MLCK by competing for its ATP-binding site and reverses the effects of agents involved in TJs disruptions [328]. Previously it was reported that ML-7 had no significant effect on total MLC2 and MLCK expression and that ML-7 mainly affects the phosphorylation of MLC2 by decreasing the activity of MLCK [112]. In the present study, expressional analysis showed that MPA treatment in the presence of ML-7 did not alter total MLC2 and MLCK expression, which was observed after MPA treatment alone. Additionally, we observed that the presence of ML-7 in the medium was able to reverse the effect of MPA on MLC2 phosphorylation (Figure 3.9). To further validate these results, cells were incubated with CD which is an actin-disrupting drug that has previously been reported to increase MLC2 phosphorylation [329]. Our results also showed that CD increased phospho-MLC2 expression; which is consistent with the previous report [329].

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Figure 3.9: Effect of ML-7 on MPA-mediated increases in MLC2, MLCK and MLC phosphorylation.

Caco-2 monolayers (21 days post-confluent) were incubated with either vehicle (DMSO), MPA, MPA+ML-7, or CD for 72 hr. Total cell proteins were isolated and equal amount of protein was loaded resolved on 1DE. Expression was analysed by immunoblot analysis using antibodies against MLC2, MLCK and p-MLC2. Beta actin was used as a control for an equal amount of protein load. Bands were quantified using the Lab image software. The data represent the mean of 4 independent experiments ± SEM. *=p< 0.05, **=p< 0.005.
3.4.9 MLCK inhibition partially prevented MPA effects on TER and permeability

To investigate whether MPA mediated TJs alteration is through effects on MLCK, we pre-treated Caco-2 monolayers with ML-7 for 1 hr and then co-incubated them with (10 µmol/L) MPA for the indicated time periods. It was previously reported that ML-7 prevents TJs disrupting agent mediated decreases in TER and increases the permeability via inhibition of MLCK [153,328]. Co-treatment with ML-7 and MPA resulted in a significant higher TER as compared to cells treated with MPA alone. Similarly, apical to basal FD4 influx was also reduced in cells co-treated with MPA and ML-7 (Figure 3.10). CD was previously reported to decrease TER and increase permeability [330]. In the following experiments, similar to MPA, CD treated cells showed significant decreases in TER and increases in FD4 influx (Figure 3.10). These findings suggest that the MPA-induced increases in Caco-2 TJ permeability are at least partly the result of a mechanism closely associated with MLCK expression and activity.

Figure 3.10: ML-7 co-treatment reversed the effect of MPA on TER and permeability.

Cells were grown to 21 days post-confluence and incubated with MPA or MPA+ML-7, or CD for 72 hr. The effects on (a) TER and (b) FD-4 influx were measured as described in the methods section. ML-7 a specific MLCK inhibitor prevented both the MPA-mediated increase in FD4 paracellular diffusion and the decreases in TER. Data are the mean ± SEM of at least four independent experiments. **=p<0.005, ***=p<0.0005.
3.4.10 Inhibition of MLCK prevented MPA mediated alteration of TJ proteins

We examined the involvement of MLCK in MPA mediated TJs regulation using immunofluorescence methods. Previously it was reported that the redistribution of TJs proteins by TJs disrupting agents can be reversed by inhibiting MLCK [215,331,332]. Immunofluorescence localization of occludin and ZO-1 showed that ML-7 could partly prevent redistribution of ZO-1 and occludin induced by MPA exposure (Figure 3.11 d-f) when compared to cells treated with MPA alone (Figure 3.11 a-c).

Figure 3.11: ML-7 co-treatment reversed the effect of MPA on distribution of TJs proteins.
Cells following 21 days post-confluency were treated with MPA, and CD or pre-treated with ML-7 followed by MPA treatment. Cells were labelled with fluorescent antibodies specific for ZO-1 and occludin. Figure shows ZO-1 (a, g, j), occludin (b, h, k) and an overlay of ZO-1 and occludin along with DAPI stained nuclei (c, i, l). Four independent experiments were performed.

ML-7 co-treatment induced reassembly of the ZO-1 and occludin at the cellular borders with reclosure of the paracellular gaps (Figure 3.11 g-i). The MPA induced disruption of TJs proteins distribution was prevented by an MLCK inhibitor (ML-7), indicating that the downstream alteration of TJs proteins is dependent on MLCK
activation. In contrast, CD, like MPA, disrupts the distribution of ZO-1 and occludin as shown by disappearance of these proteins from the paracellular membrane (Figure 3.11 j-l)

We also performed immunoblot analysis for occludin protein expression. Results showed that ML-7 was able to reverse the effect of MPA on occludin expression by increasing its expression by 1.92 fold as compared to cells treated with MPA alone. CD treatment showed a 3.2 fold decrease in occludin protein as compared to DMSO control (Figure 3.12).

![Image of immunoblot analysis](image.png)

**Figure 3.12: Effect of ML-7 co-treatment with MPA on occludin protein expression in Caco-2 cells.** Caco-2 cell monolayers following 21 days post-confluency were incubated with DMSO, MPA (10 µmol/L), MPA (10 µmol/L) + ML-7 (10 µmol/L) or CD (10 µmol/L) for 72 hr. Protein extracts were immunoblotted for occludin and β-actin. Densitometric measurement was done with the Lab image software. Values are means ± SEM (n = 4).

### 3.5 Discussion

Intestinal cells form a crucial physical and functional barrier, which regulates the movement of water, electrolytes, nutrients, and xenobiotics [333]. The gastrointestinal tract is directly involved in the metabolism and transport of various endogenous and exogenous compounds [334]. Several intestinal diseases are characterized by barrier dysfunction including inflammatory bowel disease, graft versus host disease, and infectious enterocolitis (reviewed in [335]). It has been previously reported that epithelial barrier defects lead to increased intestinal
permeability and the development of diarrhoea in human patients with bowel diseases [336] and in mouse models [337].

MPA associated gastrointestinal adverse effects are a major concern in transplantation medicine and diarrhoea is the most frequent unwanted clinical outcome following treatment with MPA regimes [338]. Previous reports showed that MPA is associated with gastrointestinal mucosal injury [60,65,311,339,340]. The effect of therapeutic concentrations of MPA on the gastrointestinal epithelial barrier is not well described. Diverse physiological and pathophysiological stimuli cause intestinal barrier dysfunction, regulated via several pathways such as those involving protein kinase C, protein kinase A, MLCK, Rho-kinase, mitogen-activated protein kinases, and phosphoinositide 3-kinase. Disturbances in these pathways can all lead to the alteration in TJs protein expression and distribution [181,303]. In a previous study, we observed a significant increase in the MLC2 expression in HEK-293 cells following MPA exposure [323]. MLC2 phosphorylation via MLCK and/or ROCK has been implicated in several barrier disorders [329].

To better understand the possible mechanism of MPA mediated TJs regulation, we used Caco-2 monolayers as a colonic model [341]. The present study demonstrates for the first time in vitro that MPA, at non-toxic and therapeutic concentrations produces a significant modulation of intestinal epithelial barrier function in Caco-2 cells. The Caco-2 cell line is widely used as an in vitro intestinal barrier cell model, which exhibits a well differentiated brush border, TJs and intestinal proteins [341,342]. MPA exposure increased TJs permeability and impaired TJ proteins (ZO-1 and occludin) expression and distribution. On the other hand, the MPA concentrations used did not cause significant apoptosis or cell death, suggesting that the effects of MPA on GI barrier function are the result of a non-cytotoxic mechanism.

Previously it was shown that MLCK activity in Caco-2 cells triggers a series of molecular processes such as induction of MLC phosphorylation, myosin-Mg\(^{2+}\)-ATPase activation, and perijunctional actin-myosin interaction which are responsible for actin filament disruption leading to Caco-2 epithelial barrier opening [328]. Several agents increase MLCK mediated MLC2 phosphorylation which disrupts tight junction proteins, leading to the increased TJs permeability implicated in barrier associated
diseases [184]. We investigated the possible disruptive role of MPA on epithelial barrier permeability and attempted to link this effect with MLCK-induced MLC-2 phosphorylation.

To demonstrate the effect of MPA and one of its active metabolites, AcMPAG, on barrier properties of this colonic model, Caco-2 cells were exposed to non-cytotoxic concentrations of MPA (10 µmol/L) and AcMPAG (10 µmol/L) followed by measurements of TER and influx of markers. Determination of TER and influx of permeability markers are widely used techniques to assess the integrity and permeability of monolayers [343] because TJs disruption can be reflected by the reduction in TER and the increase in influx of permeability markers [335].

Our data revealed that MPA and AcMPAG increased Caco-2 cell monolayer permeability as shown by decreases in TER and increases in FD4 influx (Fig 1). These findings are in agreement with another report on the effects of MMF (an ester prodrug of MPA) on the barrier function of small bowel and distal colon of Wistar rats [344].

TJs proteins, ZO-1, and occludin are protein markers which are widely used to investigate TJs integrity [184,345]. These proteins maintain structure and function of TJs integrity which are vital for normal intestinal architecture [97,148]. The disturbance in the distribution and expression of these proteins has been observed in intestinal barrier disorders [150,313]. In the present study, we investigated the effect of MPA and AcMPAG on the distribution and expression of ZO-1 and occludin. We found that exposure of Caco-2 monolayers (21-days post-confluency) to therapeutic, non-cytotoxic concentrations of MPA and AcMPAG for 72 hr led to a decrease in the expression of occludin proteins, as evidenced by Western blot analysis (Figure 3.7). Under normal conditions ZO-1 and occludin are generally present at the pericellular boundary, and distributed homogeneously, presenting a characteristic feature of intact TJs structure. Disruption and redistribution of TJs proteins has been reported previously in several studies that suggested that alteration in these proteins can lead to hyperpermeability [184,346]. MLCK mediated MLC-2 phosphorylation (involved in modulation of ZO-1 and occludin morphologically and biochemically) can induce an increase in TJs permeability [184,184].
Furthermore, we also demonstrated that MPA and AcMPAG exposure changed the distribution of ZO-1 and occludin proteins, as revealed by a discontinuous pattern of immunofluorescent staining of these TJs proteins (Figure 3.5). To investigate whether MLCK was involved in MPA modulation of TJs, we used a specific MLCK inhibitor, ML-7 which is a selective antagonists of MLCK [347]. Previously it was reported that inhibition of MLCK mediated MLC phosphorylation by ML-7 can prevent or reverse TJs barrier losses induced by several agents such as TNFα, Cytochalasin B, and ethanol [112,153,328,348]. To investigate the effect of MPA on MLCK activity, we pre-incubated cell monolayers with ML-7 followed by MPA exposure. Results showed that ML-7 could at least partially reverse the MPA mediated decrease in TER as well as the increase in FD4 influx. Additionally, ML-7 was able to prevent the MPA induced redistribution and decrease in expression of ZO-1 and occludin proteins.

Treatment with CD, a known stimulant of MLCK and actin-depolymerising agent [329] which was used as positive control for the effects of MPA treatment, also showed a decrease in TER and increase in paracellular flux (Figure 3.10). Previously, it was reported that CD was able to increase MLCK activity and MLC2 phosphorylation [329], which our results confirmed. In addition, we found that CD was able to alter the expression and distribution of TJs proteins which is consistent with results of a previous study of CD treated epithelial cells [349]. These results showed that both CD and MPA decreased TER and disrupted the actin cytoskeleton. The present study revealed that inhibition of MLCK activity by ML-7 significantly prevented the MPA mediated increase in MLC2 phosphorylation with no significant effect on total MLCK and MLC2 expression.

MLC2 phosphorylation has a key role in maintaining TJs integrity by regulating actomyosin contraction [153]. Several pathways were described previously which regulate the phosphorylation of MLC2; among them Rho-kinase and MLCK signalling are widely studied in the context of barrier defects [191]. MLCK is involved in the regulation of barrier function by phosphorylation of MLC2 in response to diverse stimuli [153,324]. ML-7 via MLCK inhibition prevents the disruption of both occludin and actin, which demonstrates the importance of MLCK activity in TJs physiology [350]. Our results suggest that increases in MLCK might be responsible for the MPA induced redistribution of ZO-1 and occludin in Caco-2 monolayers.
3.6 Conclusion

The present study indicates that MPA and its active AcMPAG metabolite at therapeutic concentrations produce functional alterations in TJs of Caco-2 cells resulting in abnormal TJs permeability, and redistribution of TJs proteins including disturbance and displacement of F-actin. These data suggest that MPA mediated increases in permeability required increased MLCK activity which could be reversed by ML-7. While requiring further investigation, MLCK inhibition by ML-7 significantly reduced the effect of MPA exposure on TJs disruption, thus suggesting a pivotal role of MLCK in regulating TJs barrier properties. These findings provide new insights into the mechanism by which therapeutic use of MPA may alter intestinal epithelial barrier functions and suggest mechanisms which may be responsible for some of the GI adverse effects associated with MPA.
4. Summary

Mycophenolic acid (MPA) is a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH), a key regulator of purine biosynthesis. MPA is frequently used as an immunosuppressant drug to prevent acute graft rejection for kidney, liver and lung transplantation. The use of MPA is associated with GI toxicity which is a problem to the patients, and a challenge for clinicians. The present study was undertaken to identify novel molecular targets of MPA using a proteomics approach (Figure 4.1). Two dimensional gel electrophoresis (2-DE) and mass spectrometry were used to identify proteome alterations in human embryonic cells (HEK-293) following exposure to therapeutic concentrations of MPA. Cells were treated for 72 hours, and total cell lysate was resolved by 2-DE followed by QTOF-MS/MS analysis of all identified differentially regulated proteins. A total of 12 proteins were differentially regulated in HEK-293 cells following exposure to MPA. Among these, 7 proteins were up-regulated (complement component 1 Q subcomponent-binding protein, electron transfer flavoprotein subunit beta, cytochrome b-c1 complex subunit, peroxiredoxin 1, thioredoxin domain-containing protein 12, myosin regulatory light chain 2, and profilin 1), while 5 proteins were down-regulated (protein SET, stathmin, 40S ribosomal protein S12, histone H2B type 1 A, and histone H2B type 1-C/E/F/G/I). Functional annotation tool analysis showed that MPA modulated proteins were mainly involved in the cytoskeleton (26%), chromatin structure/dynamics (17%), and energy production/conversion (17%). Considering both putative functions and their clinical significance, peroxiredoxin-1 (Prdx-1) and myosin light chain 2 (MLC2) were selected for Western blot and real time PCR analysis. Both proteins showed up-regulation at mRNA as well as at protein level following MPA exposure.

MLC2 is known to be involved in several functions including tight junctions (TJ) regulation. Epithelial barrier disruption by phosphorylation of MLC2 has been implicated in several bowel diseases. Since MPA treatment often causes diarrhea when used clinically, we hypothesized that MPA regulated epithelial TJ by modulation of MLC2. To test this hypothesis, we investigated the effect of MPA on the expression of MLC2 in two colonic cell lines, HT-29 and Caco-2. Increased MLC2 expression was observed in both cell lines following MPA exposure. These findings suggest that the increase in MLC2 expression after exposure to MPA is not a cell specific effect. Moreover, we observed similar up-regulation of MLC2 expression in
whole cell lysates prepared from MMF treated rats, which implies that MPA has similar effects both in vitro and in vivo.

We then used Caco-2 cells grown for 21 days post confluence to develop polarize monolayers to conduct physiological, expressional and microscopic analysis to establish the possible role of MPA in disruption of TJ (Figure 4.1). MPA exposure caused a time and dose dependent decreases in transepithelial resistance (TER), and increases in the FITC-dextran 4 KDa (FD4) paracellular influx in these Caco-2 monolayers. In addition, we found that AcMPAG (a pharmacologically active metabolite of MPA) was also able to cause decreases in TER and increases in FD4 influx. These MPA and AcMPAG mediated increases in permeability were not due to cellular toxicity, as shown by the fact that no significant apoptosis or cell death was observed. In MPA and AcMPAG treated cells, we also found altered expression and distribution of TJ proteins (ZO-1 and occludin).

Since MLC phosphorylation is a key modulator of TJ disruption; we investigated whether MPA also increased MLC2 phosphorylation. Using immunoblot analysis we found that MPA significantly increased MLC2 phosphorylation. We then investigated whether MPA mediated increases in MLC2 phosphorylation was through effects on MLCK. Immunoblot analysis revealed that MPA increased MLCK expression both at mRNA and protein levels. To further confirm that MLCK was the key player in MPA mediated MLC2 phosphorylation and its associated TJ disruption, we pre-incubated cells with ML-7 (a specific MLCK inhibitor), and observed that ML-7 was able to partially prevent the MPA mediated increase in MLC2 phosphorylation. Furthermore, we found that ML-7 partially reversed MPA mediated decreases in TER, and increases in FD4 paracellular influx. ML-7 also prevented the MPA associated disruption of the distribution and expression of TJ proteins (Figure 4.2). These findings suggest that MPA may regulate TJ function via MLCK-driven MLC2 phosphorylation. However, these results do not exclude the possibility that other pathways may also be involved in MPA induced regulation of TJ function.

Taking together findings of the present studies showed that therapeutic concentrations of MPA can modulate the expression of important proteins which are crucial for various cellular functions. MPA may modulate epithelial TJ integrity via MLC2 phosphorylation. These findings will be helpful to understand the molecular mechanisms of MPA-induced proteome alterations, including proteins that are
involved in disruption of TJ. Further studies are needed to clarify the mechanism(s) and consequences of MPA mediated disruption of TJs, especially in *in vivo* models, to know whether these TJ barrier changes are responsible for the GI adverse events associated with MPA treatment.

**Figure 4.1: A proteomic approach for identification of novel MPA molecular targets.**

HEK-293 cells were cultured, treated with MPA, whole cell lysates was resolved using 2-DE, and silver stained. Protein spots were densitometrically analysed and differentially expressed proteins were subjected to in-gel digestion and identified by QTOF-MS/MS. The up-regulation of MLC2 by MPA was further confirmed by immunoblot analysis. The functional involvement of MLC2 in MPA mediated barrier defects was determined by physiological assays such as TER and paracellular influx of FITC-dextran using Caco-2 cells monolayers. In addition, the expression and distribution of TJ proteins (ZO-1 and occludin) were also investigated using immunoblotting and immunofluorescence microscopy.
Figure 4.2: Proposed model of MPA mediated TJ disruption.

MPA increased MLCK-mediated MLC2 phosphorylation in Caco-2 cell monolayers. MLC2 phosphorylation altered the expression and distribution of TJ proteins (ZO-1 and occludin) that have been identified as a key factor in the development of barrier defects seen in several intestinal diseases. MLC2 phosphorylation also alters the distribution of F actin filaments and the associated TJ disruption results in decreases in TER and increases in paracellular influx. In the present study we observed that MPA disrupted TJ, was associated with increased MLCK expression, and MLC2 phosphorylation. We therefore propose that MPA associated TJ disturbance is dependant on MLCK-driven MLC phosphorylation that leads to decreased expression and redistribution of TJ proteins. Pretreatment with ML-7 (a specific inhibitor of MLCK) partially prevented the MPA mediated increase in MLC2 phosphorylation, disturbance of TJ proteins, and increase in permeability. We hypothesized that the observed increase in paracellular permeability following MPA treatment is due to TJ disruption caused by MLC2 phosphorylation, which mediates alterations in the expression and distribution of TJ proteins.
5. References


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98


Figure 5.1: A graphical representation of relative abundance (% volume) of all differentially regulated proteins.

Relative abundance of the proteins differentially expressed in DMSO and MPA treated HEK-293 cells. Results shown as mean of four independent experiments (*p < 0.05 or **p < 0.005).
Table 5.1: MS/MS analysis table of all differentially regulated proteins.

Spot no: the spot identification number on 2-DE; Accession no: Swiss-prot protein identification number; Sequence coverage: the percentage of the protein's sequence represented by the peptides identified by MS/MS; MS/MS analysis: peptides sequences (bold & red) identified for a particular protein, including MS/MS queries, and the MS/MS spectra of an exemplary peptide.

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MS/MS Fragmentation of DPQHDLDR

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MS/MS Fragmentation of TIAQDYGVLK.

MS/MS Fragmentation of DPQHDLDR

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MS/MS Fragmentation of TIAQDYGVLK.

MS/MS Fragmentation of DPQHDLDR
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**MS/MS Fragmentation of LLLLPGELAK**

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**MS/MS Fragmentation of STITSREIQTAVR**

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**MS/MS Fragmentation of LGEWVGLCK**
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51 LGKDRSSFY VNQLTGGGK CSEOIDSLLG DGEFSMDLR T KSTGGAPTFN
101 VTVKTDKTL VLLMKEGWH GGLINKK CYE MASHLRISOY

Start - End  Observed    Mr(expt)   Mr(calc)      Delta   Miss  Sequence
39 - 54      822.4751  1642.93     1642.9294     0.0062     0  K.TFVNITPAEVGVLVGK.D
76 - 89      813.3810  1624.7474  1624.7403     0.0072     0  R.DSLLQGGEFSMDLR.T
92 - 105     690.3588  1378.7030  1378.7093 -0.0062     0  K.STGGAPTFTNFVTK.L
106 - 116     609.5624  1217.7102 1217.7053     0.0049     1  K.TDKTLVLLMGKR.E
117 - 127     576.3310  1150.6474 1150.6458     0.0016     1  K.EGVHGGLINKKC
128 - 136     583.7573  1165.5000 1165.5008 -0.0008     0  K.CYEMASHLR.R

MS/MS Fragmentation of TFVNITPAEVGVLVGK
7. Acknowledgements

My sincere gratitude to Prof. Dr. Michael Oellerich and PD. Dr. Abdul Rahman Asif for their constant encouraging guidance, support, and invaluable suggestions. They provide me inspiring energy and have always been ready for both scientific and social discussions during this period. Their creativity and expertise in research, patience, and motivational skills are exceptional.

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Special thanks to Hazir Rahman and Misbah Tauseef for giving me critical advice during our discussions and helped me to turn results into science. They were with me through the thick and thin of this long and arduous PhD journey.

I was fortunate to work with great colleagues Dr. Darinka Petrova, Dr. Christoph Eberle, Dr. Gunner Brandhorst, Prof. Dr. Nico Von Ahsen, Dr. Lutz Binder, Dr. Raees Ahmed for their invaluable support and perceptive comments. I am deeply indebted to my former colleagues Saima Zafar, Saadia Zahid, Bharat Singh and Dr. Saagarika Biswas for their always being there and helping me in more ways than one probably know. I would like to express my appreciation to our proteomics laboratory technicians, especially Christina Wiese, Christa Schultz, Susanne Goldmann for their technical support and expertise. I also wish to thank Ulrike Bonitz, Rainer Andaq and Sandra Hartung, for creating a supportive and pleasant work atmosphere.

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Last but not the least; words are insufficient to express my thanks to my parents, sisters and brother for all their undying love, constant support, encouragement and blessings throughout my PhD course. My warmest thanks belong to my wife and children for their understanding, never-ending support, love and sharing my affection.
8. Curriculum Vitae

Name: Muhammad Qasim

Date and Place of birth: 25th December 1978, Mardan, Pakistan

Nationality: Pakistani

Educational background

2008 – continue PhD (Biology) from Georg-August University, Goettingen, Germany.

2004 – 2006 M.Sc (Microbiology and Molecular Genetics) from University of the Punjab, Lahore, Pakistan.

2000 – 2003 B.Sc (Medical Laboratory Technology) from University of the Punjab, Lahore Pakistan

Professional experience


2004 - 2005 Research Scholar, Department of Microbiology, Children Hospital, Lahore, Pakistan.

2006 - 2008 Medical Technologist (Clinical scientist), Department of Microbiology, Federal Post Graduate Medical Institute, Shaikh Zayed Hospital Lahore, Pakistan.

2008 – to date Lecturer Microbiology, Department of Microbiology, Kohat University of Science and Technology, Kohat, Pakistan (study leave).

Publications


Abstarcts published


Research presentations


8. Qasim M. Epithelial tight junction regulation by Mycophenolic acid. Symposium of Transporttage 2011 at the Department of Physiology and Patho physiology, University Medical Center, Goettingen, Germany (22nd to 23rd Oct 2011) (Oral).

Distinction and award

1. Second position in the institute in BSc Medical Lab Technology (2003), University of the Punjab, Lahore, Pakistan.

2. PhD scholarship for Germany under the faculty development scholarship program from the Kohat University Science and Technology, Pakistan (2008-2011).