# INVESTIGATION OF THE EFFECTS OF OCHRATOXIN-A ON GLOBAL PROTEIN SUMOYLATION

by

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*to my beloved family...*

# ABSTRACT

## INVESTIGATION OF THE EFFECTS OF OCHRATOXIN-A ON GLOBAL PROTEIN SUMOYLATION

Ochratoxin A (OTA), a secondary metabolite produced by especially Aspergillus and Penicillium species, is found as a contaminant in foods such as daily consumed corn flakes, coffee, meat and dairy products. OTA is a potential carcinogenic toxin whose mode of action is not fully understood, yet. It is known that OTA deregulates MAPK/Erk1-2 and PI3K/Akt signaling pathways responsible for the vital cellular activities. Therefore, identification of the mechanisms underlying OTA's carcinogenic and toxic effects is of great importance. SUMOylation is one of the post-translational modifications playing crucial roles in cellular homeostasis and the regulation of the cell's vital functions. There is no study examining the effects of OTA on the global protein SUMOylation changes and contributions of these changes on OTA-induced activations of MAPK/Erk1-2 and PI3K/Akt signaling pathways. In this Master's project, first we showed that OTA induces oxidative stress in immortalized human proximal tubule epithelial HK-2 cell line. Next, we demonstrated that OTA treatment leads to alterations in global protein SUMOylation and activation of PI3K/Akt and MAPK/Erk1-2 pathways, which might be linked to OTA-induced oxidative stress. Moreover, we showed that OTA-induced MAPK/Erk1-2 pathway activation is not affected when global SUMOylation is inhibited using ML-792. In contrast, our data portrayed that global SUMOylation is essential for phosphorylation of Akt under OTA exposure. Taken all together, our data suggests that SUMOylation may play a key role in cellular survival under OTA exposure. Finally, we constructed stable SUMO-1- and SUMO-2-overexpressing HK-2 cell lines to eliminate low efficiency for immunoprecipitation. By using these cell lines we will be able to detect possible SUMO targets under OTA exposure via Mass Spectrometry Analysis.

# ÖZET

## OKRATOKSİN-A'NIN GLOBAL PROTEİN SUMOYİLASYONUNA ETKİSİNİN İNCELENMESİ

Özellikle *Aspergillus* ve *Penicillium* türleri tarafından üretilen ikincil bir metabolit olan Okratoksin A (OTA), günlük tüketilen mısır gevreği, kahve, et ve süt ürünleri gibi gıdalarda kontaminant olarak bulunur. OTA, etki şekli henüz tam olarak anlaşılmamış potansiyel bir kanserojen toksindir. OTA'nın, hayati hücresel aktivitelerden sorumlu MAPK/Erk1-2 ve PI3K/Akt sinyal yollarını deregüle ettiği bilinmektedir. Bu nedenle OTA'nın kanserojen ve toksik etkilerinin altında yatan mekanizmaların belirlenmesi büyük önem taşımaktadır. SUMOyilasyon, hücresel homeostazda ve hücrenin hayati fonksiyonlarının düzenlenmesinde önemli roller oynayan translasyon sonrası modifikasyonlardan biridir. OTA'nın global protein SUMOyilasyon değişiklikleri üzerindeki etkilerini ve bu değişikliklerin MAPK/Erk1-2 ve PI3K/Akt sinyal yollarının OTA kaynaklı aktivasyonlarına katkılarını inceleyen bir çalışma bulunmamaktadır. Bu Yüksek Lisans projesinde, önce OTA'nın ölümsüzleştirilmiş insan proksimal tübül epitelyal HK-2 hücre hattında oksidatif stresi indüklediğini gösterdik. Daha sonra, OTA maruziyetinin, OTA kaynaklı oksidatif stres ile bağlantılı olabilecek olan global protein SUMOyilasyonunda ve PI3K/Akt ve MAPK/Erk1-2 yollarının aktivasyonunda değişikliklere yol açtığını gösterdik. Ayrıca, ML-792 kullanılarak global SUMOyilasyonu inhibe edildiğinde OTA kaynaklı MAPK/Erk1-2 yolu aktivasyonunun etkilenmediğini gösterdik. Buna karşılık, sonuçlarımız, OTA maruziyeti altındayken Akt'nin fosforilasyonu için global SUMOyilasyonun gerekli olduğunu gösterdi. Bütün sonuçlar göz önüne alındığında, verilerimiz SUMOyilasyonun OTA maruziyeti altında hücresel hayatta kalmada önemli bir rol oynayabileceğini düşündürmektedir. Son olarak, immün-çökeltme için düşük verimliliği ortadan kaldırmak amacıyla stabil SUMO-1- ve SUMO-2-aşırı ifade eden HK-2 hücre hatları oluşturduk. Bu hücre hatlarını kullanarak, OTA maruziyeti altındaki olası SUMO hedeflerini Kütle Spektrometrisi Analizi yoluyla tespit edebileceğiz.

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# LIST OF SYMBOLS

| | |
|---|---|
| $g$ | Gram |
| $h$ | Hour |
| $kDa$ | Kilodalton |
| $L$ | Liter |
| $M$ | Molar |
| $mg$ | Miligram |
| $mM$ | Milimolar |
| $ng$ | Nanogram |
| | |
| $\alpha$ | Alpha |
| $\beta$ | Beta |
| $\mu g$ | Microgram |
| $\mu l$ | Microliter |
| $\mu M$ | Micromolar |
| | |
| $\circ C$ | Degree Celcius |

# LIST OF ACRONYMS/ABBREVIATIONS

| | |
|---|---|
| APS | Ammonium Persulfate |
| BEN | Balkan Endemic Nephropathy |
| BSA | Bovine Serum Albumin |
| $ddH_2O$ | Double-distilled water |
| DMEM | Dulbecco's Modified Eagle's Media |
| DNA | Deoxyribonucleic Acid |
| Erk1-2 | Extracellular Signal-Regulated Kinase 1-2 |
| EtOH | Ethanol |
| FBS | Fatal Bovine Serum |
| GFP | Green Fluorescence Protein |
| HK-2 | Human Kidney 2 |
| MAPK | Mitogen Activated Protein Kinase |
| MEK | Mitogen-activated Protein Kinase Kinase |
| NAC | N-acetyl-L-cysteine |
| OTA | Ochratoxin A |
| PBS | Phosphate Buffer Saline |
| PCR | Polymerase Chain Reaction |
| PI3K/Akt | Phosphoinositide 3-kinase/Protein Kinase B |
| PVDF | Polyvinylidene fluoride |
| PYO | Pyocyanin |
| RNA | Ribonucleic Acid |
| ROS | Reactive Oxygen Species |
| RT | Room Temperature |
| SDS | Sodium Dodecyl Sulfate |
| SUMO | Small Ubiquitin-like Modifier |
| TBS-T | Tris Buffer Saline-Tween 20 |
| VHC | Vehicle |

# 1. INTRODUCTION

Ochratoxin A ($C_{20}H_{18}O_6NCl$) is a secondary metabolite produced by certain species of *Penicillium* and *Aspergillus* fungi. OTA has a phenylalanine group attached to a dihydroisocoumarin molecule by a peptide bond [1] (Figure 1.1), and due to its lipophilic structure, OTA can easily pass through the membrane [6].

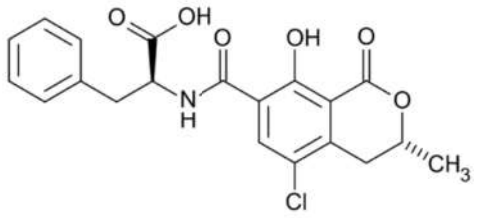


Figure 1.1. Structure of OTA [1].

OTA can be found as contaminant in many food and feed commodities such as wheat, hazelnut, pea, coffee, beer, wine, meat and meat products, milk that are consumed frequently by humans and animals in daily life. Thus, compared to other contaminants OTA exposure of human and animals is relatively high. One dose of OTA can remain in the human circulatory system for about 35 days (800 hours) due to the long half-life of the molecule [7]. Nephrotoxic, immunotoxic, neurotoxic, and carcinogenic effects of OTA have also been demonstrated in rodents [8].

OTA accumulates in kidney cells over time due to its longevity and reabsorption from proximal tubules. OTA is one of the potential toxins that cause Balkan Endemic Nephropathy (BEN). BEN is usually associated with renal tumors and death in the ages of 40s and 50s. In addition, several studies have led OTA to be classified as a potential carcinogen (2B) by the International Agency for Research on Cancer [9].

Even though the molecular mechanisms of the toxic and carcinogenic effects of OTA are not yet fully understood, there is plenty of evidence supporting non-genotoxic epigenetic effects of OTA. For example, it is known to cause an increase in Reactive Oxygen Species

(ROS) and protein oxidation, deregulation of cell signal transduction pathways, apoptosis and/or cell cycle disruption [10–12]. It has been reported that the toxic accumulation of aggregates that may occur because of protein oxidation causes secondary inhibition of the lysosome system and causes a vicious cycle that promotes further aggregation and cell death [13].

### 1.1. Ochratoxin A and Reactive Oxygen Species

ROS are some of the most active chemicals that can be produced during cellular respiration. Super-oxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl ($HO^-$) radicals are examples of ROS that can occur inside the cell. Exposure of cells to ionizing radiation, consumption of fried food products or cigarette smoking and certain xenobiotics cause ROS formation. Moreover, metabolic degradation and/or processing of other xenobiotics can cause formation of reactive oxygen species. Oxidative stress occurs when the amount of ROS produced in the cell rises above the basal level or the antioxidant capacity decreases [14].

Oxidative stress is involved in the pathogenesis of many diseases or important biological activities (Figure 1.2). One of the most important effects of oxidative stress on intracellular proteins is the oxidation and the degradation of proteins [15]. Main targets of free radicals are usually amino acid specific. Especially, sulfur-containing amino acids like cysteine and methionine are sensitive to ROS attacks [2].

OTA has been shown to increase oxidative stress in our previous works [10, 11] and by other research groups [16, 17]. Increased oxidative stress can cause disruption of several cellular pathways such as proliferation, survival, differentiation, and protein modifications like phosphorylation, ubiquitination and SUMOylation. There are studies in the literature showing that ROS production and oxidative stress can affect SUMOylation [3].

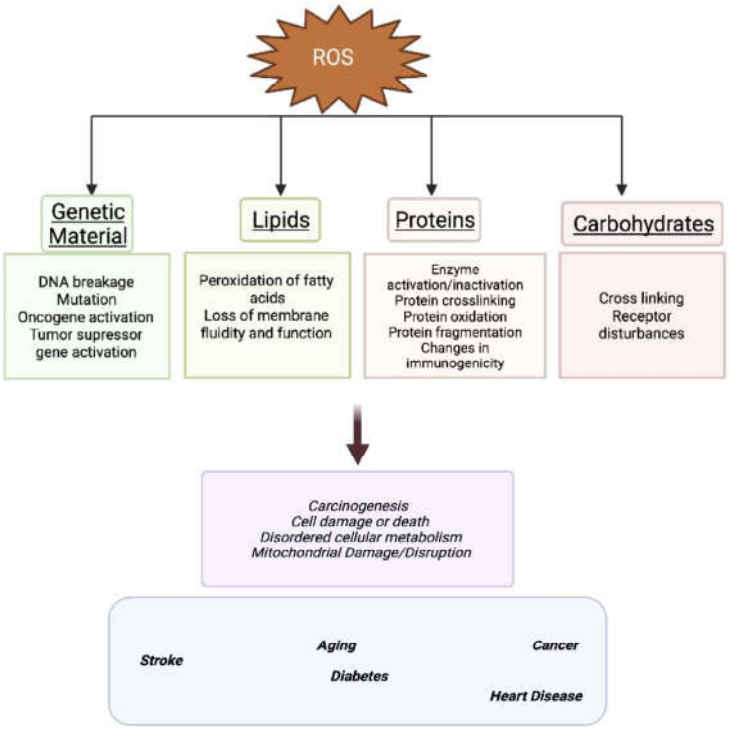


Figure 1.2. Reactive oxygen species and related biological activities (modified from [2]).

## 1.2. SUMOylation

After translation, proteins may undergo some modifications. These "post-translational modifications" often change the ultrastructural and functional properties of a protein, causing substantial downstream signaling effects [18]. Alterations in post-translational modifications such as disruption in phosphorylation, ubiquitination and SUMOylation can lead to abnormalities and alterations in protein folding, function or binding partners of the proteins.

It has been shown that SUMOylation provides regulatory mechanisms in cell cycle, cellular signaling and transcription [19]. Small Ubiquitin-like Modifiers (SUMO) are involved in post-translational processes such as stabilization of proteins, regulation of their localization or gaining function. SUMOs are reversibly attached to certain lysine amino acids of target proteins and can affect protein-protein interaction, protein stability, and intracellular localization [20]. SUMOylation has been associated with neuropathic and cardiovascular diseases as well as cancer [21, 22].

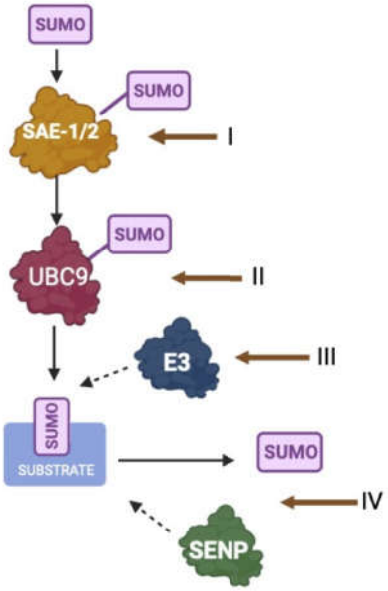


Figure 1.3. SUMO Pathway (modified from [3]).

SUMO has 4 known paralogs: SUMO-1, SUMO-2, SUMO-3, and SUMO-4. SUMO-2 and 3 are considered as one (SUMO-2/3) as they have 97% identity in their amino acid sequences. It has been observed that SUMO-4 emerges during placental development [23].

Specialized enzymes are involved in the SUMO pathway; E1 as SUMO-activating enzyme (SAE1/2), E2 as conjugating enzyme (Ubc9) and E3 as SUMO ligase (PIAS3) responsible for SUMO activation, whereas SENPs deSUMOylation [24–26](Figure 1.3). SUMO protein is activated by SAE-1/2 (I) and creates conjugations with particular substrate by Ubc9 (II). To stabilize this conjugation E3 ligase (III) sometimes cooperates with E2 enzyme. SUMOylation is a reversible mechanism and SENPs play role in deSUMOylation of the substrate (IV).

Most SUMOylated proteins have a 4-amino acid peptide motif: ψ -K-x-D/E where ψ represents a hydrophobic amino acid, K represents the lysine amino acid suitable for SUMO conjugation, X represents any amino acid, D or E represents the acidic amino acids. The motif of interest provides the specificity required for Ubc9.

Oxidative stress triggering SUMOylation after chemical agent treatment has also been reported in many studies [27, 28]. Environmental factors such as osmotic and oxidative stress have been shown to increase SUMOylation via SUMO-2/3 but have little effect on SUMO-1 [29]. In studies examining global SUMOylation under oxidative stress, it has been reported that SUMO-2/3 SUMOylation increases as a result of high-dose hydrogen peroxide ($H_2O_2$; 100 mM), but low-dose $H_2O_2$ (1 mM) inhibits the formation of disulfide bridges in the catalytic cysteine regions of E1 and E2 enzymes [29]. Disulfide bridges between E1 and E2 enzymes inhibits SUMOylation reversibly [3]. Thus, the inhibition of formation of the disulfide bridges causes increase in SUMOylation. Furthermore, because $H_2O_2$ has been shown to affect the activity of a variety of kinases and phosphatases, it is possible that oxidative stress will affect the phosphorylation of SUMO targets and hence, affect their SUMOylation. HIPK2 [30] and TP53INP1 [31] are two examples of particular targets that have been identified to be modified by SUMOylation in response to oxidative stress.

One way of controlling SUMOylation is the regulation of protein kinase cascades in response to extracellular signals [32]. Reciprocally, it has to be noted that changing SUMOylation patterns might also play role in the regulation of different cellular pathways [33]. Inducing oxidative stress in cells using hydrogen peroxide, for example, resulted in higher amounts of SUMO-2/3 modified p53, although p53/SUMO-1 conjugates were unaffected [34]. Moreover, the MAPK/Erk1-2 and PI3K/Akt pathways are the most common cascade-mediated routes [35] and whose activities are associated with SUMOylation [36].

## 1.3. Signal Transduction Pathways and SUMOylation

### 1.3.1. Mitogen Activated Protein Kinases (MAPKs) / Erk1-2

MAPK signaling pathway provides the production of appropriate genomic or physiological responses to changing environmental conditions by amplifying the signals they receive from extracellular milieu or by converging different signals [37]. After being activated, the MAPK pathway follows three stages; MAPKKK (MAPK Kinase Kinase) phosphorylates MAPKK (MAPK Kinase), MAPKK phosphorylates MAPK [4] (Figure 1.4). MEK1 and

MEK2 are phosphorylated by activated Raf proteins from specific serine amino acids. Activated ERKs phosphorylate many proteins, such as nuclear and cytoplasmic kinases, phosphatases, transcription factors, and cytoskeletal proteins. The ERK pathway regulates proliferation, survival, cell migration, angiogenesis, chromatin remodeling, and apoptosis [4].

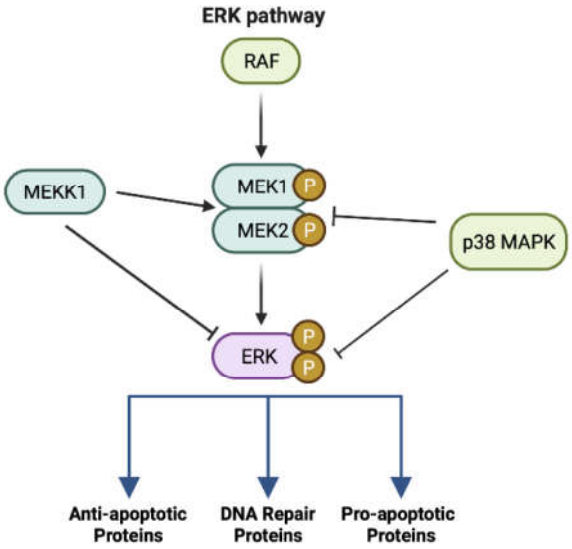


Figure 1.4. MAPK/Erk1-2 pathway (modified from [4]).

There are studies investigating the relation of MEKs with SUMOylation. In a study examining the effect of MEK SUMOylation on ERK phosphorylation, it was shown that MEK1 and SUMO-MEK conjugate were equally phosphorylated by Raf-1N, but SUMO-MEK had a lower activity in ERK phosphorylation than MEK-1 [38]. DeSUMOylation has also been noted to rescue MEK activity. In the same study, it was stated that MEK SUMOylation down-regulates the cellular functions performed by the MAPK/Erk1-2 signaling pathway.

### 1.3.2. Phosphoinositide 3-Kinase (PI3K) / Akt

The PI3K/Akt pathway is responsible for the regulation of cell metabolism, survival, and proliferation signals [5]. Malignant tumor formation and kidney cancer have been observed as a result of overactivation of the PI3K/Akt pathway [39]. Akt is a serine/threonine kinase, also known as PKB (Protein Kinase B), and is responsible for the phosphorylation of numerous proteins. Induced ROS levels could also activate PI3K and leading to phosphory-

lation of Akt via PDK1 and pave the way for tumorigenesis [40] (Figure 1.5.). Additionally, among other proteins phosphorylated by Akt, there are proteins involved in apoptosis and survival pathways [41].

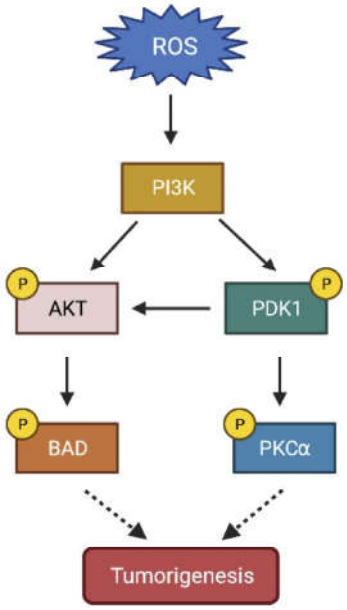


Figure 1.5. PI3K/Akt pathway and tumorigenesis (modified from [5]).

In a study on albino mice, phosphorylations of Epidermal Growth Factor Receptor (EGFR), Erk1-2, p38 and Akt were observed, as well as tumor formation in mice whose skin was exposed to OTA [42]. Similarly, in our previous studies, it was shown that OTA treatment causes sustained activation of the PI3K/Akt and MAPK/ERK pathways by two different mechanisms [11]. It has been observed that activation of these two pathways, can lead to cell survival or apoptosis in HK-2 cells after OTA treatment.

In addition to phosphorylation, Akt can be post-translationally modified at the lysine 276 (K276) amino acid residue which is found in a SUMOylation motif [43]. As a result of mutations in K276, a decrease in the growth and proliferation of cells and a decrease in the kinase activity of Akt were observed. In support of these results, a decrease in Akt SUMOylation has been reported as a result of mutations in K276 [44]. Again, in the same study, it was shown that the half-life of Akt decreased to 1.5 hours from 5 hours due to inhibition of SUMOylation, which is known to play a role in the stabilization of proteins.

# 2. HYPOTHESIS AND PURPOSE

As a food contaminant, OTA is an agent classified as highly toxic and possible carcinogen for humans. The mechanisms of action of OTA in the cell are not yet fully understood. However, based on studies, it is thought that OTA accumulates in kidney proximal tubule cells, increases oxidative stress, disrupts cellular signal transduction pathways and might be leading to nephrotoxicity and kidney cancer [45].

Being one of the post-translational modifications, SUMOylation is involved in various vital functions such as cellular homeostasis, regulation of transcription, cellular stress responses, and cell cycle regulation. It has been reported that SUMOylation is induced in many stress conditions such as DNA damage, heat shock and oxidative stress [46].

In one hand, our studies have shown that PI3K/Akt and MAPK/Erk1-2 pathways [11] as well as autophagy and the Ubiquitin-Proteosome System (UPS) [10] are activated in the human kidney proximal tubule cell line HK-2 after OTA treatment. On the other hand, it has been shown that the Ubc9 enzyme, which is involved in the SUMOylation process, is highly expressed in the kidneys [47], and it has been clearly shown that SUMOylation and/or deSUMOylation play a role in nephropathic diseases such as renal dysgenesis, renal carcinoma, acute kidney injury, podocyte apoptosis and stress response [25, 48, 49].

Based on these findings, we hypothesize that OTA exerts its toxic and/or carcinogenic effects in part by triggering changes in the SUMOylation pattern of proteins via creating oxidative stress.

We have 3 aims to test our hypothesis:

*(I) Detection of OTA-induced oxidative stress and determination of its association with global SUMOylation pattern*

*(II) Investigation of the effect of altered SUMOylation pattern on OTA-induced signal transduction pathways*

*(III) Detection of possible SUMOylation target proteins under OTA exposure*

# 3. MATERIALS

Table 3.1. Cell Culture Solutions

| **Solution** | **Provider** |
|---|---|
| DMEM:F12 without phenol red | PAN Biotech, Germany |
| Fetal Bovine Serum (FBS) | Gibco, Fisher Scientic, USA |
| Opti-MEM | Gibco, Fisher Scientic, USA |
| Penicillin/Streptomycin (100X) | PAN Biotech, Germany |
| Trypsin-EDTA (0.5 mM EDTA, 0.025% Trypsin) | PAN Biotech, Germany |
| GenoxxoFect Transfection Reagent | Genaxxon Bioscience, Germany |
| L-Glutamine PAN | Biotech, Germany |

Table 3.2. Plasmids and Providers

| **Plasmid** | **Provider** |
|---|---|
| GFP-SUMO-1 and GFP SUMO-2 | Kindly provided by Assoc. Prof. Umut Şahin |
| pLEX307 and pDONR207 | Kindly provided by Prof. Dr. Arzu Çelik Fuss |
| pCMV-VSV-G | Kindly provided by Assoc. Prof. NC Tolga Emre |
| psPAX2 | Kindly provided by Assoc. Prof. NC Tolga Emre |
| pMEV-2HA | Biomyx |

Table 3.3. Adapter primer sequences for Gateway cloning

| **Primer** | **Sequence** |
|---|---|
| AttB-F | GGGGACAAGTTTGTACAAAAAAGCAG GCTTAATCACTAGAAGCTTTATTGCGGTAGT |
| AttB-R | GGGGACCACTTTGTACAAGAAAGCTGGGTTGAAG CGGCCGCCCG |

Table 3.4. Kits and Enzymes

| **Kits** | **Provider** |
|---|---|
| Halt$^{TM}$ Protease Inhibitor Cocktail (100X) | Thermo, USA |
| Pierce$^{TM}$ BCA Protein Assay Kit | Thermo, USA |
| ECL | Advansta, USA |
| PageRuler Prestained Protein Ladder | Thermo, USA |
| Midi RTA Transfer Kit | Bio-Rad, USA |
| PVDF (0.22 μm) | Bio-Rad, USA |
| PrimeStar GXL DNA Polymerase | Takara, Japan |
| Restriction Endonucleases | Thermo, USA |
| Total ROS/Superoxide Detection Kit | Enzo, USA |
| XTT Cell Viability Assay | Roche, Germany |
| PierceTM HA-Tag Magnetic IP/Co-IP Kit 40rxn | Thermo, USA |

Table 3.5. Chemicals

| **Chemical** | **Provider** |
|---|---|
| Acetic Acid | Merck, USA |
| Acrylamide/Bisacrylamide | Neofroxx, Germany |
| Agarose | GeneOn, Germany |
| Ammonium Persulfate (APS) | Sigma-Aldrich, USA |
| Ampicillin | Roche, Switzerland |
| Ampicillin | Fluka, USA |
| APS | AppliChem, Germany |
| B-Mercaptoethanol | Merck, USA |
| Boric Acid | Sigma-Aldrich, USA |
| Bovine Serum Albumin (BSA) | Santa Cruz Biotechnology, USA |
| Bromophenol Blue | Fluka, USA |
| EDTA | AppliChem, Germany |
| Ethanol | Merck, USA |
| Ethidium Bromide | Sigma-Aldrich, USA |
| Glycerol | Sigma-Aldrich, USA |
| Glycine | Applichem, Germany |
| HEPES | Sigma-Aldrich, USA |
| Kanamycin | Emsure, Germany |
| LB Broth | Caisson Laboratories, USA |
| LB-Agar | Sigma-Aldrich, USA |

Table 3.6. Chemicals cont.

| **Chemical** | **Provider** |
|---|---|
| Methanol | Merck, USA |
| Ochratoxin A | Sigma-Aldrich, USA |
| Phosphate Saline Buffer (PBS) | MPBio, USA |
| Puromycin | Sigma-Aldrich, USA |
| Sodium Chloride (NaCl) | Fisher Scientific, USA |
| Sodium Dodecyl Sulfate (SDS) | AppliChem, Germany |
| Sodium Fluoride | Merck, USA |
| Sodium Hydroxide | Sigma-Aldrich, USA |
| TEMED | Sigma-Aldrich, USA |
| Tris-Base | AppliChem, Germany |
| Tris-Cl | AppliChem, Germany |
| Tween 20 | Sigma-Aldrich, USA |

Table 3.7. Western Blot Solutions and Buffers

| **Solutions/Buffers** | **Content** |
|---|---|
| Lysis Buffer | 150 mM NaCl<br>1% NP40<br>1% SDS<br>50 mM Tris pH 7.4 |
| 4XProtein Loading Dye | 200mM TrisHCl pH 6.8<br>8% (w/v) SDS<br>40% (w/v) 100% Glycerol<br>4% (w/v) -mercaptoethanol<br>50 mM EDTA<br>0.08% (w/v) Bromophenol Blue |
| 8% Resolving Gel | 375 mM TrisHCl pH 8.8<br>0.1% (w/v) SDS<br>Acrylamide:Bisacrylamide (8% w/v)<br>0.05% (w/v) APS<br>0.005% (w/v) TEMED |

Table 3.8. Antibodies

| Antibody | Provider | Source | Dilution |
|---|---|---|---|
| ACTIN (D6A8) | Cell Signalling Technologies, USA | Rabbit | 1:2000 |
| AKT (pan)(C67E7) | Cell Signalling Technologies, USA | Rabbit | 1:1000 |
| HA | Santa-Cruz Biotechnology, USA | Mouse | 1:1000 |
| Mouse IgG, HRP | Cell Signalling Technologies, USA | Rabbit | 1:3000 |
| p-AKT (S473)(D9E) | Cell Signalling Technologies, USA | Rabbit | 1:1000 |
| p-p44/p42(T202/Y204) | Cell Signalling Technologies, USA | Rabbit | 1:1000 |
| p44/42(Erk1/2) | Cell Signalling Technologies, USA | Rabbit | 1:1000 |
| Rabbit IgG HRP | Cell Signalling Technologies, USA | Rabbit | 1:3000 |
| SUMO-1 | Cell Signalling Technologies, USA | Rabbit | 1:1000 |
| SUMO-2/3 | Cell Signalling Technologies, USA | Rabbit | 1:1000 |

Table 3.9. PCR Conditions

| Temperature $^{o}$C | Time | Cycle |
|---|---|---|
| 95 | 10 sec | 30 |
| 68 | 20 sec | cycles |

Table 3.10. PrimeSTAR GXL PCR Mix

| Reagent | Volume/Amount (1X) | Final Concentration |
|---|---|---|
| 5X PrimeStar GXL Buffer | 10 µl | 1X |
| dNTP Mixture (2.5 mM each) | 4 µl | 0.2-0.3 µM |
| attB-F | 1.25 µl | 0.25 µM |
| attB-R | 1.25 µl | 0.25 µM |
| Diluted plasmid DNA | 11 µl | 10 ng |
| PrimeSTAR GXL DNA Polymerase | 2 µl | 1.25 U/50 µl |
| Sterile Water | 30.5 µl | up to 50 µl |

# 4. METHODS

## 4.1. Cell Culture

Immortalized normal kidney-derived proximal tubular epithelium HK-2 cell line was purchased from the American Type Culture Collection (ATTC, Manassas, USA). HK-2 cells were cultured in DMEM:F12 1:1 (PAN, w/o phenol red) medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin (100U/mL)-streptomycin (100 μg/mL) at 37°C, 5% $CO_2$ and a humidified atmosphere. HK-2 cells were grown in 100 mm cell culture plates and subcultured into new plates every three days.

## 4.2. OTA Treatment

Sterile stock OTA solution (10 mM in EtOH) was added to the cell growth medium to achieve the desired concentrations. After the cells have reached approximately 95% of confluency in complete medium, they are treated with different concentrations of OTA or 0.1% EtOH (vehicle of OTA) in the medium containing 5% FBS and penicillin (100 U/mL)-streptomycin (100 μg/mL) for different time periods as necessary.

## 4.3. Cell Viability Tests

Viability measurements of cells grown in DMEM:F12 were made with the XTT Cell Proliferation Assay Kit (Roche, Germany). Cells were plated in 96-well plates in 30 x $10^3$ density, treated with different doses of OTA and left to incubate throughout the experiment. XTT solutions were prepared by mixing at a ratio of 1:50 (Reagents A:B) and 50 μl of this mixture was added to each well. After four hours of incubation, absorbance values were measured at 420 nm and 655 nm wavelengths. Absorbance values were associated with metabolic activity/viability.

### 4.4. Detection of Oxidative Stress and ROS

When the density of passaged cells was 75%, the cells were detached by Trypsin (0.25%, PAN) treatment and seeded in 4-well Ibidi plates at approximately 2 x $10^4$ cells per well. When the confluency of the cells reached 90-95%, the medium was changed from DMEM:F12 with 10% FBS to DMEM:F12 with 5% FBS. Two hours later, cells were treated with 10 μM OTA or the vector 0.1% EtOH for indicated time periods. As the positive control, 2 μM pyocyanin was given 30 minutes before imaging. After treatments, cells were incubated for 30 minutes in ROT/Superoxide Detection Solution. At the end of the incubation, medium was aspirated, and the cells were visualized under fluorescence microscopy at 490 nm and 550 nm without fixation.

### 4.5. Preparation of Cell Protein Lysates

Cells were lysed using cell lysis buffer containing protease inhibitors (20 mM HEPES-KOH (pH 7.4), 50 mM NaCl, 1% (v/v) NP-40, 1 mM EDTA (pH 8.0), 1% SDS (w/v) , Protease Inhibitor Cocktail (1X), dd$H_2O$) on the plates. Cells in lysis buffer were incubated for 5 minutes at -20 ºC for better lysis. Lysates were collected with sterile scraping spatulas and transferred to sterile microcentrifuge tubes. The samples were kept on ice for 30 minutes and then homogenized by passing them through insulin syringes. The samples were centrifuged at 18700 g for 15 minutes at +4 °C. Supernatants are taken into new tubes and stored at -20 °C. The protein concentration of samples were measured with Pierce Protein Quantification Assay following the manufacturer's instructions. Different concentrations of Bovine Serum Albumin (BSA) standards were used for the construction of the standard curve.

### 4.6. Western Blot Analysis

HK-2 cells (2.2 x $10^6$) were seeded in 100 mm culture plates. The next day, cells were treated with 0.1% EtOH (OTA vehicle) or 10 μM OTA and incubated for 1, 3, 6, 12 and 24 hours. At the end of the incubation, the cells were lysed using cell lysis buffer and collected by homogenizing with the help of 1 cc insulin syringes. The homogenates were

centrifuged at 18700 g for 15 minutes, and the supernatants taken into new, sterile tubes for analysis. The protein concentrations of the lysates were measured by BCA protocol and Pierce Protein Quantification Assay kit, and approximately 40-50 μg of sample was loaded and resolved on polyacrylamide gels (8% Resolving, 4% Stacking). Then, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 25 V in 20 minutes using Bio-Rad Trans-Blot semi-dry transfer device. Membranes were blocked with 5% skim-milk in TBS-T (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.01% Tween-20) for 1 hour. After the membranes were washed with TBS-T, membranes were incubated with primary antibodies (1:1000 dilutions of Anti-SUMO-1 [CST 4930S], -SUMO-2/3 [CST 4971S], -pERK [CST 9101S], -tERK [CST 4695S], -pAkt [CST 4060S], -tAkt [CST 4691S], 1:2000 dilution of -Actin [CST 8457S], 1:500 dilution of -HA [Santa Cruz sc-57592]) in 5% BSA containing TBS-T overnight at 4°C. The next day, the membranes were washed within TBS-T 3 times for 5 minutes each and incubated within HRP-conjugated secondary antibody (CST, 1:3000) for 1 hour at room temperature. After incubation, the membranes were washed within TBS-T and protein bands were visualized using Advansta ECL Western Blot Substrate and digital imaging system (SynGene). Band intensities were measured with the ImageJ image analysis software.

### 4.7. Chemical Inhibition of SUMOylation

In order to better understand the link between SUMOylation and OTA-induced signaling pathways, ML-972 (Medkoo 407886), a global SUMOylation inhibitor, was used. To profile the pattern changes after ML-792 treatment, HK-2 cells were grown for 3 days in DMEM:F12 medium containing 10% FBS. Cells were seeded at 1 x $10^6$ cells in 60 mm plates. HK-2 cells were incubated with ML-792 (1 μM) for 24 hours, and a sublethal dose of OTA (10 μM) for 1, 3, 6, 12 and 24 hours. Protein lysates were collected from HK-2 cells and the decrease in global SUMOylation, activity changes of PI3K/Akt and MAPK/Erk1-2 kinases were examined by Western blot analysis.

### 4.8. Plasmid Isolation

GFP-SUMO-1 and GFP-SUMO-2 plasmids were gifts from Assoc. Prof. Umut Şahin (Bogazici University). For the growth of bacteria containing plasmids, LB Medium (Lennox, Caisson 12150002) was used. Twenty μl antibiotic ampicillin (1 mg/ml) was added to 20 ml which the bacteria are resistant. Bacteria from glycerol stocks were inoculated in a small amount with the tip of the pipette tip. Inoculated bacteria taken into the media were incubated at 37°C for 16-18 hours with shaking at 200 rpm.

At the end of the incubation, a modified alkaline lysis plasmid isolation technique containing cetyltrimethylammonium bromide (CTAB), a cationic detergent, was used. First, the bacteria were pelleted by centrifugation at 3900 g for 10 minutes at 4°C . After discarding the supernatant, 500 μl of Solution I (0.25 M Tris, 0.05 M EDTA (pH 8.0) was added to the bacterial pellet. It was pipetted until it appeared homogeneous. The mixture was taken into a clean tube. Then 500 μl of Solution II (0.1% SDS, 0.2 N NaOH) was added, mixed by gentle inversion, and incubated at room temperature for maximum 5 minutes. After incubation, 500 μl of Solution III (3 M KAc, 2 M Acetic Acid) was added. When cloudiness was obtained, the tubes were centrifuged at 18700 g for 10 minutes. Next, 1400 μl of supernatant was taken into a clean tube and 5 μl of RNase A (20 μg/μl stock) was added to prevent RNA contamination. The samples were incubated at 40°C for 20 minutes with shaking at 600 rpm. At the end of the incubation, 125 μl of 50 mM CTAB stock solution was added to the samples and centrifuged at 18700 g for 10 minutes. The supernatant was discarded, and the pellet was dissolved within 600 μl of 3M NaOAc) and transferred to a new tube. Next, 300 μl of 2-propanol (100% isopropanol) was added to the samples and centrifuged at 18700 g for 10 minutes. The supernatant was discarded, and 1 ml of ice-cold 70% ethanol was added to the samples. The samples were centrifuged at 18700 g for 10 minutes. The supernatant was discarded. The tubes were allowed to dry in order to avoid ethanol contamination. Plasmids were dissolved in 50 μl of dd$H_2O$. The concentration was measured using NanoDrop.

### 4.9. Isolation of Plasmids with NucleoBond Xtra Plus

NucleoBond Xtra Plus - Midi Prep kit was used to isolate the plasmid DNAs. After growing in antibiotic containing LB media, the bacteria were precipitated by centrifugation at 3900 g for 15 minutes at 4°C and the supernatant was discarded. Next, 8 ml of Resuspension Buffer (RES) was added to pellets. Meanwhile, column was equilibrated with 10 ml Equilibration Buffer (EQU). Eight ml of Lysis Buffer (LYS) was added to bacterial resuspension and gently inverted upside-down for 5 times and incubated at room temperature for maximum 5 minutes. After incubation, 8 ml of Neutralization Buffer (NEU) was added and inverted until the mix became colorless. Before adding lysates into column filter, to prevent clogging, sample was inverted 3 more times and was added to column filter. Sample was filtered from column filter by gravity force. Column filter was washed with 6 ml EQU buffer, flow-through and column filter were discarded. Column was washed with 8 ml Wash Buffer (WASH) by gravity and flow-through was discarded. Column was transferred to a new falcon and washed with 5 ml Elution Buffer (ELU). To the eluate 0.7 volume of isopropanol was added and vortexed well. Mix was incubated at room temperature for 2 minutes. In the meantime, 50 ml syringe's plunger was pulled out and the finalizer was attached to tip of the syringe. Sample was added into the syringe and plunger was carefully attached again and slowly sample was filtered. Flow-through was discarded. First finalizer and then plunger were pulled out again. Finalizer was attached and 2 ml ice-cold 70% ethanol was added into syringe and slowly filtered again. Finalizer and plunger were detached and finalizer was attached again. While finalizer's tip was barely touching a clean tissue paper, plunger was strongly pushed. Finalizer was out and plunger was pulled out, finalizer was attached again and plunger was strongly pushed again. This step was repeated approximately 6 times until there is no more ethanol was seen on tissue paper. Finalizer was detached and attached to 1 ml syringes after plunger of 1 ml syringe was detached. Two hundred Tris Buffer (TRIS) was added into the syringe and plasmid was eluted slowly into a sterile 1.5 ml tube. Concentration and purity were measured by NanoDrop.

### 4.10. Agarose Gel Extraction

NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) was used for gel purification of DNA fragments. The weight of the bands cut from the gel with a scalpel were taken. NT1 buffer was added to the gels in the tube at a ratio of 2:1 (w/v), which allows the DNA fragment to dissolve in the melting gel. The mixtures were incubated at 50°C for 5-6 minutes until agarose pieces were melted. The solutions were taken into the columns and tubes included in the kit. Samples were centrifuged at 11000 g for 30 seconds to separate the DNA bound to the columns from unwanted residues. The flow-throughs were discarded. Afterwards, 700 µl of NT3 buffer (100% ethanol added) was added to the columns for washing and centrifuged at 11000 g for 30 seconds and flow-throughs were discarded. Tubes were centrifuged at 11000 g for 1 minute to dry column's membrane. The columns were transferred to a clean tube and incubated for 1 minute at room temperature after addition of 15-30 µl of elution buffer. After incubation, samples were collected by centrifugation at 11000 g for 1 minute. The purity and concentration of DNAs were measured NanoDrop.

### 4.11. Construction of Plasmids

In order to construct HA-tagged SUMO1 and SUMO2 expression plasmids pMEV-2HA (Biomyx) plasmid was used. GFP-SUMO plasmids were cut with BglII and KpnI. Since, pMEV-2HA plasmid does not contain BglII site, we used a compatible enzyme BamHI and KpnI for digestion. For each reaction, 5 µl Fast Digest Buffer (10X) and 2 µl of each enzyme were added. Next, 5 µg pMev and GFP-SUMO plasmids were added. Volume was completed with $ddH_2O$ up to 50 µl. The samples were incubated for 1 hour at 37 ºC using the incubation protocol in BioRad Thermal Cycler. Enzyme digestion was checked by loading samples on 2% agarose gel. Concentration and purity measurements were performed by NanoDrop.

For ligations, vector DNAs and insert DNAs were mixed in 1:1 and 1:3 molar ratios. After calculations, 1 µl of 10x Ligation Buffer and 0.5 µl of T4 DNA Ligase were added to each sample. Sample volumes were made up to 10 µl with water and incubated for 1 hour

at room temperature. In addition, negative control (no insert) and positive transformation control (uncut plasmid) samples were also prepared and tested.

### 4.12. Transformation

*Escherichia coli (E. coli)* Top10 strain was used as competent cells for transformation. Frozen competent cells from -80 ºC were incubated on ice for 10 minutes and 5 µl of the ligation products were added into the center of the competent cells. After incubation on ice for 30 minutes, cells were heat-shocked at 42 °C for 45 seconds. In order to reduce the heat shock stress and ensure their proliferation with new plasmid DNA, 1 ml of LB medium was added to transformed cells and further incubated at 37 °C for 1 hour with shaking at 230 rpm. After the incubation, cells were spread on pre-mixed LB Agar plates containing Kanamycin and were incubated at 37°C overnight. Petri dishes with bacterial growth were stored at +4 °C.

### 4.13. Transfection

GenaxxoFect Transfection Kit was used for transfection. HK-2 cells were seeded in a 100 mm petri dish the day before transfections. Cells with densities of 90-95% on the day of transfection were detached with trypsin and collected by centrifugation. Cells were re-suspended 2.2 x$10^6$ per ml. In-tube transfection mix was prepared with 20 µl of transfection agent and 600 µl of dilution buffer per plate. Eight µg of plasmid construct per well was diluted in 600 µl of dilution buffer. The transfection mix and plasmid dilution were mixed by rapid pipetting and incubated at room temperature for 20 minutes. Cells were added to the transfection complex and pipetted and homogeneously mixed complex was seeded into 100 mm plates.

### 4.14. HK-2 Stable Cell Line Construction with Gateway Technology

To construct stable HK-2 cell lines overexpressing SUMO-1 and SUMO-2/3, we used Gateway Technology. First, attB sites were added to the beginning and the end of the

fragments by using PrimeSTAR GXL DNA Polymerase (Takara Bio, R050A) and adapter primers. After PCR samples were run on 0.8% agarose gel and fragments were excised by sterile scalpel. After purifying DNA products from agarose, BP cloning was performed by using Gateway™ BP Clonase™ II Enzyme mix (Thermo) following the manufacturer's instructions. For transformation, *E. coli* DH5[U+237A] cells were used and grown on agar plates containing Gentamicin (1 mg/ml) at 37 ºC overnight. Next day, colonies were inoculated into LB Broth containing Gentamicin and incubated overnight at 37 ºC. Cloning was confirmed by enzyme digestion with HindIII and EcoRI. After confirmation, to perform LR cloning we used Gateway™ LR Clonase™ II Enzyme mix (Thermo). After LR cloning, plasmids were transferred into Stbl3 cells and grown on agar plates containing Ampicillin at 37 ºC for overnight. Grown colonies were selected and inoculated within Ampicillin containing LB Broth at 30 ºC.

### 4.15. Virus Production in HEK293FT Cells

HEK293FT cells were seeded into 100 mm cell culture plates as 8 million/plate, one day prior to transfection. Chloroquin (Cq) was added as 10 μM final concentration in 9 ml DMEM:F12 (containing 10% FBS, Pen/Strep and L-Glu). Cells' mediums were aspirated and medium with Cq was added. Cells were incubated in 37 ºC. In a sterile tube, 4 μg pVSVG and 7.5 μg pSPAX2 plasmids, 62.5 μl $CaCl_2$ were added. Next, volume was completed up to 500 μl with sterile water. Five hundrer microliter HBSS (2X, transfection grade) was added drop by drop while mixing. Ten micrograms of GFP-containing plasmid as positive control, pLEX307-HA-SUMO-1 and pLEX307-HA-SUMO-2 plasmids were added into separate tubes. To each tubes, 500 μl master mix was added. Final mixes were incubated for 13 minutes. After incubation, plasmid-containing mixes were added onto cells drop by drop and incubated for 6 hours. After 6 hours, mediums were changed into complete DMEM:F12 without Cq and incubated further for 48 hours. To collect the virus, 0.45 μm filters and 10 ml syringes were attached for each. Five hundred microliter virus-containing mediums were filtered and collected in 50 ml falcons and aliquoted. Aliquotes were stored in -80 ºC.

### 4.16. Transduction and Selection of HK-2 Cells

HK-2 cells were seeded into 6-well plates with several confluencies between 10-20% for both transduction and control sets. Next day, 500 µl lentivirus and 1:4000 dilution of polybrene (1000x stock) for each well were mixed. Medium were aspirated from wells and polybrene-lentivirus mix was given to HK-2 cells for transduction. Cells were incubated for 6 hours. At the end of the incubation, lentivirus were aspirated and to each well complete DMEM:F12 was added. Cells were incubated further for 48 hours. Afterwards, 8 µg/ml Puromycin (1 mg/ml stock) was prepared in complete DMEM:F12 for each well of both transduced and untransduced cells. Medium was changed into puromycin-containing medium and cells were incubated within until there were no more untransduced cells. Medium was refreshed every 3 days. Finally, selected cells were trypsinized and subcultured into 100 mm plate.

### 4.17. Statistical Analysis

To analyze our data GraphPad Prism 8 was used as analysis software. The data was shown as avarege ± standard deviation. Comparisons were with ANOVA with Bonferroni correction. For all of the tests, significance criteria was p¡0.05. All experiments were repeated independently, at least three times. ns: nonsignificant, *p¡0.05, **p¡0.01, ***p¡0.001, ****p¡0.0001.

# 5. RESULTS

## 5.1. Detection of OTA-induced oxidative stress and determination of its association with global SUMOylation pattern

Sublethal dose is concentration of the toxin that is insufficient to cause death. Before treating cells with the toxic reagent, sublethal concentration must be determined to induce cellular stress but do not cause large-scale death. To analyze cellular viability and cytotoxicity, XTT assay can be used. In this assay cellular metabolism converts tetrazolium salt to formazan and amount of formazan is quantitied. This quantification is correlated with amount of viable cells. In one of our previous studies [11], after HK-2 cells were treated with 0.1, 1 and 10 μM OTA for 24 hours and viability of cells was seen to decrease approximately 35% for 10 μM whereas lower doses decreased viability insignificantly. Thus in the same study [11], sublethal dose of OTA for HK-2 cells was determined as 10 μM. To determine cell viability in HK-2 cells after OTA treatment in a time-dependent manner, HK-2 cells were exposed to 10 μM OTA for 1, 3, 6, 12 and 24 hours and cell viability was examined (Figure 5.1).

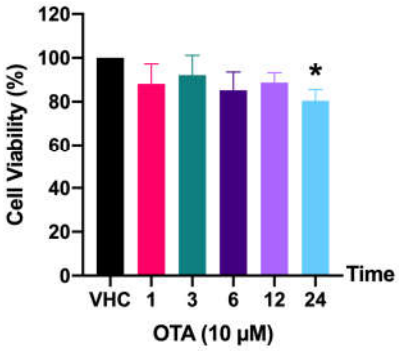


Figure 5.1. OTA (10 μM) treatment decreases cell viability in 24 hours.

It has been shown in the literature and in our previous studies that OTA induces oxidative stress in various cells. However, different cell types and lines may produce different molecular responses. Therefore, it was planned to confirm that OTA causes oxidative stress in the human proximal tubule cell line HK-2.

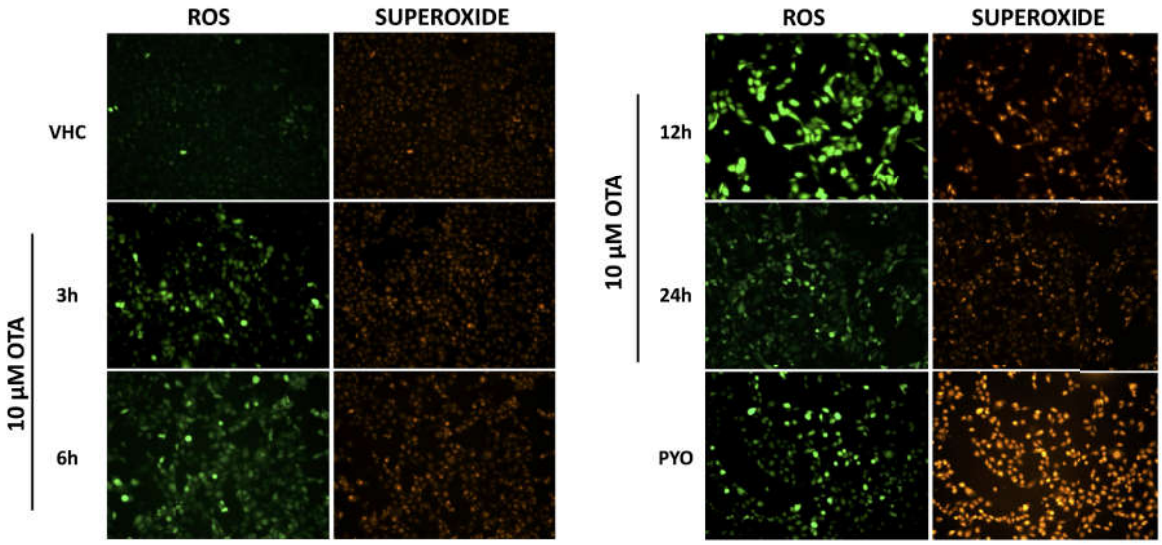


Figure 5.2. OTA treatment induces oxidative stress in HK-2 cells in a time-dependent manner.

Pyocyanin is a zwitterion containing a phenol group, giving weakly acidic properties. Oxidative stress induction of pyocyanin is due to its ability to increase the levels of intracellular ROS, particularly superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) [50]. In order to confirm that OTA triggers oxidative stress in HK-2 cells, the change in the amount of reactive oxygen species (ROS) under OTA exposure was determined with the Total ROS/Superoxide Detection Kit. Pyocyanin was used as positive control to confirm the induction of oxidative stress and absolute ethanol was used as vector control (VHC) to demonstrate the effect of OTA solvent. Cells were exposed to OTA (10 µM) for 3, 6, 12, 24 hours or ethanol (0.1%) or to pyocyanin for 30 minutes. Images were captured using a green filter compatible with Fluorescein (Ex/Em: 490/525 nm) for the total amount of ROS and an orange filter compatible with Rhodamine (Ex/Em: 550/620 nm) for the total amount of superoxide. As a result of the analysis, it was observed that the total amount of ROS and superoxide in HK-2 cells exposed to OTA gradually increased between 3-12 hours and decreased at 24 hours (Figure 5.2).

Oxidative stress and increased ROS are among the stresses that cause cytotoxicity. It has been reported that about 50 proteins involved in stress response signaling pathways are also among SUMO targets [51, 52].

In line with this information, we predict that OTA-induced oxidative stress could lead to changes in the SUMOylation pattern in the HK-2 cell line. To test this hypothesis, HK-2 cells were treated with either absolute ethanol as vector control (VHC) or sublethal dose of OTA (10 μM) for the indicated time periods. Cells were lysed with cell lysis buffer and the lysates were harvested. Fifty μg of samples were resolved on a 8% SDS-PAGE gels and visualized by Western blot analysis using SUMO-1 and SUMO-2/3 primary antibodies.

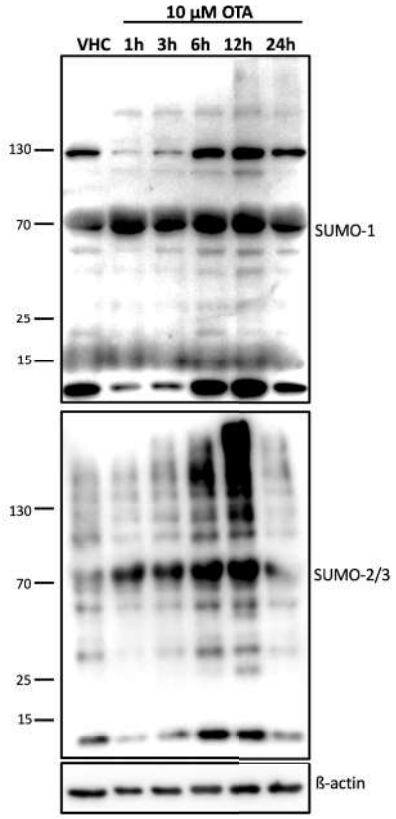


Figure 5.3. OTA alters global protein SUMOylation pattern in HK-2 cells in a time-dependent manner.

Results are showing that SUMOylation patterns change over time in HK-2 cell line treated with OTA. More SUMO-2/3-conjugated proteins were observed in cells exposed to OTA for 12 hours compared to the vector control and cells exposed for 1, 3, 6 hours. This effect decreases between at 12-24 hours (Figure 5.3) In addition, we see that alterations in SUMO-2/3 were more distinct than SUMO-1 as SUMO-1 is more readily available to conjugations under physiological conditions whereas SUMO-2/3 is that of under stress.

For further analysis to understand OTA and oxidative stress association with SUMOylation, we aimed to decrease amount of Reactive Oxygen Species in HK-2 cells by using a ROS scavenger, N-acetyl L-cysteine (NAC) dissolved in $ddH_2O$. Sublethal dose of NAC was determined in one of our previous studies [10] as 10 mM. HK-2 cells were treated with 10 μM OTA alone or in combination with 10 mM NAC for the indicated time durations. Upon NAC treatment, phosphorylation of Erk and Akt proteins were decreased but global SUMOylation was not altered significantly (Figure 5.4).

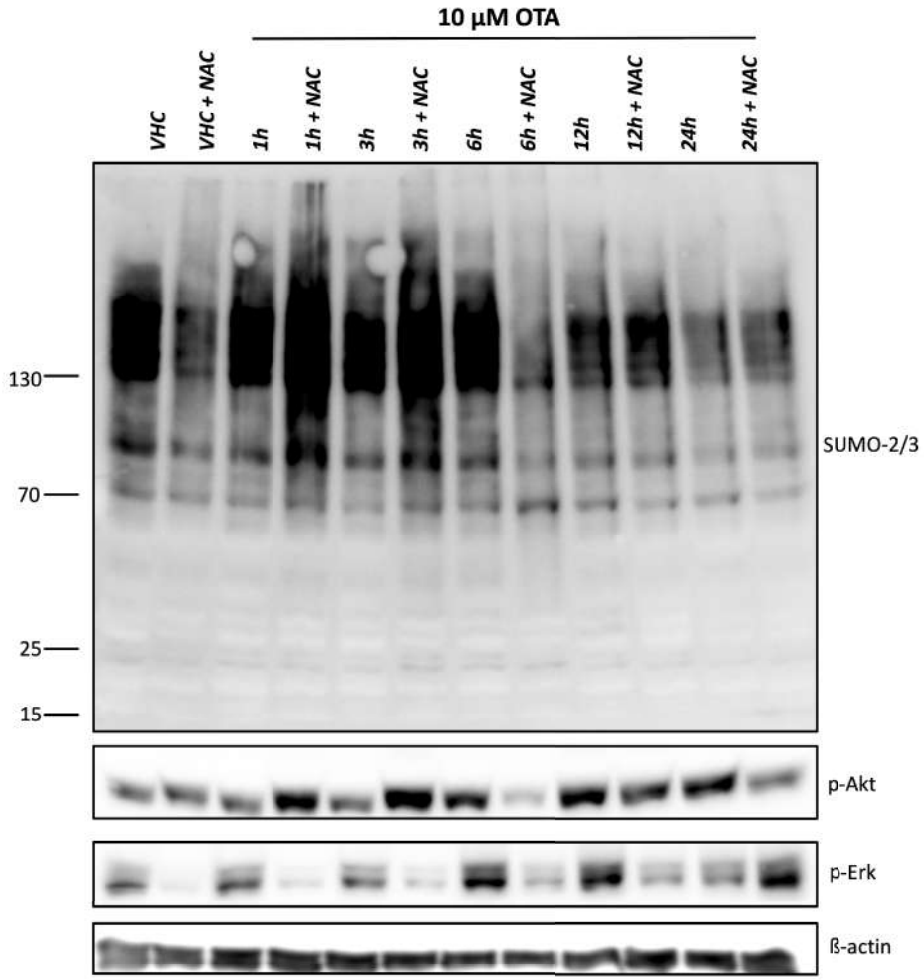


Figure 5.4. OTA treatment in combination with NAC, altered SUMOylation pattern and phosphorylation of Erk and Akt proteins insignificantly.

To better understand the nature of relationship between OTA and SUMOylation, HK-2 cells were treated with ML-792, SUMO-activating Enzyme (SAE) Inhibitor to inhibit global SUMOylation process. First, to find the sublethal dose of ML-792, HK-2 cells were exposed

to ML-792 at 0, 0.2, 0.3, 0.5 and 1 μM doses for 24 hours. The viability of HK-2 cells was checked by XTT Assay after inhibition of SUMOylation (Figure 5.5).

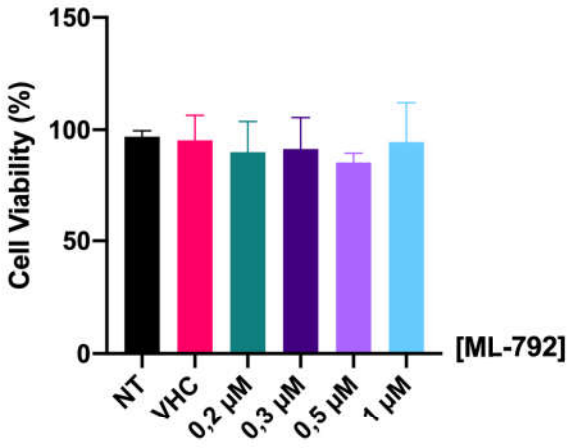


Figure 5.5. Different concentrations of ML-792 do not change cell viability significantly.

Hormesis phenomenon is a term expressing the biphasic dose response, which is characterized by a beneficial effect on the cell due to treatment with a low-dose agent and a toxic effect when treated with the same agent at a high dose [53]. As a result of XTT assay analysis, a slight but statistically insignificant increase in viability was observed in cells exposed to ML-792 at low doses compared to the control group. To confirm that the dose and the time frames used for ML-792 treatment indeed inhibits SUMOylation, Western blot analysis was performed with SUMO primary antibodies.

ML-792 greatly decreased SUMOylation in a dose-dependent manner in HK-2 cells (Figure 5.6). Based on XTT assay and Western blot analyses, effective sublethal dose of ML-792 was determined as 1 μM. Afterwards, HK-2 cells were exposed to 1 μM ML-792 for 24 hours, 10 μM OTA and 0.1% ethanol or DMSO as vehicle for 1, 3, 6, 12, and 24 hours. At the end of the experiment, it was seen that ML-792 significantly decreased SUMOylation in HK-2 cells under OTA toxicity (Figure 5.7 and Figure 5.8).

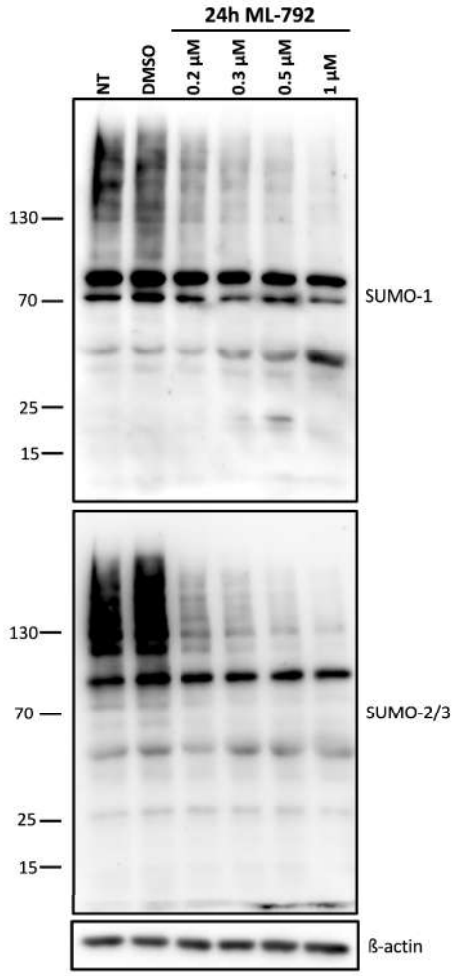


Figure 5.6. Global SUMOylation is inhibited upon ML-792 treatments in a dose-dependent manner.

## 5.2. Investigation of the effects of altered SUMOylation patterns on OTA-induced signal transduction pathways

SUMOylation can be controlled by the regulation of protein kinase cascades that are activated in response to extracellular signals [32]. The MAPK/Erk and PI3K/Akt pathways are the most common central routes [35]. They are involved in many vital activities in cellular processes such as cell proliferation, differentiation, apoptosis, and regulation of cell viability. Therefore, we aim to examine whether the activity of these pathways is correlated with global SUMOylation pattern alterations under OTA exposure.

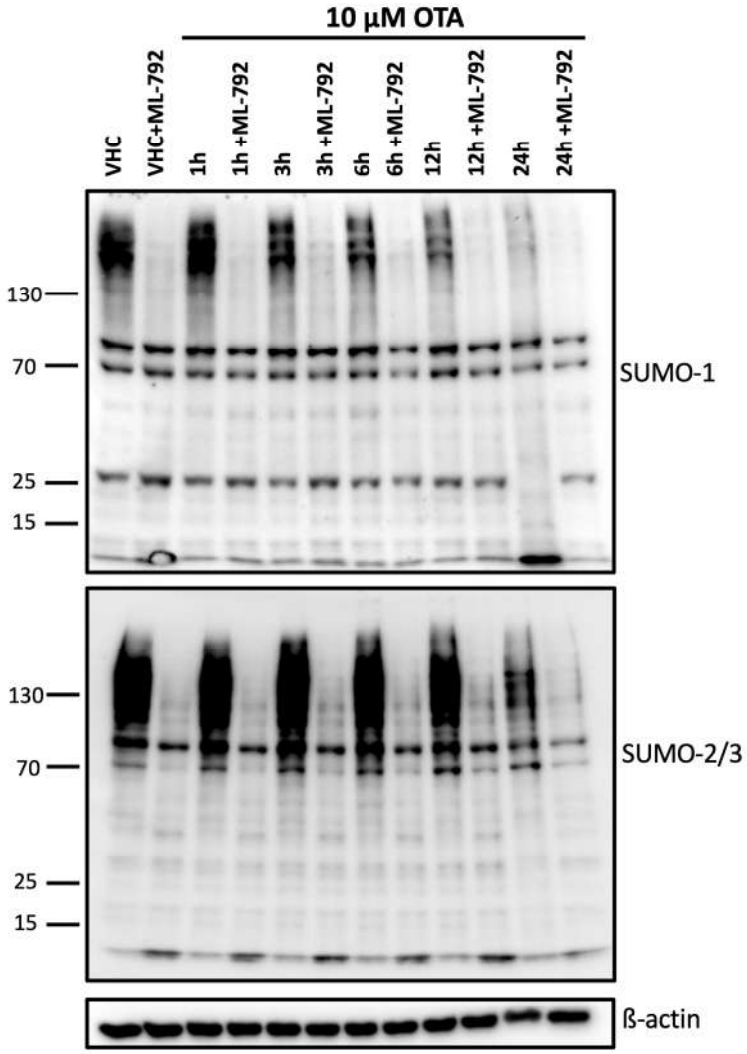


Figure 5.7. OTA-altered SUMOylation is inhibited upon ML-792 treatments.

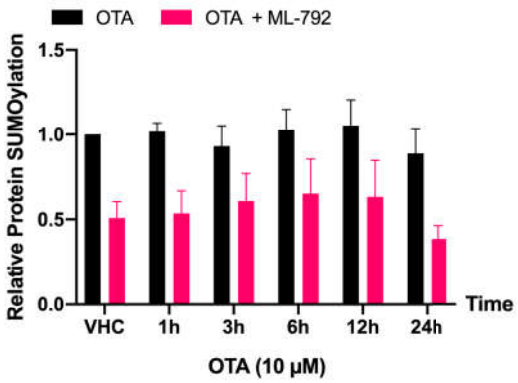


Figure 5.8. Relative protein SUMOylation is decreased upon ML-792 treatments.

For this purpose, HK-2 cells were exposed to sublethal dose (10 μM) of OTA for different time periods to understand how OTA affects the phosphorylation of Erk and Akt, which are downstream effectors of MAPK and PI3K pathways, respectively. The phosphorylation levels of proteins Erk (p-Erk) and Akt (p-Akt) were examined by Western blotting by utilizing phosphor-specific antibodies against each protein and comparing to total Erk and total Akt levels, respectively (Figure 5.9).

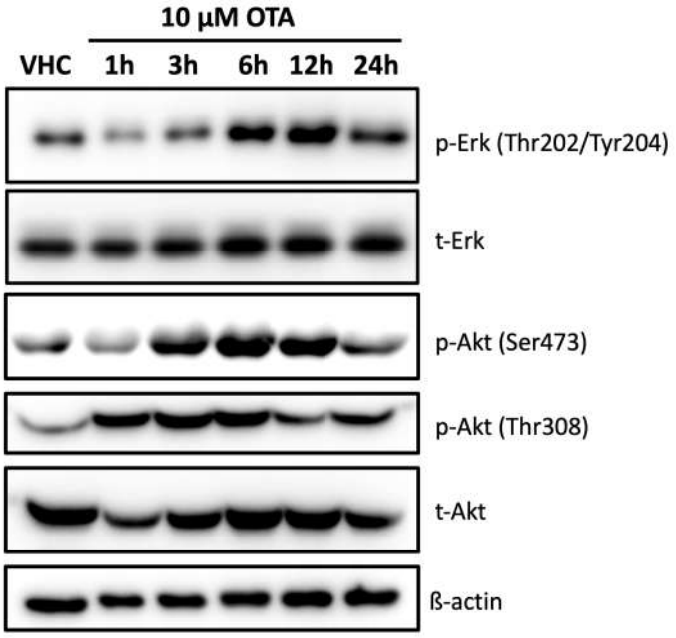


Figure 5.9. OTA treatment induces phosphorylation of Erk and Akt in HK-2 cells in a time-dependent manner.

Upon OTA treatments, phosphorylation of Akt and Erk proteins are increased in a time-dependent manner. Erk phosphorylation can be seen earlier than Akt phosphorylation under OTA-induced oxidative stress. However, even though there is a slight decrease in phosphorylation of both proteins after 12 hours, activated forms can still remain until 24 hours.

U0126 acts by selectively blocking Erk phosphorylation via inhibiting the kinase activity of MEK1/2 [54]. Although SUMOylation and MAPK/Erk1-2 pathways are shown to be associated with each other, the molecular mechanisms underlying is not yet clearly understood. To determine whether the inhibition of OTA-induced MAPK/Erk activity can affect

SUMOylation in HK-2 cells, the pathway was inhibited with 10 μM U0126, and the pattern changes in SUMOylation were examined. Inhibition of MAPK/Erk1-2 pathway did not alter global SUMOylation significantly (Figure 5.10).

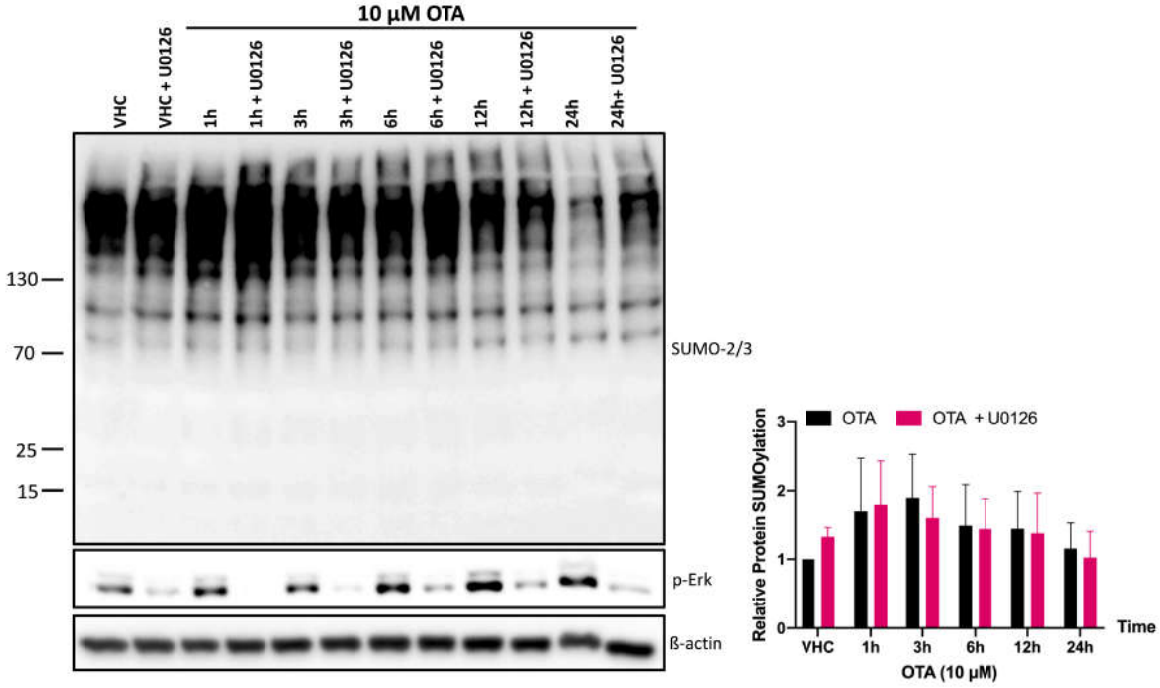


Figure 5.10. Inhibition of MAPK/Erk1-2 pathway does not alter global SUMOylation significantly upon OTA treatment in HK-2 cells.

Wortmannin is a potent irreversible inhibitor of the PI3K pathway that binds the ATP-binding site of p110 kinase domain, preventing the conversion to PIP3 [55]. By preventing PIP3 conversion, PDK1 activation is inhibited thus Akt phosphorylation does not occur. To answer how SUMOylation and PI3K/Akt pathway are affecting each other, we inhibited PI3K/Akt pathway in HK-2 cells by treating with 1 μM Wortmannin. At the end of treatments, results were visualized by Western blot analysis using SUMO-2/3 antibody. As opposed to, U0126 inhibiton of MAPK/Erk, inhibiting PI3K/Akt pathway with Wortmannin slightly increased SUMO-2/3 conjugations in HK-2 cell line upon OTA exposure all periods (Figure 5.11).

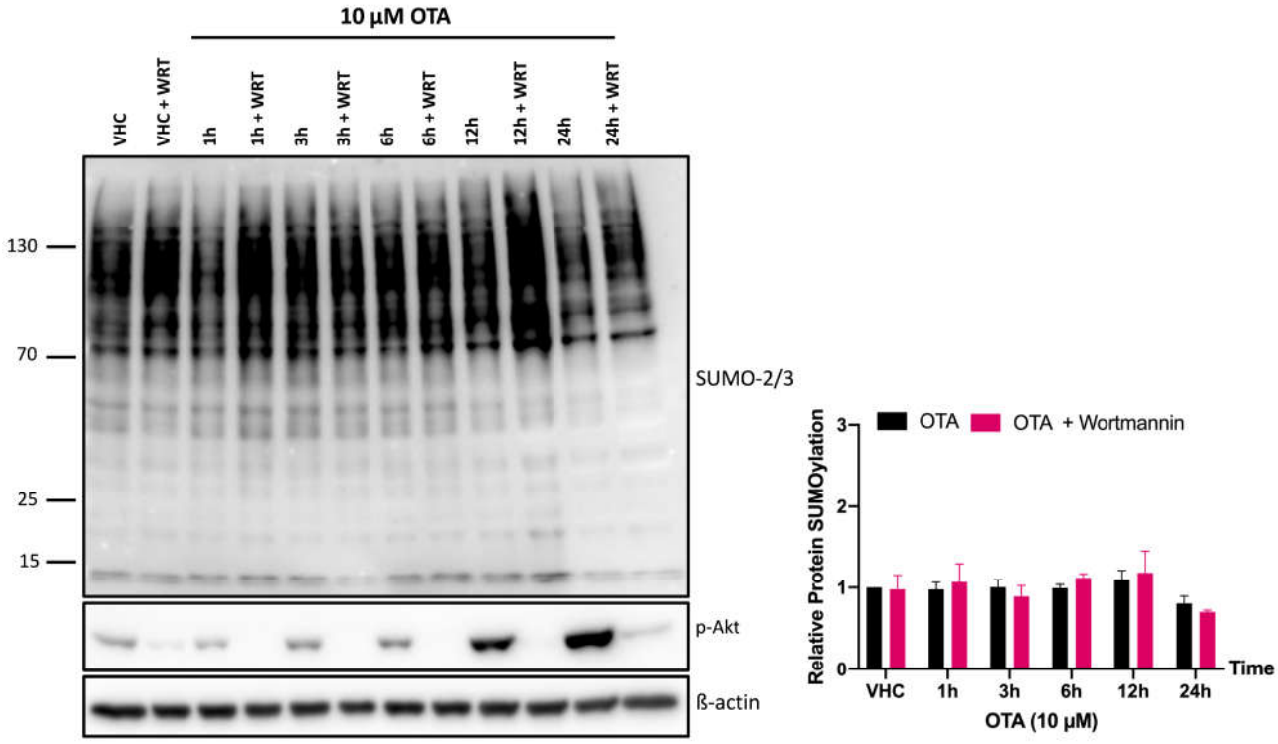


Figure 5.11. Inhibition of PI3K/Akt pathway does not alter global SUMOylation significantly upon OTA treatment in HK-2 cells.

Reciprocally, SUMOylation was inhibited in HK-2 cell lines to better examine association(s) with signal transduction pathways. To examine whether ML-792 affects basal activities of MAPK and PI3K pathways, we treated HK-2 cells with several doses of ML-792 (Figure 5.12) and showed that ML-792 treatment did not affect basal Akt and Erk phosphorylation status in a dose-dependent manner.

Afterwards, HK-2 cells were treated with previously determined effective sublethal dose of ML-792 (1 µM) and OTA (10 µM). Protein expression changes in MAPK/Erk1-2 and PI3K/Akt pathways in HK-2 cells under SUMO-inhibited conditions were detected primarily by total Akt, total Erk, and activation changes via phosphorylated-Akt (p-Akt) and phosphorylated-Erk (p-Erk) antibodies. OTA treatments slightly increased phosphorylation of Erk1-2, but surprisingly, Akt phosphorylation was abolished under SUMOylation-inhibited conditions (Figure 5.13).

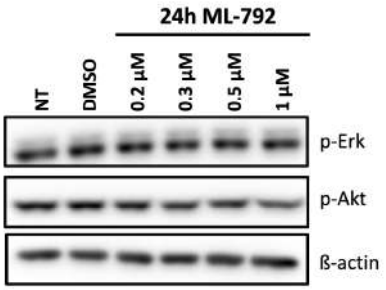


Figure 5.12. ML-792 treatment does not affect basal Akt and Erk phosphorylation status.

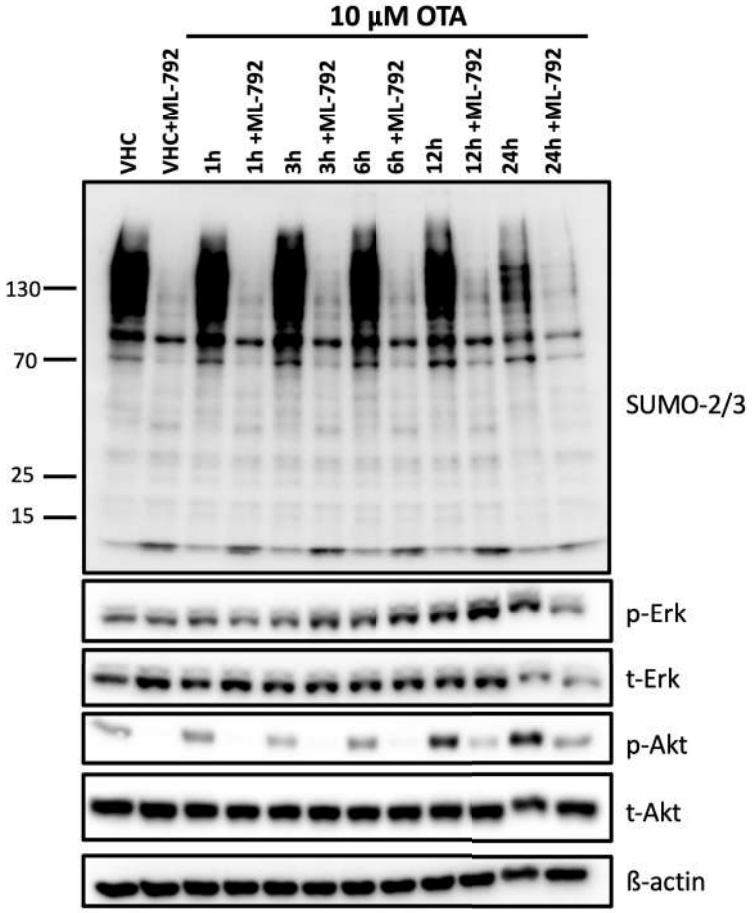


Figure 5.13. OTA-induced Akt phosphorylation is abolished when SUMOylation is inhibited.

In order to confirm whether inhibition of SUMOylation affects cell survival via Akt phosphorylation upon OTA exposure, we performed cell viability test with OTA only, ML-792 only and OTA + ML-792 combined test groups. Cell viability was greatly decreased when SUMOylation-inhibited HK-2 cells were exposed to 10 μM OTA in a time-dependent manner (Figure 5.14) supporting that SUMOylation might have a key role in cell survival by inducing Akt phosphorylation.

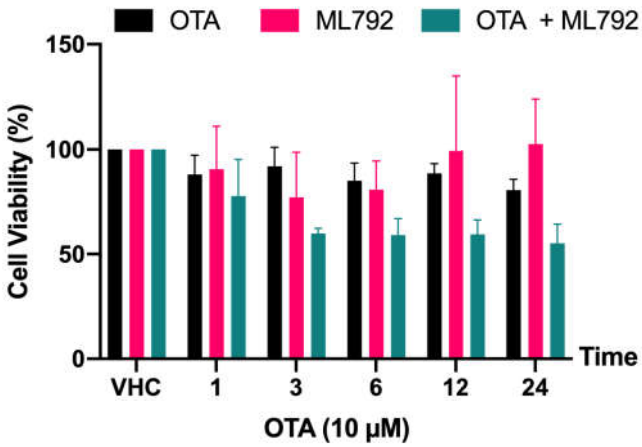


Figure 5.14. OTA further decreases cell viability when global SUMOylation is inhibited by ML-792.

### 5.3. Detection of possible SUMOylation target proteins under OTA exposure

Based on the changes seen from our results so far, we aim to identify SUMOylated or deSUMOylated proteins. Since SUMO is a reversible modification, purification of the conjugates was carried out in denaturing buffers to inhibit the activity of SUMO proteases. The use of these buffers also inhibits the purification of non-covalent SUMO-binding proteins.

To achieve this aim, SUMO-1 and SUMO-2 plasmids were prepared for assays. GFP-SUMO-1 and GFP-SUMO-2 plasmids were kindly provided by Assoc. Prof. Umut Şahin (Bogazici University, Department of Molecular Biology and Genetics). Plasmids were digested with BglII and KpnI restriction enzymes. As destination vector, pMev-2HA plasmid was used. Empty pMev-2HA plasmid, SUMO-1 and SUMO-2 fragments were extracted from agarose gel by using NucleoSpin Kit. Because pMev-2HA plasmid does not have BglII site, its compatible enzyme BamHI was used for digestions and subsequently for ligations.

To verify the reconstructed plasmids, HK-2 cells were transfected with 3 colonies selected from pMEV-2HA-SUMO-1 and -SUMO-2. Expressions and hence the transfections were verified by Western blot analyses using the HA primary antibody. Among selected colonies, pMev-2HA-SUMO-1 Colony-2, pMev-2HA-SUMO-2 Colony-3 were the ones that significantly increased SUMOylation in HK-2 cells (Figure 5.15).

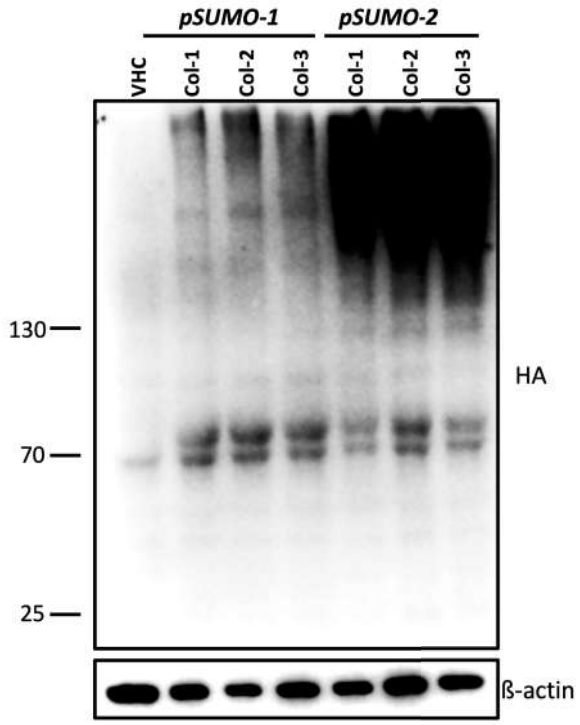


Figure 5.15. Transfection with pMEV-2HA-SUMO-1 and pMEV-2HA-SUMO-2 induces SUMOylation in HK-2 cells.

Afterwards we transfected HK-2 cells and treated them with 10 μM OTA for 1,3,6 and 24 hours and performed immunoprecipitation (IP) analysis using Magnetic HA-Tag IP/Co-IP Kit followed by Western blotting utilizing HA, total Akt and phospho-Akt antibodies. But unfortunately, we could not determine our results clearly probably due to inefficiency of transient transfection or inefficient anti-HA antibody binding.

Because transient transfection is not as efficient as stable one to perform immunoprecipitation and followed by Mass Spectrometry analysis, we wanted to construct a stable HK-2 cell lines overexpressing SUMO-1 and SUMO-2. To reach this aim, we used Gateway

cloning method. Since our pMEV plasmids do not contain any *att* sites, we generated primer adapter sequences to add *attB* sites both to beginning and end of SUMO sequences. Insertion of adapters were confirmed by PCR. We extracted SUMO-1 and SUMO-2 DNA fragments containing *attB* sites from agarose gel by using NucleoSpin Purification Kit. Extracted SUMO fragments were inserted into pDONR207 plasmid via BP cloning and transformed into *E. coli* DH5α cells. Colonies were grown on agar plates and were picked and transferred into LB Broth with Gentamicin. Insert-containing colonies were verified by restriction digestion. After confirmation, 2HA-SUMO sequences were cloned into pLEX307 plasmid by LR cloning from pDONR207 plasmid. Plasmids were transformed into *E. coli* STBL3 cells and incubated in Ampicillin containing agar plates. After incubation, pLEX307-2HA-SUMO-1 plasmid was confirmed from colonies.

Next, we transfected HEK293FT cells, which are ideal for generating high-titer lentivirus with pLEX307-2HA-SUMO-1 and pLEX307-2HA-SUMO-2 plasmids and GFP-containing plasmid for positive control for viral production. After 2 days of incubation, virus were collected. HK-2 cells were seeded transduced with lentivirus. For transduction control, nontransduced HK-2 cells were seeded into another plate and both sets were treated with puromycin containing medium until there were no more transduced cells in the wells. Finally, transduced cells were collected and seeded for expansion. Stable HK-2 cell lines will be treated with OTA (10 μM) for 1,3,6,12 and 24 hours or with ethanol (0.01%). Next, we will pull down SUMO-conjugates with HA-tag immunoprecipitation and perform mass spectrometry analysis to identify possible SUMO targets.

# 6. DISCUSSION

OTA is a mycotoxin that can be found on daily consumptions of both human and animals. Due to its structure, OTA can be reabsorbed from proximal tubules of kidneys. Moreover, OTA is highly stable and it can accumulate in kidneys and can cause nephropathy and/or kidney cancer. Several studies have suggested that OTA is one of the most potent factors in the etiology BEN [56]. How OTA causes nephropathy and carcinogenic transformation is not fully understood yet.

Oxidative stress induction may lead to alterations in several pathways causing disruptions in cell homeostasis. Previous studies in our laboratory have demonstrated that OTA causes sustained activation of PI3K/Akt and MAPK/Erk signal transduction pathways in the HK-2 cell line [10, 11]. It was shown that activation of PI3K/Akt pathway pushes cells to survival whereas MAPK/Erk1-2 pathway drives cells to apoptosis upon OTA exposure [11]. As mentioned earlier, high ROS and superoxide levels in cells can damage proteins involved in apoptosis and survival pathways, leading to tumorigenesis and carcinogenesis. Cellular oxidative stress can be observed due to increased ROS and superoxide levels and/or decreased antioxidant capacity. Furthermore, it is biologically plausible to expect that both the PI3K/Akt and MAPK/Erk1-2 pathways may be affected by the OTA-driven altered SUMOylation pattern.

Based on the data and literature obtained so far by our and other research groups, we hypothesized that OTA might induce toxicity and/or carcinogenic effects in part by altering SUMOylation pattern via inducing oxidative stress therefore by affecting signal transduction pathways.

To test our hypothesis, we aimed to (i) show that OTA can induce oxidative stress in HK-2 cell line, (ii) show OTA-induced oxidative stress affects SUMOylation, (iii) demonstrate the association between SUMOylation and signal transduction pathways, (iv) determine the potential substrates of SUMOylation under OTA exposure.

Our experiments have shown that OTA induces changes in global SUMOylation pattern in a time-dependent manner. Costa et al [16] has reported that in Vero cells (green monkey kidney cells) 30 μM OTA slightly increased ROS level in 30 minutes and 24 hours. In another, it was reported that OTA increased ROS in the HepG2 cell line in 1 hour [57]. In the same study [57], it was shown that the increase in ROS induced by OTA decreased with quercetin, a ROS scavenger. Similarly, in our experiments oxidative stress is increased mostly at 12 hours after 10 μM OTA treatment as shown in Figure 5.2.

Oxidative stress was shown to increase SUMOylation by several agents. Sahin et al. [58], showed that global SUMOylation was increased by oxidative stress in a time-dependent manner upon arsenic treatment. Similarly, Manza et al. [59], has reported that, several agents that can induce oxidative stress also induced changes in SUMO-1 and SUMO-2/3 patterns. Moreover, in COS-7 cells oxidative stress induced by $H_2O_2$ changed SUMO-2/3 pattern as shown by Saitoh et al [29]. Likewise, we have seen changes in global protein SUMOylation patterns in HK-2 cells after 10 μM OTA treatment in a time-dependent manner. In addition, Saitoh et al [29] has shown that activation of SUMO-1 pathway was less prominent than is that of SUMO-2/3 under stress. In our studies, under OTA-induced oxidative stress SUMO-2/3 were more readily available for conjugation than SUMO-1 as well. When HK-2 cells were exposed to 10 mM NAC and 10 μM OTA simultaneously, Erk1-2 phosphorylation was decreased in early period whereas Akt phosphorylation and SUMOylation were increased at that period. Six hours upon NAC and OTA treatments, p-Akt and SUMO-2/3 levels showed slight decrease whereas p-ERK levels showed increase. Unfortunately, we were able to perform this assay only once and these results are insignificant.

The extracellular regulated kinases Erk1-2 are responsible for a wide variety of cellular processes such as growth, differentiation, and apoptosis. The regulation of Erk1-2 is dependent on its upstream kinases which are known as Ras/Raf/MEK. Kubota et al [60] has demonstrated that MEK is one of the SUMO-1 targets. They showed that, SUMOylation of MEK leads to inactivation and Erk1-2 phosphorylation cannot occur. Inhibition of SUMO-1 rescues MEK-1 activity and Erk1-2 phosphorylation is induced. In our previous studies [10, 11] the proposed OTA's mode of action suggested that the upstream effector of

MAPK/Erk1-2 is not known and we hypothesized that SUMOylation might be the link between OTA and MAPK/Erk1-2 pathway. But, unfortunately, inhibition of SUMOylation via ML-792 did not cause any significant changes in Erk phosphorylation in HK-2 cells. Additionally, inhibition of MAPK/Erk1-2 pathway slightly but insignificantly altered SUMO-2/3 conjugations upon OTA treatment (Figure 5.10). These results suggest that SUMOylation might not be related to MAPK/Erk1-2 pathway under OTA exposure.

The PI3K/Akt pathway is one of the key controlling molecular processes of cell proliferation and survival. Akt is an anti-apoptotic kinase that causes cell cycle arrest and prevents cellular death when overexpressed. Akt inhibits pro-apoptotic proteins, which regulates the programmed cell-death pathway. Dysregulations in this pathway have been linked to a variety of disorders, including tumorigenesis and cancer. PI3K/Akt signaling has been demonstrated to be disrupted in variety of cancers, including breast, ovarian, and pancreatic. Horiguchi et al [60] has indicated that Akt has relatively high frequency of activation among patients with Renal Cell Carcinoma (RCC). In the same study, the author also noted that there were significant connections between increased Akt activation and metastasis and tumor grade [60]. The discovery that PTEN mutations are common in RCC provides additional evidence for the importance of PI3K/Akt. In human RCCs, PTEN protein and gene expression has been described as decreased [61, 62], absent [63], mutated [64], or deleted [65]. Because PTEN inhibits PI3K activity, a negative change in this protein levels results in constitutive PI3K activation and these tumors may be particularly vulnerable to PI3K/Akt inhibitors. In our experiments, we showed that inhibition of PI3K/Akt pathway by Wortmannin slightly but insignificantly increased SUMOylation. Remarkably, when we inhibited SUMOylation by ML-792, Akt phosphorylation was abolished upon OTA exposure suggesting that, SUMOylation might be essential for the activity of PI3K/Akt pathway cell survival under OTA toxicity.

In this Master's project, we show that OTA induces oxidative stress in HK-2 cells and alters global protein SUMOylation in a time-dependent manner. Our data suggest that OTA-induced MAPK/Erk1-2 pathway activation may not be related to SUMOylation. Thus, how OTA affects MAPK/Erk1-2 pathway should be investigated further. But, our results

indicate that SUMOylation might be playing a key role in cell survival by interacting with Akt. The suggested mechanisms of how OTA toxicity affects signal transduction pathways by disrupting SUMOylation is shown in Figure 6.1 .

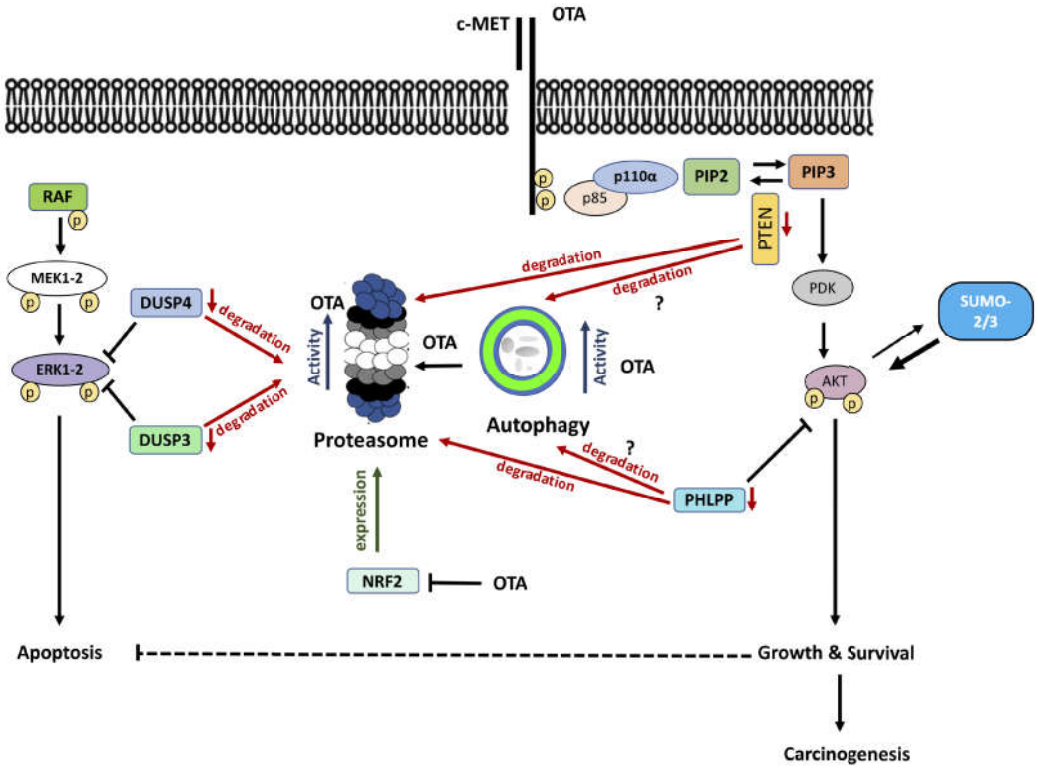


Figure 6.1. Suggested mechanism of signal transduction pathways and SUMOylation communication under OTA toxicity.

In future studies, the detection of potential SUMO targets by Mass Spectrometry will be performed to clarify the possible proteins involved in this relationship. In line with the results we will further clarify, the mechanism of OTA-driven kidney cancer paving way towards identification of new agents and targets for future therapeutic studies.

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